

Dissecting PTEN function in cancer

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To my family and friends

По лесу идет голодная обезьяна и вдруг видит пальму с бананами. Начинает ее трясти, но плоды не падают.

Голос научного руководителя говорит:

— Подумай!

Обезьяна подумала, нашла палку, бросила сбила гроздь бананов и наелась.

Идет голодный аспирант по лесу, видит ту же пальму с бананами. Начинает трясти, бананы не падают. Голос научного руководителя говорит:

— Подумай!

Аспирант:

— Да чего тут думать-то?! Трясти надо, трясти!

Популярный аспирантский анекдот 1970-х годов

Declarations

This thesis, written by Marina Fedorova, presents and describes the original research conducted solely by the author. It has not been submitted in any form for another degree/diploma at any other university. Any published and unpublished research that led to the development of this thesis has been acknowledged and suitably referenced and a complete list of references is provided in the bibliography.

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Abstract

PTEN downregulates a number of essential biological processes such as cell proliferation, survival and growth. Alterations of PTEN levels can lead to cancer, and PTEN is indeed one of the most frequently disrupted tumour suppressor genes in human cancers. Thus, anti-cancer treatment strategies focusing on PTEN deficiency could prove very beneficial. As such, a wide range of therapeutic approaches targeting cancers with various PTEN genotypes is currently at preclinical and clinical stages. Complete loss of PTEN expression by cells is less tumourigenic, more treatable and can be targeted by “lethal sensitivity” (increased sensitivity of PTEN deficient cells to radiation, cytotoxic drugs or treatment with inhibitors of downstream proteins). At the same time, cells with partial PTEN loss can be a target for “pro-senescence therapy” (activation of irreversible cell cycle arrest). Herein, we explore PTEN requirements for lethal sensitivity (focusing on Skp2 and NLK inhibition). Towards this goal, we have created isogenic PTEN wild type, heterozygous and null cells using CRISPR/Cas9 technology and introduced wild type or various functionally impaired forms of PTEN into those lines using CRISPR/Cas9 knock-in and Sleeping Beauty transposon system. Synthetic lethal sensitivities to inhibition of Skp2, TTK and PARP or to curcumin treatment did not provide robust responses. Using chemical genetics, an analogue sensitive version of the kinase NLK was constructed and using this for genetic engineering of human cells enabled the validation of the synthetic lethality between NLK and PTEN to be established. These results provide valuable insights into the cancer biology of PTEN and the reagents and techniques developed within this study will enable greater characterisation of anti-cancer therapies based on the genetic status of PTEN.

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Nomenclature and abbreviations

Abbreviation	Description
2D-GE	Two-Dimensional Gel Electrophoresis
4-OHT	4-hydroxytamoxifen
aa	Amino acid
AAVS1	Adeno-associated virus integration site 1
AcNBP	Autographa californica nuclear polyhedrosis virus
Akt	Serine/threonine-specific protein kinase / protein kinase (PKB)
AML	Acute myeloid leukaemia
AP	Alkaline Phosphatase
AS	Analogue sensitive
ASV16	Chicken retrovirus
ATCC	Diploid colorectal carcinoma epithelial immortalised cells
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
AZ3146	TTK inhibitor
AZD2281	PARP inhibitor
BAD	Pro-apoptotic protein
BCIP	5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
BCL-X _L	Apoptosis-inhibitory protein
BIM	Pro-apoptotic member of the BCL-2 protein family
bp	Base pair
bpV(phen).	Bisperoxovanadium (bpV) compound
bpV(pic)	Bisperoxovanadium (bpV) compound
BRCA	Gene provides instructions for making a protein that acts as a tumor suppressor
BSA	Bovine serum albumin
BstEII	Eco911
Caco-TC7	Colon carcinoma
Cas9	CRISPR Associated 9
CAT	Chloramphenicol Acetyl Transferase
CBP	Cystein-based phosphatases
CCV	Clathrin-coated vesicles
CDC42	Cell division control protein 42 homolog
CDK	Cyclin-cyclin dependent kinase
cDNA	Complementary DNA - double-stranded DNA synthesized from a single stranded RNA
CETSA	Cellular Thermal Shift Assay
CHK2	The serine/threonine kinase
CK2	Ser/Thr kinase
CMV	Cytomegalovirus
COBALT	Constraint-based Multiple Protein Alignment Tool
COX-2	Cyclooxygenase-2
CRISPR	Clustered Regularly at Interspaced Short Palindromic Repeats

crRNA	CRISPR RNA
CS	Cowden Syndrome
CSPD	Stationary phase-induced, stress response protein in the CspA family of Escherichia coli
CxATP	N ⁶ -cyclohexyl-ATP
D1	Cyclin
DARTS	Drug Affinity Responsive Target Stability
DCM	Dichloromethane
DDR	DNA damage response
DIG	Digoxigenin
DLD1	Human colorectal carcinoma
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
Dox	Doxycycline
DSB	DNA double strand break
DSBR	DNA Double strand break repair
E	Endosomes
EDTA	Ethylenediaminetetraacetic acid
eIF-4E	Eukaryotic initiation factor
EMC	Enzyme mismatch cleavage
ER	Endoplasmic reticulum
EtOAc	Ethyl acetate
ETS-2	Transcriptional regulator
FAK	Focal adhesion kinase
FAS	Type-II transmembrane protein that belongs to the tumor necrosis factor family
FDG	Fluorescein-β-D-galactopyranoside
FDG-C12	5-dodecanoylamino fluorescein di-b-D-galactopyranoside
FOXO	Forkhead box protein O
FRT	Flippase recognition target
FRT-Flp	Site-directed recombination technology
G1/S	Cell cycle pathway
gBlocks	Computer program
GC	Golgi complex
gDNA	Genomic DNA
GFP	Green fluorescent protein
Gly (G)	Glycine G
GOI	Gene of interest
gRNA	Guide RNA
GSK3β	Glycogen synthase kinase-3β
GST	Glutathione-S-transferase
H-pic	Picolinic acid
H1047R	Expression of the p110 mutant
HAUSP	Herpesvirus-associated ubiquitin-specific protease
HCC	Hepatocellular Carcinoma

HCT116	Human colon cancer cells
HDR	Homology-directed recombination
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HepG2	Hepatoma
HIF-1 α	Hypoxia-inducible transcription factor-1 α
HL60	Promyelocytic leukemia
HPLC	High Performance Liquid Chromatography
HRP	Horse Reddish Peroxidase
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IGF	Insulin-like growth factors
I κ B	NF- κ B inhibitor
IKK	I κ B kinase
IR	Ionising radiation
ITR	Inverted Terminal Repeats
KI	Knock In
KO	Knock Out
LacZ	Gene that encodes β -galactosidase
LC	Liquid Chromatography
LE	Late endosomes
LNCaP	Androgen-sensitive human prostate adenocarcinoma cells
Ly	Lysosomes
MAGI-1,2,3	Members of the membrane-associated guanylate kinase family
MAGI2	Membrane-associated guanylate kinase inverted 2
MAPK	Mitogen-activated protein kinase
MAST205	Ser/Thr kinase
MBP	Myelin basic protein
MDM2	Murine double minute 2
MEF	Mouse embryonic fibroblasts
MEK1	Meiotic chromosome-axis-associated kinase
MFI	Median fluorescence intensity
Mi	Mitochondria
miRNA	Micro RNA
MMAC1	Phosphatase and tensin homologue mutated in multiple advanced cancers
MRC-5	Human normal diploid lung fibroblasts, immortalised with SV40
mRNA	Messenger ribonucleic acid
MTM1	Myotubularin
mTOR	Mammalian target of a bacterial kinase rapamycin
mTORC1	Mammalian target of rapamycin complex 1
MUG	4-methylumbelliferyl-b-D - galactopyranoside
N	Nucleus
NADH	Reduced Nicotinamide Adenine Dinucleotide
NaOAc	Sodium acetate
NBT	Nitro-blue tetrazolium chloride
ND	Not determined
NF- κ B	Nuclear factor κ -light-chain-enhancer of activated B cells

NGG	PAM Cas9 binding motif
NHEJ	Non-homologous end joining
NHL	Non-Hodgkin's lymphoma
NLK	Nemo Like Kinase
NP40	Nonyl phenoxypolyethoxyethanol
OH-pic	3-hydroxypicolinic acid
OMFP	3-O-methylfluorescein phosphate
OVA	Ovalbumin
P-Akt(Ser473)	Polyclonal Antibody for studying Akt1
P-Akt(Thr308)	Monoclonal Antibody for studying Akt1
p21 ^{CIP1}	CDK inhibitors
p27 ^{KIP1}	CDK inhibitors
PAGE	PolyAcrylamide Gel Electrophoresis
PAM	Protospacer-Adjacent Motif
PARP	Poly(ADP-ribose) polymerase
pBabePuro	Retroviral expression vector derived from the Moloney murine leukemia virus.
pcDNA3	Mammalian expression vector with the CMV promoter and a neomycin-resistance marker
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
PDK1	Phosphoinositide-dependent kinase 1
PDZ domain	Common structural domain of 80-90 amino-acids found in the signaling proteins of bacteria, yeast, plants, viruses and animals
pEGFP	Vector for fusing EGFP to the N-terminus of a partner protein
PEST	Two amino-acid sequences rich in proline (P), glutamic acid (E), serine (S) and threonine (T)
pFRT/lacZeo	Vector for establishing Flp-In Cell Lines that contain a single FRT site
pGEM-T	Plasmid
PH	Pleckstrin homology
PI3K	Phosphatidylinositol-3 kinase
PI4K	Phosphatidylinositol-4 kinase
PI5K	phosphoinositide-5 kinase
PICS	PTEN induced cellular senescence
PINK1	mitochondrial serine/threonine-protein kinase
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PIP4K	Phosphatidylinositol 5-phosphate 4-kinase
PIP5K	Phosphatidylinositol 4-Phosphate-5 kinase
PKB	Ser/Thr protein kinase Akt
PLK4	Polo Like Kinase 4
PM	Plasma membrane
PMS	Phenazine Methyl Sulfate
PMSF	Phenylmethylsulfonyl fluoride
PNK	T4 Polynucleotide Kinase

PNT1A	Human diploid post pubertal normal adult prostatic epithelial cells, immortalised with SV40
pOG44	Flp-Recombinase Expression Vector
polyA	Polyadenylation
pOTB7	cDNA cloning vector
pRb	Retinoblastoma protein
PtdIns	Phosphatidylinositol
PtdIns(3,4,5)P ₃	Phosphatidylinositol (3,4,5)-trisphosphate
PTEN	Phosphatase and tensin homologue
PTP	Protein tyrosine phosphatases
PTPMT1	Protein Tyrosine Phosphatase, Mitochondrial 1
RAC1	Ras-related C3 botulinum toxin substrate 1
Rheb	Ras homologue enriched in brain
RNAi	RNA interference
RNAse A	Ribonuclease A
rpS6	40S ribosomal protein S6
RTK	Receptor tyrosine kinases
SA-β-Gal	Senescence β-Galactosidase
SAHF	Senescence-associated heterochromatin foci
SB	Southern blotting
Ser (S)	Serine
SGK	Serum and glucocorticoid-inducible kinase
SH2	Src-homology 2
siRNA	Small interfering RNA
STE	Sodium Chloride-Tris-EDTA
SV	Synaptic vesicles
SV40	Simian Virus 40
T7E1	T7 Endonuclease I
TAE LB	Tris-acetate loading buffer
TALEN	Transcription Activator-like Effector
TEP1	TGFβ-regulated and epithelial cell-enriched phosphatase
TGN	Trans-Golgi membranes
THF	Tetrahydrofuran
Thr (T)	Threonine
TNF	Tumor necrosis factor
tracrRNA	Trans-encoded crRNA
TRIS	Organic compound with the formula (HOCH ₂) ₃ CNH ₂
TSC-2	Tuberous sclerosis 2
TTK	Dual specificity protein kinase
Tyr (Y)	Tyrosine
U87-MG	Human primary glioblastoma cell line
V	Vanadium
WB	Western Blotting
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
ZFN	Zinc-finger nucleases
γ-H2AX	Phosphorylated histone H2AX

1. Introduction

1.1. Cancer and its Causes - an Overview

Cancer has become one of the main healthcare problems for humanity in the 20th and 21st centuries. In 2012, 14 million new cancer cases and 8.2 million deaths worldwide were registered (around 13% of all cases of death) (Ramalakshmi, Muthuchelian 2011, Ferlay et al. 2016, World Health Organization 2015). Fortunately, over the last century, significant progress has been achieved in treating cancer, but only in specific classes. Approximately 200 different classes of cancer are currently known and the causes of each class of cancer can vary from individual to individual (Bamford et al. 2004). Cancer has been defined as the mutation-driven, uncontrolled growth and division of cells and results from the accumulation of genetic alterations (mutations) over time (Temin 1988).

Not all DNA damage leads to cancer even though cancer is triggered by damage to DNA. Errors in the DNA sequence are common events but most are either harmless as they affect non-essential parts of the genome or are accurately repaired by highly efficient DNA damage systems that recognise abnormalities in DNA sequences and subsequently block further cell division until this DNA damage is fully repaired (Ramalakshmi, Muthuchelian 2011). However, if irreversible mutation happens in genes responsible for the DNA repair, cancer often results. Indeed, it has been shown that the malfunction of DNA repair systems underlies many cancers (Peltomaki 2003, Loeb, Loeb & Anderson 2003).

A lack of DNA repair mechanisms then leads to the accumulation of un-repaired mutations in various genes, including those controlling cell proliferation and growth (i.e. tumour suppressor genes and oncogenes) (Loeb, Loeb & Anderson 2003). In the normal non-mutated condition, oncogenes encode proteins, the majority of which are activated only when a cell has to grow or enter mitosis. Whereas, if an oncogene mutates, it can encode a protein in a constantly active conformation that sequentially can provoke a cell to enter a continuous proliferative state (Vogelstein, Kinzler 2004).

On the other hand, if a single cell has errors in DNA sequences after division, proteins encoded by tumour suppressor genes can activate a temporary cell cycle arrest (quiescence) for cells to repair these errors or trigger an irreversible cell cycle arrest (senescence). In cases where there is too much DNA damage, these proteins self-destruct by activating the pro-apoptotic pathway for the mutated cells. Thus, inactivating mutations of tumour suppressor genes take away these blocks in cell proliferation (Miyake et al. 2011).

The biggest challenge of cancer biology is to study mutations in tumour suppressor genes (even though many cancers are caused by mutations in oncogenes), because they are more difficult to treat.

1.2. Overview

Phosphatase and tensin homologue (PTEN) is a key tumour suppressor that can dephosphorylate 3-phosphoinositides and phosphoproteins (Myers et al. 1997). The lipid targets of PTEN play key roles in basic intracellular processes such as cell survival, growth, proliferation, motility and membrane trafficking. PTEN was also revealed to be a negative regulator in these processes and, therefore, its malfunction contributes to cancer (Myers et al. 1998, Maehama, Dixon 1998). It is also known that PTEN function is suppressed in many cancers (Steck et al. 1997) and in fact, *PTEN* is one of the most frequently disrupted and mutated tumour suppressor genes causing cancer (Yin, Shen 2008, Liu, Bankaitis 2010). For these reasons, PTEN has been the focus of cancer research for almost two decades.

This introduction gives an overview of a family of inositol phospholipids and their downstream pathways, before focusing on PTEN regulation, structure and activity. *PTEN*'s role as a tumour suppressor gene in various pathways is discussed followed by the introduction of approaches to therapy centred on PTEN: pro-senescence treatment and lethal sensitivity.

1.3. Inositol Phospholipids

Inositol phospholipids have been intensively studied for the past fifty years. Cockcroft *et al.* and Vanhaesebroeck *et al.* have written an excellent overview of the molecular biology of these lipids (Cockcroft 2000, Vanhaesebroeck et al. 2001), and this thesis provides a concise review of the relevant material.

1.3.1. Overview of the Inositol Phospholipids family

Inositol phospholipids were first identified by Mabel and Lowell Hokin in 1955. The authors detected the turnover of a minor membrane lipid as a result of acetylcholine stimulation in pancreatic tissue. This provided a basis for this lipid in membrane signalling. The lipid was later assigned to the phosphatidylinositol (PtdIns) class (Cockcroft 2000, Hokin, Hokin 1955, Zhang, Majerus 1998, Di Paolo, De Camilli 2006).

PtdIns is the primary building block for inositol phospholipids (Vanhaesebroeck et al. 2001). It is composed of diacylglycerol and D-*myo*-inositol-1-phosphate, linked via a phosphate group (Figure 1.1).

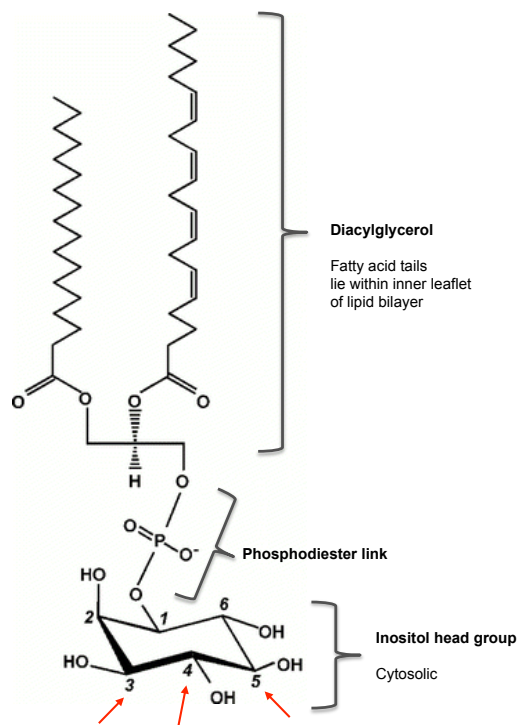


Figure 1.1. Phosphatidylinositol chemical structure. The inositol ring may be combinatorially phosphorylated at positions 3, 4 and 5 (highlighted with red arrows), which results in seven possible products referred to as phosphoinositides.

Three out of five hydroxyl groups on the inositol ring may be reversibly phosphorylated in cells (positions 3, 4 and 5 in all combinations, Figure 1.1). The phosphorylated products are referred to as phosphoinositides. There are seven phosphoinositide species in mammalian cells: phosphatidylinositol 3-phosphate (PtdIns(3)P), PtdIns(4)P, PtdIns(5)P, phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P₂), PtdIns(4,5)P₂, PtdIns(3,4)P₂, and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P₃) (Liu & Bankaitis 2010).

PtdIns(4)P is by far the most abundant of the singly phosphorylated species (90-96%), with PtdIns(3)P and PtdIns(5)P each comprising only 2-5% each (Rameh, Cantley 1999, Cockcroft 2000, Vanhaesebroeck et al. 2001, De Matteis, Godi 2004, Di Paolo, De Camilli 2006, Liu, Bankaitis 2010). PtdIns(4,5)P₂ accounts for over 99% of the total doubly phosphorylated PtdIns, whereas PtdIns(3,4)P₂ and PtdIns(3,5)P₂ each account for nearly 0.2%. Finally, PtdIns(3,4,5)P₃ is present at similar levels to PtdIns(3,4)P₂ and PtdIns(3,5)P₂ (Vanhaesebroeck et al. 2001). However, levels of 3-phosphorinositides can vary significantly and indeed this variation is essential for cell signalling. These molecules function as important mediators or second messengers in converting an extracellular signal (that is a 'first messenger' in this case) into a specific cellular response. They achieve this by altering the conformation and activity of specific intracellular signalling molecules. Thus, their levels will depend on the cell environment and serve to regulate cell behaviour (Vanhaesebroeck et al. 2001, Rommel, Vanhaesebroeck & Vogt 2010).

All phosphoinositides display compartmentalised distribution in cellular membranes. After being synthesised in the endoplasmic reticulum (ER), PtdIns moves to other membranes via transfer proteins or by vesicular transport (Di Paolo, De Camilli 2006) and the various phospho-species accumulate in distinct membrane systems. For example, in mammalian cells PtdIns(3)P is concentrated on endocytic membranes whilst PtdIns(4)P is restricted to trans-Golgi (TGN) membranes and PtdIns(4,5)P₂ is primarily found on the plasma membrane. PtdIns(3,5)P₂ is associated with multivesicular bodies (MVBs), late endosomes and lysosomes. Finally, PtdIns(3,4,5)P₃ has a high abundance on the inner area of the plasma membrane as well as on intracellular organelle membranes and in the nuclear matrix (Liu, Bankaitis 2010). This distribution is illustrated in Figure 1.2.

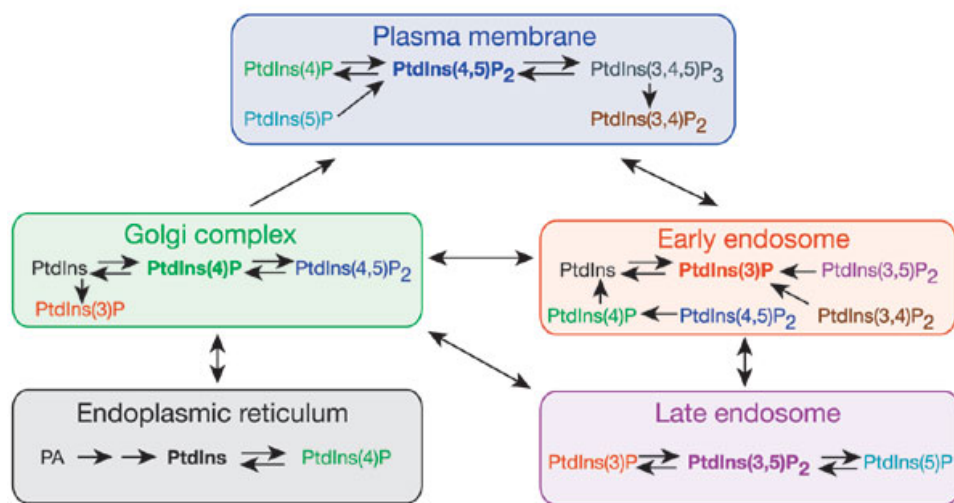


Figure 1.2. Specific subcellular distribution and turnover of various phosphoinositides in mammalian cells. PA, phosphatidic acid. (Figure modified from ref. (Di Paolo, De Camilli 2006)).

This compartmentalisation is key for lipid specificity in cell signalling (De Matteis, Godi 2004). The compartmentalisation of phosphoinositides as well as their highly dynamic turnover is only possible due to their phosphorylation or dephosphorylation by phosphoinositide kinases and phosphatases, respectively. These enzymes are located at highly specific organelles (De Matteis, Godi 2004). Phosphoinositide kinases and phosphatases are classified on the basis of the hydroxyl groups they target on the inositol ring. Kinases PI3K; PI4K and PIP4K; PI5K and PIP5K phosphorylate 3-OH, 4-OH, 5-OH positions of the inositol head group, respectively. In a similar manner, 3-, 4-, 5-phosphatases dephosphorylate phosphoinositides at the 3-OH, 4-OH, 5-OH positions of the inositol ring. The localisation of phosphoinositide kinases and phosphatases at different organelles and their activity in phosphoinositide turnover are presented in Figure 1.3.

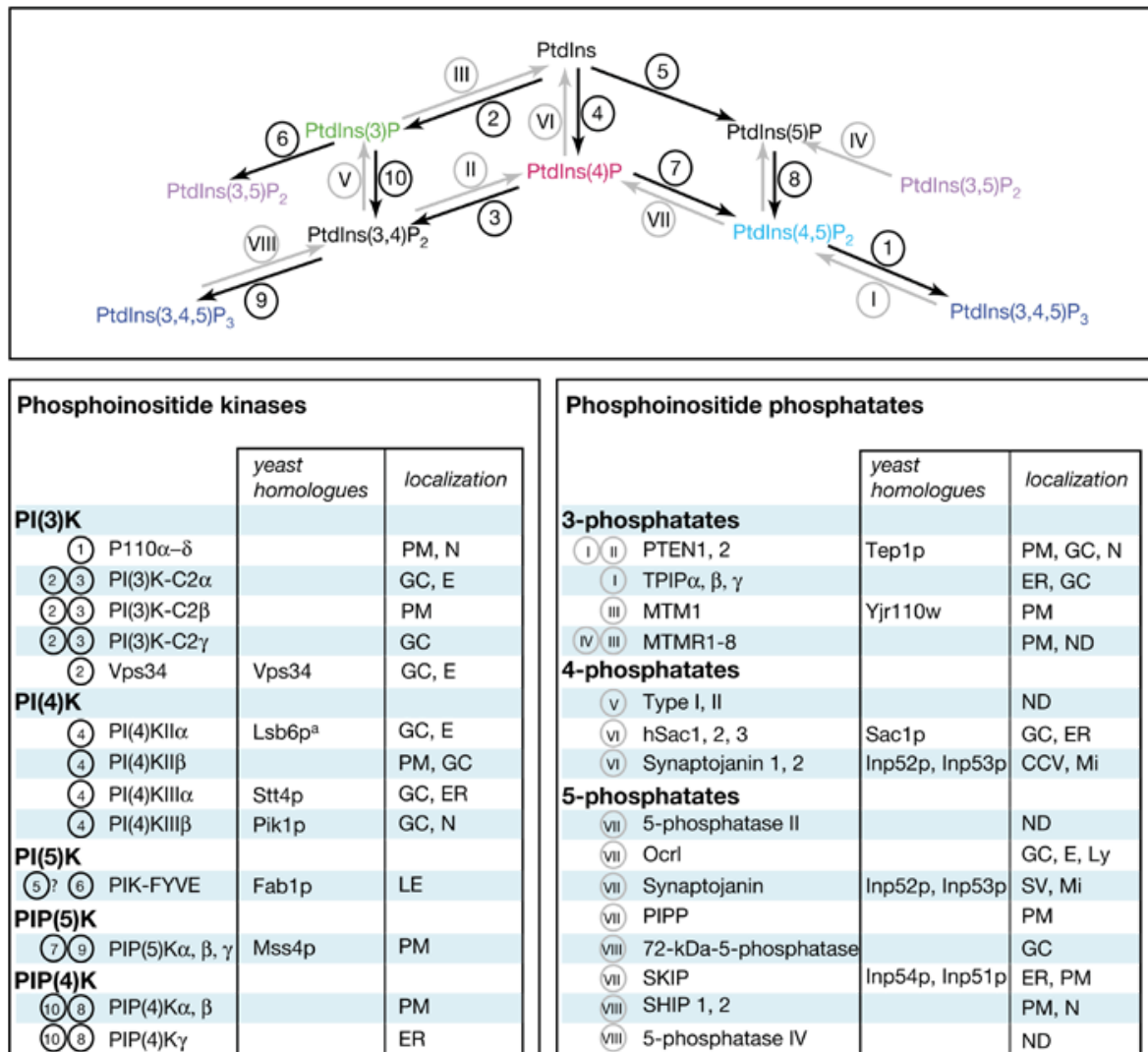


Figure 1.3. Turnover of phosphoinositides in yeast and eukaryotes. Phosphoinositides synthesis (black arrows) and hydrolises (grey arrows) are regulated by phosphoinositide kinases and phosphatases respectively. Their intracellular localisation is given in the table. Abbreviations used: ER, endoplasmic reticulum; GC, Golgi complex; PM, plasma membrane; E, endosomes; LE, late endosomes; Ly, lysosomes; N, Nucleus; SV, synaptic vesicles; CCV, clathrin-coated vesicles; Mi, mitochondria; ND, not determined. (Figure taken from ref. (De Matteis, Godi 2004)).

The fact that phosphoinositides and their regulators are broadly distributed in the cell indicates their central role in cell signalling and transport. Thus, we will next describe the major intracellular functions of various phosphoinositides.

1.3.2. Role of Phosphoinositides in Intracellular Processes

1.3.2.1. Role in Cell Transport and Vesicles Regulation

For both intracellular traffic and communication with their environment, cells often employ vesicles to carry nutrients, proteins, enzymes and cell waste materials (collectively referred to as cargo). The formation and reorganisation of vesicles are essential for all types of

intracellular traffic, and various phosphoinositides are involved at certain stages of these processes (Cremona et al. 1999, Simonsen et al. 2001). For instance, transporting cargo between cell compartments and phagocytosis are controlled by PtdIns(4,5)P₂ (Rameh, Cantley 1999, Martin 2001)); while vesicle fusion with other membranes and exocytosis are regulated by PtdIns(3)P and PtdIns(4)P (Rameh, Cantley 1999).

1.3.2.2. Role in Cell Survival

In both vertebrates and invertebrates, cell growth and survival are primarily regulated by the PI3K – Akt signalling pathway. Therefore, we will discuss the PI3K – Akt – dependent cell survival and cell growth mechanisms.

Ser/Thr protein kinase Akt (otherwise known as protein kinase B or PKB), activated by PI3K, negatively regulates the apoptotic pathway and enhances cell survival. Activated Akt phosphorylates various target proteins, including cytosolic pro-apoptotic protein BAD (BCL-2-associated death promoter) (Vivanco, Sawyers 2002). This is one of the principal mechanisms through which Akt protects cells from death. Being unphosphorylated, BAD is bound to the apoptosis-inhibitory protein BCL-X_L which keeps it in an inactive state and promotes cell death by apoptosis. Once phosphorylated by active Akt, BAD preferably binds to a second protein (14-3-3 protein) and as a result, releases BCL-X_L. This in turn blocks apoptosis and enhances cell survival (Cockcroft 2000, Vivanco, Sawyers 2002, Steelman et al. 2011). This pathway is illustrated by Figure 1.4.

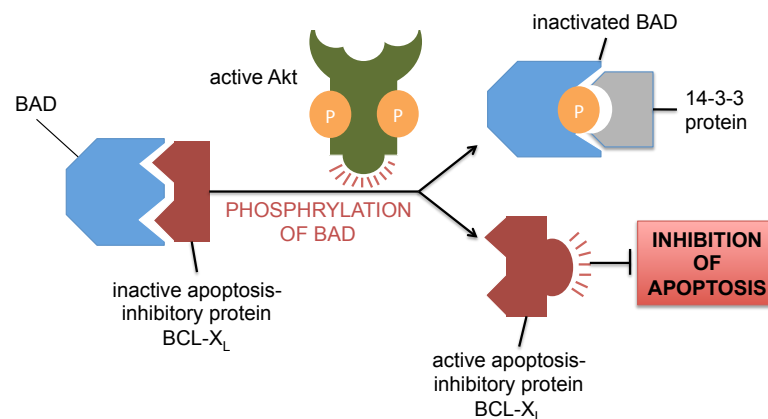


Figure 1.4. Akt – BAD – dependent cell survival pathway.

Through other anti-apoptotic mechanisms, activated Akt phosphorylates and thus inhibits pro-death protein FOXO3 (a member of the forkhead family of transcription factors) (Steeleman et al. 2011) and a pro-apoptotic protease, caspase-9 (Vivanco, Sawyers 2002). In a similar manner, Akt phosphorylates and inhibits FOXO1 and consequently suppresses production of FOXO1 downstream targets. These targets include pro-apoptotic proteins FAS ligand and BIM

(Vivanco, Sawyers 2002). Akt can also directly phosphorylate pre-existing BIM and thereby inactivate it (Steelman et al. 2011).

Akt also indirectly affects the activity of two essential regulators of cell survival: the pro-apoptotic tumour suppressor p53 and the transcriptional regulator NF- κ B. In the former case, Akt phosphorylates and inhibits p53 binding protein MDM2, which promotes p53 degradation. NF- κ B prevents apoptosis and Akt positively regulates it through phosphorylation of I κ B kinase, IKK, which in turn promotes degradation of the NF- κ B inhibitor, I κ B (Vivanco, Sawyers 2002, Steelman et al. 2011).

In order to activate all these components of the cell-survival machinery, Akt must itself be activated. This requires both phosphorylation at threonine (Thr-308) and serine (Ser-473) residues and correct localisation at the plasma membrane. Both of these processes are PI3K – dependent (Rameh, Cantley 1999, Vivanco, Sawyers 2002).

PI3K is composed of two subunits: the p85 regulatory subunit and the p110 catalytic subunit. Such extracellular molecules as insulin-like growth factors (IGFs) send survival signals to a cell through binding to certain cell-surface receptors (receptor tyrosine kinases or RTKs). These in turn autophosphorylate on tyrosine. These phosphotyrosine residues bind the Src-homology 2 (SH2) domain on the p85 subunit, consequently bringing PI3K to the plasma membrane, i.e. bringing it to the site of its substrate PtdIns(4,5)P₂. As a result, the p110 subunit phosphorylates PtdIns(4,5)P₂ yielding PtdIns(3,4,5)P₃ (Vivanco, Sawyers 2002, Steelman et al. 2011). PtdIns(3,4,5)P₃, in turn, functions as a key second messenger in complex activation pathway of Akt. PtdIns(3,4,5)P₃ also functions as a docking site at the plasma membrane for Akt and another Ser/Thr protein kinase, PDK1 (phosphoinositide–dependent kinase 1 or PKB kinase). Akt and PDK1 bind to PtdIns(3,4,5)P₃ through their binding PH domains specific only to this phosphoinositide. The binding of Akt to PtdIns(3,4,5)P₃ in the plasma membrane causes a conformational changes exposing Thr-308 and Ser-473 for subsequent phosphorylation (Rameh, Cantley 1999). These changes to the localisation and conformation of Akt brings (i) Thr-308 on its surface into contact with neighbouring PDK1, and (ii) Ser-473 – into contact with a third Ser/Thr protein kinase mTOR in complex 2. mTOR (first identified as a mammalian target of a bacterial metabolite rapamycin) exists as two multiprotein complexes: sensitive to rapamycin mTOR complex 1 (mTORC1) is sensitive to rapamycin whilst mTOR complex 2 (mTORC2) is insensitive. mTORC1 contains the protein Raptor and is activated by fully phosphorylated Akt. It plays a significant role in cell growth regulation and will be discussed in the next section. mTORC2 contains the protein Rictor, and helps to activate Akt by phosphorylating it at Ser-473 (Steelman et al. 2011).

After phosphorylation by mTORC2 and PDK1, Akt dissociates from the membrane and subsequently activates its downstream targets, including those discussed above (Vivanco, Sawyers 2002, Manning, Cantley 2007). Akt activation is illustrated on Figure 1.5.

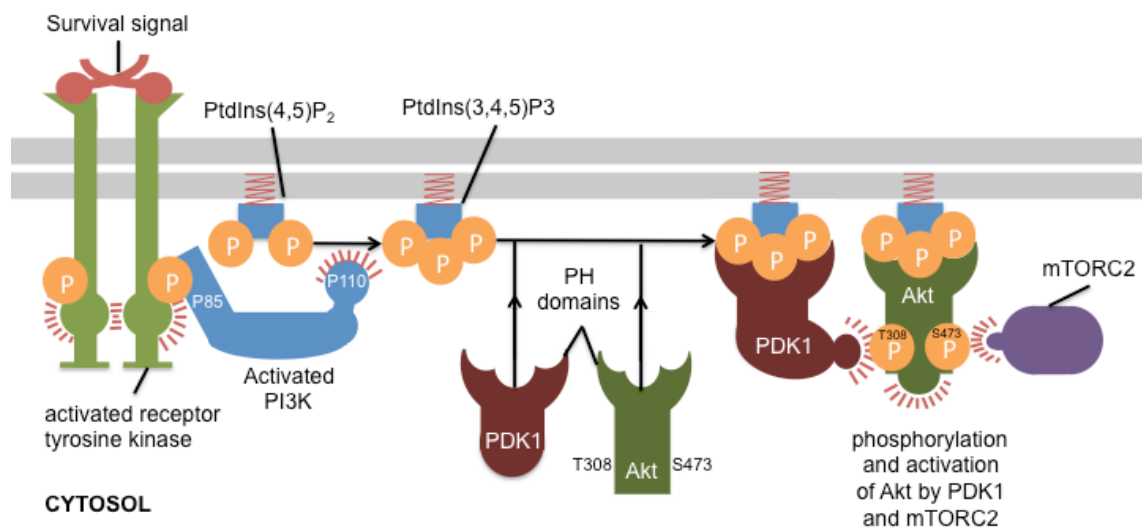


Figure 1.5. PI3K – dependent Akt activation by PDK1 and mTORC2.

It should be mentioned that there are several cell survival pathways known which are PI3K – dependent – Akt – independent, however they are less fully described than the PI3K - Akt pathway. In one such pathway, PI3K regulates the activity of the serum and glucocorticoid-inducible kinase (SGK). This kinase shows strong homology to Akt and has similar functional effects on cell survival (Vivanco, Sawyers 2002, Engelman 2009).

1.3.2.3. Role in Cell Growth

As discussed above, cell growth is regulated by mTORC1 by stimulating protein synthesis, nutrient uptake, ribosome production and inhibiting protein degradation. mTORC1 activation by Akt is indirect: first Akt phosphorylates and inactivates tuberous sclerosis 2 (TSC-2 or hamartin). Being active, TSC-2 in complex with TSC-1 (also known as tuberin) inhibits the small G-protein Rheb. In contrast, when TSC-2 is inactivated through Akt phosphorylation, Rheb activates mTORC1 (Figure 1.6). Eventually, active mTORC1 phosphorylates components of the protein synthesis machinery that influences downstream cell growth signalling pathways (primarily, translation) (Engelman 2009, Steelman et al. 2011, Chappell et al. 2011). For example, active mTORC1 activates a protein kinase referred to as p70S6K, which phosphorylates the ribosomal protein S6. This protein regulates the ribosomal translation of mRNAs that primarily encode ribosomal components and also indirectly activates translation such factors as eukaryotic initiation factor eIF-4E by inactivating its inhibitor, 4EBP-1 (eIF-4E binding protein 1). In this manner, PI3K – Akt – dependent activation of mTORC1 increases

ribosome production, which is vital for elevated protein synthesis during cell growth (Steelman et al. 2011).

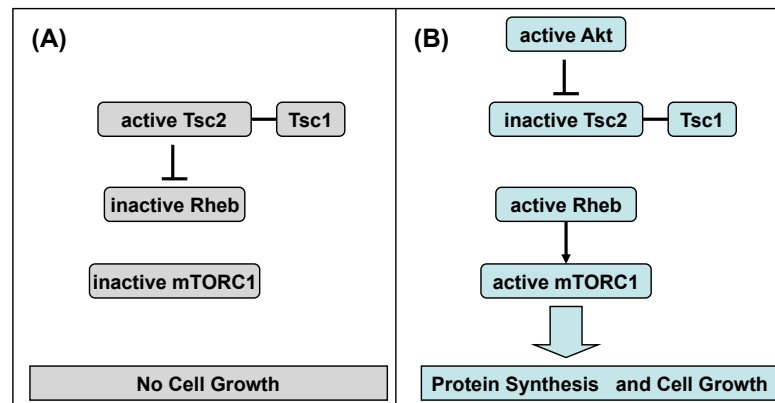


Figure 1.6. Akt – dependent activation of mTORC1. **(A)** In the absence of Akt inhibition, Tsc2 inactivates Rheb leaving mTORC1 inactive. **(B)** When activated, Akt phosphorylates and inhibits Tsc2 triggering mTORC1 activation.

As well as this, mTORC1 is also responsible for energy accumulation in growing cells. It increases glycolysis by stimulating glucose uptake (Alberts et al. 2008). For instance, mTOR regulates the translation of the hypoxia-inducible transcription factor-1 α (HIF-1 α), which itself controls the glycolytic pathway by influencing expression of glucose-sensing molecules such as glucose transporters. HIF-1 α also upregulates expression of various growth factors, thus inducing cell growth (Edinger, Thompson 2002, Buller et al. 2008).

As critical regulators of cell growth and survival, PI3K and Akt play key roles in cancer development. As discussed above, the PI3K – Akt signalling pathway is activated in response to growth factors, and therefore promotes cell growth. However, where mutations occur, Akt can be activated even without extracellular signals from growth factors, and this can result in cancer.

For example, expression of oncogenic forms of PI3K (isolated from chicken retrovirus ASV16, which causes hemangiosarcomas) or the constitutively active PI3K (isolated from lymphoid transformed cells) increases PtdIns(3,4,5)P₃ levels leading to hyperactivation of Akt and its downstream pathways. This promotes cell survival and cell growth even in the absence of extracellular signals (Rameh, Cantley 1999). Further supporting evidence includes the fact that the p110 catalytic subunit of PI3K was found to be mutated in 30% of the most common cancer types, including breast, colon, prostate and endometrial cancers. Besides this, it has been shown that expression of the p110 activating mutant (H1047R) in mouse lungs resulted in adenocarcinomas (Steelman et al. 2011). Studies of Akt mutants provided similar results:

dominant negative Akt blocks cell survival (Dudek et al. 1997) whereas a constitutively active mutant prevents cell death (Li et al. 1998).

1.3.2.4. Role in Cell Proliferation

As well as regulating cell growth and cell survival, activated Akt also plays a role in cell proliferation. It does so by influencing interactions between cyclin/CDK (cyclin dependent kinase) complexes and CDK inhibitors (Vivanco, Sawyers 2002). For example, cyclin D1 is required for the G1/S cell cycle transition. Activated Akt has been shown to phosphorylate glycogen synthase kinase-3 β (GSK3 β) and thus inactivate it. Non-phosphorylated and active GSK3 β binds to cyclin D1, phosphorylating and targeting it for ubiquitin-mediated degradation. When phosphorylated by Akt, GSK3 β is inactive and hence cyclin D1 levels increase within the cell.

Akt can also downregulate CDK inhibitors such as p21^{CIP1} (also known as p21^{WAF1}) and p27^{KIP1} (Vivanco, Sawyers 2002). It has also been shown that when activated by Akt, mTOR ensures that a cell divides only when nutrients and energy levels are at required levels (Steelman et al. 2011). Further, several studies have found that suppressing PI3K or Akt activity provokes cell cycle arrest (Vanhaesebroeck et al. 2001, Vivanco, Sawyers 2002).

Taken together, these findings confirm that the PI3K – Akt pathway plays an important role in promoting proliferation.

1.3.2.5. Role in Cell Motility

PI3K also regulates other intracellular pathways that lead to cytoskeletal reorganisation, membrane polarisation and ruffling. These processes are vital for cell migration and chemotaxis (cell motility along a chemical gradient) (Vanhaesebroeck et al. 2001).

As an example, PI3K controls activation of the small GTP-binding proteins RAC1 and CDC42. When they are activated, these proteins play a crucial role in promoting cytoskeletal rearrangements and thereby allow cells to move (Rameh, Cantley 1999, Cockcroft 2000). The mechanism for RAC1 and CDC42 activation is PtdIns(3,4,5)P₃ – dependent and Akt – independent (Vivanco, Sawyers 2002). PtdIns(3,4,5)P₃ binds to the PH domain of the nucleotide exchange factor Vav and subsequently changes its conformation. Active Vav binds to and activates RAC1 and CDC42 (Rameh, Cantley 1999).

1.3.3. Summary

Some of the main PI3K – Akt dependent pathways discussed above are illustrated by Figure 1.7.

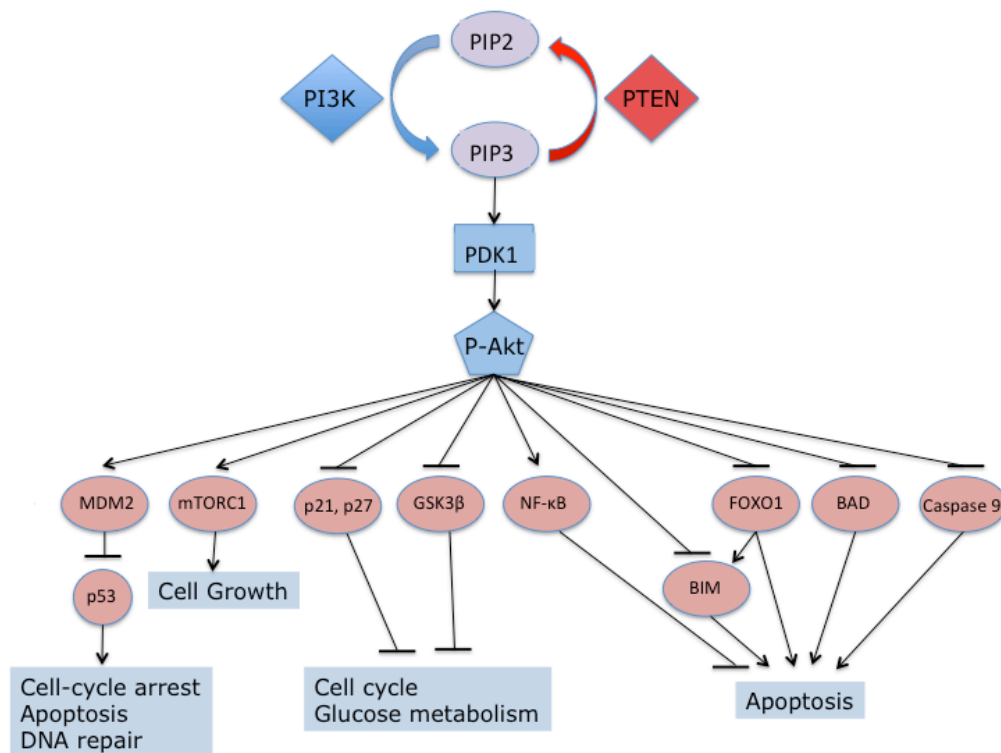


Figure 1.7. PI3K - Akt role in cell signalling.

In this section, we have discussed a number of intracellular pathways regulated by a class of membrane lipids, phosphoinositides. Since these pathways play essential roles in controlling cell behaviour, any malfunction in the regulation of these processes can result in severe disorders, e.g., cancer. In the following section, we will examine how phosphoinositide phosphatase PTEN plays a significant role in the regulation of the above pathways.

1.4. PTEN

1.4.1. PTEN Structure and Activity

The *PTEN* gene (phosphatase and tensin homologue) is also referred to as *MMAC1* (mutated in multiple advanced cancers) or *TEP1* (TGFβ-regulated and epithelial cell-enriched phosphatase). The gene was initially identified as a candidate tumour suppressor gene on human chromosome 10q23 (Steck et al. 1997, Myers et al. 1998). It was suggested that the *PTEN* gene encoded a phosphatase belonging to the tyrosine phosphatase family as well as to the cysteine-based phosphatase family. This was due to its having a similar homology to members of these families. As evidence, it was demonstrated that PTEN dephosphorylates multiple protein substrates, including focal adhesion kinase (FAK), platelet-derived growth

factor receptor (PDGFR), Shc exchange protein and transcriptional regulators ETS-2 and Sp1 on serine, threonine and tyrosine residues (Myers et al. 1998, Steelman et al. 2011).

Further research, however, confirmed that PTEN also demonstrates phosphoinositide phosphatase activity (also referred to as lipid phosphatase activity). This appeared to be far more significant than any protein phosphatase activity (Myers et al. 1998, Maehama, Dixon 1998, Cockcroft 2000). As a phosphoinositide phosphatase PTEN targets 3-phosphoinositides, and $\text{PtdIns}(3,4,5)\text{P}_3$ in particular, as its primary substrate *in vivo* (Yin, Shen 2008, Liu, Bankaitis 2010, Steelman et al. 2011).

PTEN is composed of four structural domains: the lipid and protein phosphatase domain (in the N-terminal region); the C2 domain; the PEST domain (two amino-acid sequences rich in proline (P), glutamic acid (E), serine (S) and threonine (T)) and the PDZ domain. The latter three make up the C-terminal region (Figure 1.8) (Simpson, Parsons 2001, Wang, Jiang 2008, Steelman et al. 2011).

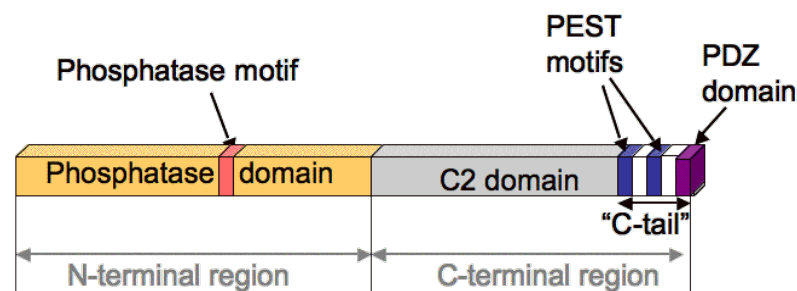


Figure 1.8. Domain organisation of PTEN. PTEN consists of 403 amino-acid residues. The N-terminal region contains the phosphatase domain (residues 15 – 186) with the phosphatase motif (residues 124-130). This precedes the C-terminal region with the C2 domain (residues 186 – 351) and the tail region (known as “C-tail”) (residues 352-403). The “C-tail” region contains two PEST motifs (residues 350-375 and 379-386) and the PDZ domain (residues 401-403).

Although tumour-suppressor activity has been primarily attributed to the N-terminal region, with its phosphatase domain, over 40% of PTEN tumourigenic mutations occur in the C-terminal region (Waite, Eng 2002). This indicates that both regions have important roles in determining PTEN activity and that each domain regulates specific cellular processes.

The phosphatase domain, with its phosphatase motif (i.e., the active site sharing homology with all protein tyrosine phosphatases), is responsible for PTEN catalytic activity. Lack of endogenous PTEN function can be found in cells expressing phosphatase mutant PTEN. Together with C2 domain the phosphatase domain is also responsible for the PTEN tumour-suppressor function. Evidence for this has been found in several mutation studies *in vitro* and cancer studies *in vivo* (Lee et al. 1999, Simpson, Parsons 2001, Steelman et al. 2011). For instance, the C124S PTEN mutant, lacking both lipid and protein phosphatase activity, fails to suppress growth in $\text{PTEN}^{-/-}$ cancer cells (Li, Sun 1998). The G129E PTEN mutant, that lacks

only lipid phosphatase activity, has been identified in patients with Cowden's syndrome (also known as multiple hamartoma syndrome). Studies have shown that this mutant failed to inhibit cell proliferation (Vivanco, Sawyers 2002). These examples suggest that the phosphatase domain is essential for PTEN tumour-suppressor activity, and that various sites within this domain regulate protein and lipid phosphatase activity.

The C2 domain is associated with PTEN lipid binding and membrane localisation. For example, mutations in the C2 domain result in a lower PTEN affinity for membranes *in vitro* (Simpson, Parsons 2001). There is also evidence that the C2 domain influences the growth- and proliferation- suppressor activity of PTEN. As a result, glioma cell lines expressing PTEN with mutations in this domain proliferate at a more rapid rate and establish anchorage-independent growth. Such substratum-independent growth is typical of transformed cells and is repressed by wild type PTEN (Georgescu et al. 1999).

The "C-tail" region, containing both the PEST and PDZ domains, has been initially suggested to be dispensable for PTEN tumour-suppressor activity. However, the "C-tail" region does have an influence on both the stability and enzymatic activity of PTEN and therefore influences tumour-suppressor activity. PEST motifs are often found in proteins with short intracellular half-lives (Simpson, Parsons 2001). Phosphorylation of the PTEN PEST motif by the Ser/Thr kinase, CK2, at Ser370, Ser380, Thr383 and Ser385 (also referred to as CK2 phosphorylation sites) can modulate PTEN turnover. PTEN mutants that cannot be phosphorylated by CK2 have decreased stability and elevated proteasome-mediated degradation compared with wild type PTEN. As well as this, CK2 inhibition results in a decrease of steady-state PTEN levels. In conclusion, phosphorylation of the PEST domain regulates PTEN half-life and PTEN protein levels in the cell (Simpson, Parsons 2001).

The PDZ domain influences protein-protein interactions through binding to consensus motifs in the C-terminal regions of partner proteins, which are essential for PTEN activity. For example, PTEN binds to the members of the membrane-associated guanylate kinase family, MAGI2 (membrane-associated guanylate kinase inverted 2) and MAGI3, through its PDZ domain. This interaction was found to favour PTEN's inhibition of Akt activity (Simpson, Parsons 2001).

In this manner all four domains are involved in the function and/or regulation of PTEN as a phosphatase, and mutations or alterations in any of the domains can result in malfunction of its tumour-suppressor activity. Further in this introduction we will discuss various intracellular pathways that can be influenced by mutated PTEN.

1.4.2. PTEN's Regulation Role in Intracellular Pathways and Cancer

As we have discussed, PI3K – mediated pathways regulate cell survival, growth, proliferation and motility in response to extracellular signals. Because PTEN is a PI3K antagonist all downstream processes that are activated by PI3K are negatively regulated by PTEN. In this

way PTEN functions as a natural tumour suppressor acting to repress cell growth, proliferation and invasion. In consequence, the loss of PTEN activity correlates with cancer development.

1.4.2.1. PTEN Influence on PI3K – Akt – dependent Pathways

PTEN inhibits Akt activation through the dephosphorylation of PtdIns(3,4,5)P₃. This inhibition results in a downregulation of all signalling processes, which are themselves Akt – dependent. Decreased PTEN activity, by contrast, which can be due to mutations or alterations in the *PTEN* gene, results in higher Akt levels. Consequently, Akt is often overexpressed/hyperactivated in cancer cells, which leads to uncontrolled cell growth, survival or proliferation. We have previously discussed the major PI3K – Akt – dependent signalling pathways. We will provide examples of pathways influenced by loss of PTEN tumour-suppressor activity and discuss their correlation with cancer.

In certain primary endometrial tumours PTEN is mutated and Akt upregulated. It has been suggested that the Akt – NF-κB – COX-2 pathway may be affected in this cancer type. As discussed previously, Akt indirectly activates NF-κB (see Section 1.3.2.2). NF-κB normally blocks apoptosis through regulation of cyclooxygenase-2 (COX-2). NF-κB and COX-2 levels are indeed been elevated in endometrial cancer cells with mutated PTEN (St-Germain et al. 2004). These results indicate that in endometrial tumours where PTEN is mutated Akt is hyperactivated which indirectly activates COX-2 through NF-κB activation. At the end of this chain, COX-2 produces elevated levels of prostaglandin E₂, a known tumour promoter contributing to an aggressive malignant phenotype through suppression of apoptosis, promotion of angiogenesis, invasiveness, inflammation and immunosuppression (Sobolewski et al. 2010). COX-2 overexpression has also been identified in a range of other cancer types including bladder, pancreas, breast, stomach, prostate, colon carcinomas, cervix, head and neck. This suggests that PTEN deregulation affects the Akt – NF-κB – COX-2 pathway across multiple human cancers (St-Germain et al. 2004).

Other PTEN – regulated and Akt – dependent pathways detected in cancer cells are mTOR – p70S6K and mTOR – 4EBP-1. As we discussed, upregulation of these pathways promotes ribosome production, which is significant for cell growth. In patients with acute myeloid leukaemia studies have found elevated levels of Akt and mTOR, together with downstream substrates p70S6K and 4EBP-1 (Chow, Minden & Hedley 2006). It is interesting to note that *PTEN*-deficient cells and *PTEN*^{+/-} mice are very sensitive to mTOR inhibitors and that p70S6K activity is lowered by these inhibitors (Podsypanina et al. 1999, Neshat et al. 2001). The p70S6K gene has also been elevated in approximately 9% of primary breast cancer cases and amplified levels of its mRNA transcripts have been found in approximately 41% of the tumours (Steelman et al. 2011). This indicates that Akt – mTOR – dependent pathways are important in PTEN-altered cells and in tumours by affecting cell growth.

1.4.2.2. PTEN Influence on PI3K – Akt – independent Pathways

Although PI3K – Akt pathways are known to be the primary direction for PTEN tumour-suppressor activity, there are multiple other pathways regulated by PTEN. These pathways also play an important role in cancer development. For example, it has been found that PTEN inhibits cell migration and spreading through the dephosphorylation of focal adhesion kinase (FAK). In this way it negatively regulates cell surface interactions with the extracellular matrix (Tamura et al. 1998, Gu et al. 1999). This mechanism allows PTEN to function as a tumour suppressor by preventing transformed cells from migrating and thus preventing tumour invasion.

Supporting this hypothesis, it has been demonstrated that levels of CDC42 and RAC1 were elevated in cells where the *PTEN* gene was deleted. These cells also exhibited increased levels of motility. As discussed previously, CDC42 and RAC1 are essential for cytoskeleton reorganisation and cell motility. In this manner, PTEN operates as a negative regulator of the pathway and loss of PTEN can result in metastasis (Liliental et al. 2000).

It has also been demonstrated that PTEN inhibits activation of the mitogen-activated protein kinase (MAPK) pathway (Gu, Tamura & Yamada 1998, Gu et al. 1999). The MAPK pathway is involved in regulating critical cell behaviour processes, in particular cell motility. This pathway is stimulated through activation of growth factor receptors and cell surface integrins during cell interaction with the extracellular matrix. The phosphatase domain of PTEN has been found to be essential for suppressing both these processes through inactivation of proteins upstream of MAP kinase such as Shc kinase, Ras and IRS-1 (Yin, Shen 2008).

By inhibiting the MAP kinase signalling pathway, PTEN regulates focal contact formation, interaction with extracellular matrix and cell spreading (Yin, Shen 2008, Simpson, Parsons 2001). PTEN's ability to dephosphorylate and inactivate FAK, as well as to phosphorylate and activate Cdc42 and Rac1, indicates that it has an important negative role in regulating dynamic cell surface interactions, cell migration and invasion (Yin, Shen 2008).

1.4.3. PTEN Mutations and Alterations in Various Cancer Types

As we have discussed, *PTEN* is a key tumour suppressor gene, which is frequently mutated in different types of cancer. Below are several examples.

Loss of heterozygosity at the *PTEN* locus in mice results in neoplasia in prostate, endometrium, intestine and mammary gland epithelia (Podsypanina et al. 1999). Loss of *PTEN* heterozygosity has also been detected in approximately 30% of patients with breast cancer (Singh, Ittmann & Krolewski 1998) and approximately 30% of those with Hepatocellular Carcinoma (HCC) (Fujiwara et al. 2000).

PTEN-deficient prostate tissue developed prostate carcinoma (Wang et al. 2003). In a similar fashion, mice with PTEN-deficient hepatocytes became hyperproliferative and developed liver adenomas in 100% of cases and 66% of these developed HCC (Suzuki et al. 2008). Similarly, mice with PTEN-deficient hematopoietic stem cells developed myeloproliferative disease. This resulted in leukemic transformation (Uddin et al. 2004).

Mutations in PTEN have been found in 40-80% of patients with endometrial cancer (Ana-MariaBamberger, Milde-Langosch 2001) and in 80% of patients with Cowden's syndrome. This syndrome is characterised by several different types of cancer conditions (Steelman et al. 2011). PTEN mutations also have been detected in acute myeloid leukaemia (AML) (Sakai et al. 1998) and non-Hodgkin's lymphoma (NHL) (Butler et al. 1999).

As well as mutations, PTEN activity can also be suppressed by several other alterations in gene expression. For example, low or absent PTEN expression and inactivation through phosphorylation have been identified in AML (Steelman et al. 2011). Low PTEN levels were also detected in approximately 26% of patients with primary breast cancers (Tsutsui et al. 2005). In general, low PTEN expression is due to methylation of the PTEN promoter (García et al. 2004). However, certain miRNAs can also regulate PTEN expression. For example, mi-214 can bind to PTEN and in this manner suppress PTEN mRNA translation (Ana-MariaBamberger, Milde-Langosch 2001). Further examples of tumours resulting from PTEN mutations or alterations, along with the proportion of occurrences that result from PTEN inactivation, are listed in Table 1.

Table 1. Reported frequency of PTEN inactivation in a range of human cancers. (Data adapted from ref. (Chalhoub, Baker 2009)).

Cancer Type	PTEN inactivation-caused cases as % of all cancer cases
Endometrial	38
Central nervous system	20
Skin	17
Prostate	14
Colon	9
Urinary track	9
Lung	8
Ovary	8
Breast	6
Haematopoietic & Lymphoid	6
Thyroid	5
Stomach	5
Liver	5
Upper aerodigestive track	4
Esophagus	1
Pancreas	1

1.4.4. Summary

Mutation and alteration in the *PTEN* gene is frequent across human cancers. The gene encodes protein and lipid phosphatase PTEN, which serves to downregulate a number of intracellular pathways responsible for cell growth, survival, proliferation and migration. Inactivation of the PTEN tumour-suppressor function through mutation, deletion or transcriptional silencing results in a wide spectrum of human cancers. Therefore, drugs that serve to reactivate PTEN could be very valuable in cancer therapy. Unfortunately however, it is extremely difficult to reactivate these genes. A paradoxical discovery has been made recently, that PTEN inhibitors have the potential to be valuable in cancer therapy. We will examine this phenomenon in more detail in the following subchapter.

1.5. PTEN Inhibition

Earlier in this report, we discussed how PTEN provides countermeasures against the action of PI3K, which lead to termination of certain downstream signalling pathways. Consequently, PTEN inhibitors suppressing the effects of PTEN have been employed by a number of researchers studying these pathways. In 2004, a series of vanadium complexes were developed with the aim to better understand signalling pathways downstream of PTEN (Schmid et al. 2004). These studies indicated that PTEN inhibition could mimic human disease. In particular, they demonstrated that treatment of diabetes type 2, brain injury and asthma could benefit from activation of PTEN-regulated downstream pathways (Mak *et al.* 2010, (Mendes-Pereira et al. 2009, Alimonti et al. 2010). It was also suggested that activation of PTEN could have potential in cancer research.

In this subchapter we will discuss vanadium-based PTEN inhibitors and examine their influence on intracellular processes. On the basis of this, we will provide some conjectures on their therapeutic potential.

1.5.1. Vanadium Complexes acting as PTEN Inhibitors

It is well recognised that vanadium complexes are isosteres of phosphoesters and phosphates, and as a result act to inhibit a broad range of phosphatases (Gordon 1991, Huyer et al. 1997, Cuncic et al. 1999, Bhattacharyya, Tracey 2001, Peters et al. 2003). Studies have shown that bisperoxovanadium (bpV) molecules (V^{+5}) inhibit the action of protein tyrosine phosphatases (Posner et al. 1994, Cuncic et al. 1999, Schmid et al. 2004). Given that the active site of PTEN shares homology with protein tyrosine phosphatases (PTPs), certain authors (Schmid et al. 2004) suggested that bpV compounds should also inhibit PTEN. This conclusion was on the basis of studying bpVs with polar N,O ligands [dipotassium bisperoxo(5-hydroxypyridine-2-

carboxyl)oxovanadate (bpV(HOpic)) and bpV(pic)] and with the neutral N,N ligands [potassium bisperoxo(bipyridine)oxovanadate (bpV(bipy)) and bpV(phen)], as illustrated by Figure 1.9.

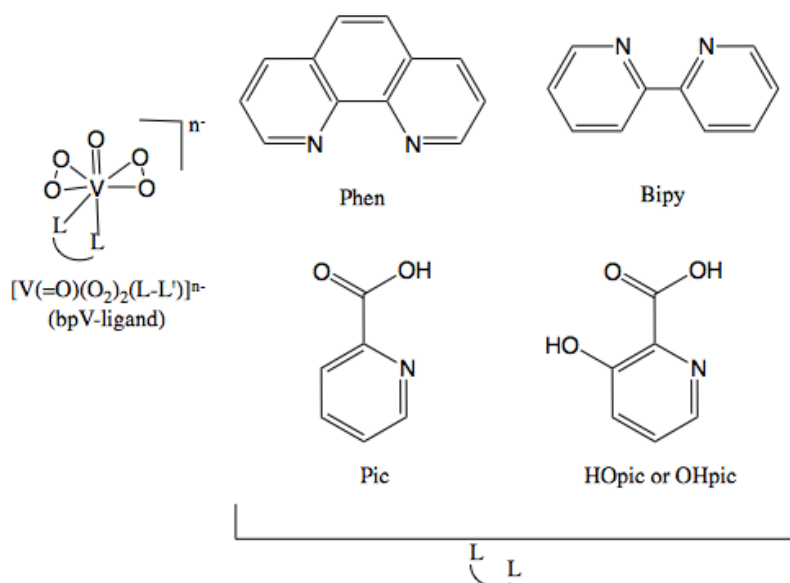


Figure 1.9. Bisperoxovanadium compounds. Representation of four bisperoxovanadium compounds studied in (Schmid et al. 2004).

All of these metal complexes have been shown to inhibit PTEN specifically out of a small panel of other PTPs and at low nanomolar doses (Schmid et al. 2004). bpV compounds worked several orders of magnitude better as an inhibitor towards PTEN than other PTPs. Indeed, they were shown to target other PTPs only at micromolar concentrations (Schmid et al. 2004). An increase in Akt phosphorylation in a PTEN-dependent manner was observed as the result of such inhibition for all four bpVs. Such effects demonstrate the preference and potency of bpVs with regards to PTEN. It was also observed that low nanomolar concentrations of the compounds do not result in any significant changes to cell viability and morphology in comparison to unstimulated cells. These results indicate that bpV compounds preferentially inhibit PTEN over PTPs *in vitro* and *in vivo* (Schmid et al. 2004).

A few years later it was demonstrated that the class of inhibitors described in (Schmid et al. 2004) also target most cysteine-based phosphatases (CBPs), with comparable affinity (Rosivatz et al. 2006). The lack of specificity motivated the authors to design a more PTEN-specific vanadium-based inhibitor. This was attempted on the basis of the difference between active sites of PTEN compared to other members of CBPs. In comparison with other CBPs and PTPs, it was observed that PTEN has a substantially wider active site cleft, and that this enables PTEN to bind with PtdIns(3,4,5)P₃ substrate. This observation was employed in the design of vanadate scaffolds that bound to a range of organic ligands, some of which demonstrated potent PTEN inhibitory activity. The authors succeeded in synthesising a range

of vanadium(IV) and vanadium(V) complexes with picolinic ligands. These are described by general formulas $V(=O)(L-L')_2$ (abbreviated as VO-ligand) and $K_n[V(=O)(O_2)_2(L-L')]^n$ (abbreviated as bpV-ligand) (Figure 1.10).

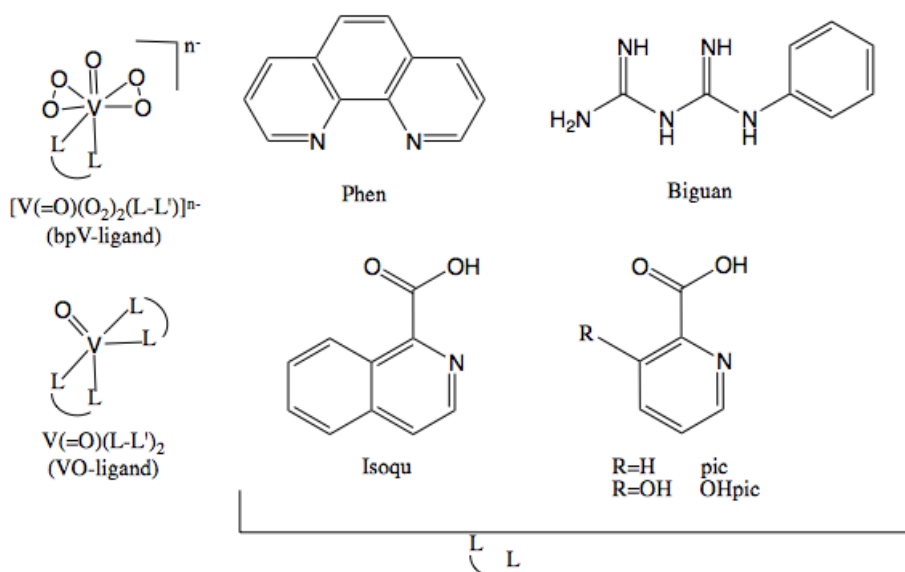


Figure 1.10. Vanadium-based compounds. Representation of the vanadium-based compounds studied in (Rosivatz et al. 2006). Ligands *phen* and *isoqu* were coordinated with bpVs only.

In an experiment reported in (Rosivatz et al. 2006), the authors tested eight vanadium complexes with ligands of various sizes with regards to their ability to inhibit the PtdIns(3,4,5)P₃-phosphatase activity of PTEN. It was concluded that the 3-hydroxypicolinate vanadium(IV) complex VO-OHpic ($[V(=O)(H_2O)(OHpic)_2]$) was the most effective PTEN inhibitor. Further, it was also concluded that nanomolar concentrations of VO-OHpic inhibit PTEN exclusively. This occurs with high specificity and potency *in vitro*. This conclusion was made by comparing the inhibitory potency of VO-OHpic with the enzyme activities of four other recombinant CBPs. *In vivo*, VO-OHpic increases PtdIns(3,4,5)P₃ levels and the phosphorylation of Akt, and it permits translocation of this kinase to the plasma membrane. Interestingly, the nanomolar VO-OHpic inhibition *in vivo* is independent of cell cycle. The cells appeared to be healthy and there were no obvious morphological changes compared with untreated cells. Combined with the results summarised above, nanomolar concentrations of VO-OHpic are likely to be selectively targeted at mammalian PTEN both *in vitro* and *in vivo*. In the following subchapters, we will discuss the structure of VO-OHpic and its inhibitory activity.

1.5.2. VO-OHpic as a PTEN Inhibitor

An understanding of VO-OHpic effect on the enzymological activity of PTEN is necessary if we are to draw conclusions regarding the effectiveness of inhibition as well as on potential areas for further research.

VO-OHpic was synthesised and isolated by Yano *et al.* (Yano *et al.* 2002) from an aqueous solution of vanadyl sulphate ($\text{VOSO}_4 \cdot x\text{H}_2\text{O}$) and 3-hydroxypicolinic acid in a molar ratio 1:2 (Figure 1.11).

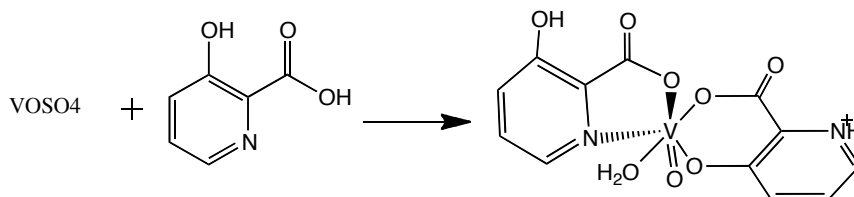


Figure 1.11. Representation of the synthesis of VO-OHpic. First reported by Yano *et al.* (Yano *et al.* 2002).

VO-OHpic is a water-soluble vanadyl complex (V^{+4}) that has a distorted octahedral geometry. The vanadium centre is coordinated to two 3-hydroxypicolinic (OH-pic) ligands, an oxo-ligand and a water molecule (Yano *et al.* 2002, Nakai *et al.* 2004, Rosivatz *et al.* 2006).

The X-ray structure of VO-OHpic was developed by Yano *et al.* and Nakai *et al.* (Yano *et al.* 2002, Nakai *et al.* 2004). These authors showed that in solid state VO-OHpic contains one N,O-coordinated and one O,O-coordinated OHpic ligand. A key finding was that although this particular geometric system was an excellent fit for the catalytic pocket of PTEN, it was too large to fit into the active site pockets of CBPs or PTPs, since these are far narrower (Rosivatz *et al.* 2006).

A key paper on the determination of the activity of VO-OHpic was published by Mak *et al.* (Mak, Vilar & Woscholski 2010), in which the authors studied inhibition of PTEN by VO-OHpic using the natural substrate $\text{PtdIns}(3,4,5)\text{P}_3$ as well as of the artificial substrate 3-O-methylfluorescein phosphate (OMFP). The goal of this study was to compare the different effects on lipids *in vivo* and *in vitro*. It was observed that PTEN's enzymatic properties are in fact independent of the choice of substrate. These findings were crucial because they led to the selection of OMFP as a suitable substrate for PTEN inhibition research and drug screening, as OMFP being far easier to work with when compared to PtdIns .

The kinetic characteristics of the PTEN inhibitor VO-OHpic were also studied in detail. These studies showed that VO-OHpic shares similar kinetic characteristics on both substrates ($\text{PtdIns}(3,4,5)\text{P}_3$ and OMFP) (Mak, Vilar & Woscholski 2010). This was demonstrated by a Lineweaver-Burk plot detailing the changes of K_m and $V_{\max}$ as a function of the concentration of VO-OHpic. According to their data, K_m increases as a function of the concentration, while

$V_{\max}$ decreases. This strongly suggests that VO-OHpic is a non-competitive inhibitor whereas many other vanadium-based complexes are competitive PTPs inhibitors (Huyer et al. 1997, Peters et al. 2003). It is important to mention that non-competitive inhibition is surprising given that the inhibitor was designed to fit in the active site and hence would be expected to be a competitive inhibitor. It therefore raises questions as to how it binds and where. Another interesting finding of (Mak, Vilar & Woscholski 2010) indicated that VO-OHpic inhibition of PTEN is a fully reversible process, thus offering greater flexibility in subsequent medical studies.

1.6. The role of PTEN Inhibition in Health and Disease

1.6.1. PTEN Inhibition in Non-Cancer Therapy

In earlier sections we discussed PTEN inhibition and how this can either cause cancer or have significant therapeutic effects. Paradoxically, PTEN inhibition could even provide a therapeutic approach for certain types of cancer. We will discuss this apparent paradox in more detail, but first we will provide an overview of potential applications of PTEN inhibition in non-cancer therapy.

As mentioned earlier, several diseases can be treated by reducing PTEN activity. It has been demonstrated by (Zhao 2007) and (Mihai et al. 2011) that PTEN inhibition enhances wound repair following an injury. During the healing process, it has been shown that Young's modulus (a measure of the stiffening of the cellular surface, or 'cell stiffness') varies according to the location within the wound. In particular, it was found that cell stiffness was lower near the edge of the wound (Mihai et al. 2011). Higher cellular stiffness reduces lamellipodia ruffling (Pelham, Wang 1997), the area of focal adhesions (Riveline et al. 2001) and increases cell contractility (Ghosh et al. 2007, Park et al. 2010). This in turn reduces the speed of cell migration. Mihai *et al* (Mihai et al. 2011) examined the impact of PTEN inhibition on the wound healing process. In their studies, PTEN inhibition was achieved by bpV(phen). They demonstrated that there was a reduction of cell stiffness in PTEN-inhibited cells, which improved the cell migratory phenotype. As well as this, increased wound closure (due to PTEN inhibition) came about as a result of more active cell migration and without having a significant impact on cell proliferation. They concluded that temporary and controlled PTEN suppression in wounds is beneficial for epithelial repair. The influence of PTEN inhibition on epithelial injuries resulting in asthma and lung pathogenesis has also been reported by Lai *et al*. (Lai, Dalton & Knoell 2007). The authors concluded that PTEN inhibition enhanced the survival of cells in lung tissues through the activation of the PI3K – Akt pathway. The authors employed bpV(phen) and bpV(pic) to achieve PTEN inhibition. Their study demonstrated that cells treated

with either of these compounds increased the rate of wound closure following mechanical injury. Similar results were observed after transfection of lung epithelia with PTEN siRNA and a dominant negative PTEN. This indicates that wound closure is enhanced when PTEN deficiency is induced.

A study carried out in 2007 focused on PTEN's influence on the pathogenesis of brain injury, brain disorders and neuroprotection (Chang et al. 2007). The authors examined the potential for PTEN to be a focus for developing a novel therapeutic strategy for central nervous system repair. According to their hypothesis, PTEN inhibition could function as a regulator of neurogenesis and axonal growth, as well as exerting neuroprotective effects.

Wound healing is not the only example demonstrating the benefits of PTEN inactivation. Another area where PTEN inhibition can have a significant impact is obesity and diabetes. It has been demonstrated (Ishihara et al. 2003, Hafen 2004, Lazar, Saltiel 2006) that PTEN performs a positive role in insulin signalling and that PTEN inactivation could be employed for drugs against diabetes. (Lazar, Saltiel 2006) showed that PTEN inactivation enhances insulin sensitivity and overcomes insulin resistance, two impacts which have a bearing on diabetes.

PTEN hyperactivation and consequent PI3K deregulation have been observed in patients with type II diabetes. These effects correlate with compromised insulin-dependent signalling. The mechanism is for insulin to bind to its tyrosine kinase receptor resulting in PI3K activation through insulin receptor substrate proteins. This activation results in an increase in glucose transport, as well as glycogen and lipid synthesis. It can therefore be concluded that PI3K downregulation by PTEN influences insulin activity. Different studies illustrate this point. For example, it has been shown that the inhibition of PI3K with pharmacological inhibitors (wortmannin, LY294002) acts to block most cellular actions of insulin. As well as this, glucose uptake can be suppressed by an overexpression of dominant-negative forms of PI3K as a result of the decrease in PtdIns(3,4,5) P_3 levels. In contrast, overexpression of constitutively active forms can mimic insulin action (Martin et al. 1996). Two other studies have demonstrated that insulin-stimulated PtdIns(3,4,5) P_3 levels were elevated as a result of a microinjection of an anti-PTEN specific antibody (Nakashima et al. 2000, Thong, Dugani & Klip 2005). As well as this, PTEN knock-out was reported to reduce the development of high-fat-induced insulin resistance and subsequently the development of diabetes (Wijesekara et al. 2005). Moreover, insulin-dependent glucose uptake *in vivo* is shown to be enhanced by the PTEN knock-down with siRNA (Tang et al. 2005).

It has previously been shown that vanadium complexes are capable of mimicking various effects similar to those of insulin, which related to the inhibition of protein tyrosine phosphatases (PTPs) (Heyliger, Tahiliani & McNeill 1985, Bevan et al. 1995, Rumora et al. 2001). It was only recently found that the effects of the vanadium complexes in fact result from PTEN inhibition (Schmid et al. 2004, Mak, Vilar & Woscholski 2010). (Rosivatz et al. 2006)

showed that PTEN inhibition with VO-OHpic at nanomolar concentrations resulted in increased glucose uptake *in vivo*. This leads to insulin sensitivity being enhanced and insulin resistance overcome. Such results have the potential to be beneficial in anti-diabetes therapeutics.

In relation to diabetes therapeutics, it has been suggested that tissue-specific suppletion of VO-OHpic in nanomolar concentrations would not increase cell proliferation or cause malignant cell growth (Rosivatz et al. 2006). This is consistent with other studies which showed that PTEN loss in pancreatic β cells is not tumorigenic (Stiles et al. 2006) and that targeted PTEN downregulation in skeletal muscle protects cells against insulin resistance and cancer development (Wijesekara et al. 2005). These results suggest that a targeted tissue-specific VO-OHpic delivery has the potential to treat diabetes without causing uncontrolled cell growth.

1.6.2. PTEN Inhibition in Cancer Therapy

It has recently been shown that PTEN inhibition or targeting PTEN deficiency could be valuable in the treatment of some types of cancer. We shall now focus on these approaches targeting cancers with various *PTEN* genotypes. We will cover PTEN-induced cellular senescence (PICS) upon PTEN inhibition with VO-OHpic and PTEN – induced tumour sensitisation (lethal sensitivity) to a range of anti-cancer drugs.

1.6.2.1. PTEN-Induced Cellular Senescence (PICS)

The first description of cellular senescence was made 50 years ago by Leonard Hayflick. He detected replicative senescence in cultured cells, that was named after him, the “Hayflick limit” (Hayflick, Moorhead 1961, Hayflick 1965, Ohtani, Mann & Hara 2009, Nardella et al. 2011). Senescence is a cellular program that triggers the terminal arrest of cell proliferation and is thought to be an endogenous anticancer response. Senescence has also been associated with certain non-cancerous pathologies, including ageing, human atherosclerosis, ischemic heart disease, liver fibrosis and cirrhosis or chronic pulmonary diseases due to cigarette smoke, (Ohtani, Mann & Hara 2009). A key function of senescence is preventing cancer development. Cellular senescence specifically opposes tumour formation *in vitro* and in various tissues *in vivo*, in both humans and mice (Campisi 2005, Michaloglou et al. 2005, Collado et al. 2005, Braig et al. 2005, Chen et al. 2005, Ohtani, Mann & Hara 2009, Collado, Serrano 2010) and can be triggered by a number of cellular stresses including oxidative stress, DNA damage, hyperactivity of oncogenes and loss of tumour suppressor genes (Campisi, Di Fagagna 2007, Ohtani, Mann & Hara 2009, Alimonti et al. 2010, Nardella et al. 2011). Since senescence has been found to be associated with loss of *PTEN* by Chen *et al.* (Chen et al. 2005), their findings have led to more research in this field.

Salmena *et al.* (2008) showed that 50% of total PTEN is insufficient for tumour suppression in mice. This phenomenon has been termed tumour suppressor *haplo*-insufficiency. It was more recently shown (Carracedo, Alimonti & Pandolfi 2011) that 80% of total PTEN is insufficient for tumour suppression in mice. This phenomenon is termed “*quasi*-insufficiency”. In humans, PTEN function can be reduced by genetic mutations, which result in a stepwise loss of PTEN protein (Salmena, Carracedo & Pandolfi 2008, Carracedo, Alimonti & Pandolfi 2011). As soon as PTEN levels near a complete loss of expression, cells trigger a cellular senescence program via p53 signalling, limiting tumour progression (Salmena, Carracedo & Pandolfi 2008). This process is termed PTEN-loss – induced cellular senescence (PICS).

Chen *et al.* (Chen et al. 2005) have analysed the correlation between the dose of PTEN and tumour progression. This work has been carried out using mouse models of PTEN loss in prostate tissue. Unexpectedly, the authors found that complete loss of PTEN did not result in a proliferative advantage. Instead it promoted a strong senescence response, which acted against tumour progression. In agreement with this, mutated PTEN that maintains partial function has been shown to have a selective advantage over mutated PTEN which has a complete loss of function. It was however demonstrated that mice with conditional PTEN inactivation in prostate tissue do develop invasive cancer eventually. Raised p53 levels, cellular senescence and slow growth were observed at early-stage tumour development in the same mice. Chen *et al.* concluded that such progression of cancer and excessive growth of cells may result from failure of the senescence mechanism. This is due to secondary mutations (for instance in p53) that arise from genomic instability, itself caused by the loss of PTEN (Salmena, Carracedo & Pandolfi 2008). This indicates that complete inactivation of PTEN suppresses tumorigenesis in the absence of other mutations. It also highlights the importance of PTEN “*quasi*-insufficiency” in tumour progression (Trotman et al. 2003, Salmena, Carracedo & Pandolfi 2008, Carracedo, Alimonti & Pandolfi 2011). At a clinical level these findings explain why complete loss of PTEN is seldom observed in cancer cases.

As a result, scientists have proposed that PTEN-deficient cancers, including most cases of prostate cancer, may benefit from treatment with PICS. This is referred to as “pro-senescence” therapy or therapy-induced senescence (Salmena, Carracedo & Pandolfi 2008, Ewald et al. 2010). This potential application of PICS in anti-cancer therapy remained uncertain, due to the fact that replicative and oncogene-induced senescence were known to be the result of DNA replication, cell hyperproliferation, and subsequent accumulation of a DNA damage checkpoint response (DDR) (Bartkova et al. 2005, Bartkova et al. 2006, Di Micco et al. 2006, Di Micco, Fumagalli & d'Adda di Fagagna 2007, Mallette, Gaumont-Leclerc & Ferbeyre 2007). Hyperproliferation and DDR could in turn induce the accumulation of damaged cells, supporting rather than blocking cancer progression. These processes unfortunately make replicative and oncogene-induced senescence types unsuitable for cancer therapy (Alimonti et al. 2010).

Since the molecular mechanism of PICS remained unexplored it was necessary to test whether hyperproliferation and DDR was the driving force in PICS *in vivo*. If this were the case, it would prevent the use of this type of senescence in anti-cancer therapy. Although initially Di Micco *et al.* proposed that this was indeed the case (Di Micco *et al.* 2006), Alimonti *et al.* later demonstrated that in fact PICS occurs in the absence of both hyperproliferation and DDR and could therefore serve as a target for pro-senescence therapy (Alimonti *et al.* 2010). The authors used $PTEN^{-/-}$ knock-out mouse embryonic fibroblasts (MEFs) and $PTEN^{+/-}$ heterozygous MEFs treated with PTEN siRNA to downregulate PTEN activity. They found that PICS occurs soon after siRNA treatment, without preceding a long-term hyperproliferative phase or establishment of DDR. They also demonstrated that in contrast to oncogene-induced senescence, PICS was observed in the complete lack of DNA replication and cellular proliferation *in vitro* (using MEFs), and lack of DDR *in vivo* (using mice model with a tissue specific $PTEN$ -null prostatic epithelial).

Consistent with these results, PICS was also shown to be regulated by p53 through the Akt-mTORC1 signalling pathway and not through DDR activation. Finally, it was also found that PICS is associated with an increase in p53 protein levels (Alimonti *et al.* 2010).

On the basis of these findings, Carracedo *et al.* categorised PICS as a unique type of cellular senescence, separating it from both replicative and oncogene-induced cellular senescences (Carracedo, Alimonti & Pandolfi 2011). The fact that PICS can be triggered in the absence of DNA damage or hyperproliferation implies it has potential in developing a pro-senescence approach to cancer therapy.

1.6.2.2. VO-OHpic influence on PICS

Drugs that trigger PICS by targeting PTEN may activate a beneficial senescence response without causing DNA replication, hyperproliferation or damage. In particular, since “quasi-insufficient” $PTEN$ does not activate the p53 response in heterozygous cells, it should be possible to employ drugs such as VO-OHpic to inhibit the remaining PTEN allele and trigger PICS (Alimonti *et al.* 2010, Ewald *et al.* 2010, Carracedo, Alimonti & Pandolfi 2011).

Consistent with this theory, (Alimonti *et al.* 2010) have confirmed that inhibition of PTEN by VO-OHpic induces senescence and reduces proliferation in $PTEN^{+/-}$ MEFs without having any visible effect on wild type cells. It is striking that VO-OHpic did not result in further senescence in $PTEN^{-/-}$ MEFs, but activated PICS in $PTEN$ hypomorphic MEFs ($PTEN(Hypo/-)$ with 30% of the total dose of PTEN protein). As a consequence, $PTEN$ heterozygous or homozygous cells are differentially vulnerable to the action of VO-OHpic.

Alimonti *et al.* also studied a human xenograft model of prostate cancer and observed that VO-OHpic treatment resulted in a significant decrease in tumour growth *in vivo*. The authors

also found that long-term VO-OHpic treatment of human prostate xenograft tumours reduced tumour size and increased survival rates of treated mice.

Finally, it has been proposed (Alimonti *et al.* 2010) that VO-OHpic's ability to trigger PICS has the potential to be beneficial for pro-senescence therapy in those cancer types where cells have lost one allele of *PTEN*. Such cancer types include the majority of prostate cancers. This would be especially useful given that prostate cancer is the second-most frequent cancer in men and the sixth-most common cause of cancer mortality in men (Ferlay *et al.* 2010).

1.6.2.3. PTEN – Induced Tumour Sensitisation

Targeting cells with total PTEN deficiency could also be potentially therapeutically beneficial as it is a common genetic aberration in several cancers (Abbotts, Thompson & Madhusudan 2014, Benafif, Hall 2015). For instance 80% of endometroid endometrial cancer (EEC) (Mutter *et al.* 2000), 36% of glioblastoma (McEllin *et al.* 2010) and 20% of prostate cancer are characterised with loss of *PTEN* (Benafif, Hall 2015). Such targeting can be performed via PTEN - induced tumour sensitisation (also referred as lethal sensitivity or synthetic lethality).

Synthetic lethality occurs when defects in two genes cause cell death, but individually these defects do not (first described 60 years ago (Dobzhansky 1946)). Based on this property, synthetic lethality provides an opportunity for the development of cancer-specific cytotoxic agents by targeting a gene that is synthetic lethal exclusively to a specific cancer mutation. This approach allows killing only of cancer cells whilst sparing normal cells and thus is intensively studied for various types of cancers (Kaelin 2005, Brough *et al.* 2011a, Lee, Swanton 2012, Li *et al.* 2014, Smith, French & Hollingsworth 2014, Liu *et al.* 2015a, Kamal, Shaik & Malik 2015, Jackson, Chen 2016).

There are number of studies showing that PTEN deficient cancer models are more sensitive to other reagents or inhibition strategies (M Dillon, W Miller 2014). Some of these reports are listed in Table 1.2.

Table 1.2. Synthetic lethality studies in PTEN^{-/-} cancer models.

Reagent/treatment	Effect on PTEN ^{-/-} cells	References
PARP inhibitors	Cell death induction	(Mendes-Pereira <i>et al.</i> 2009)
PNKP inhibitors	Sensitise to irradiation	(Merenuik <i>et al.</i> 2013)
mTOR inhibitors	Sensitise to irradiation and cell growth arrest	(Neshat <i>et al.</i> 2001, Cao <i>et al.</i> 2006, Kim <i>et al.</i> 2013)
Methylxanthine Caffeine (PI3K inhibitor)	Sensitise to irradiation	(Sinn <i>et al.</i> 2010)
S6 kinase 1 inhibitors	Apoptosis induction	(Tandon <i>et al.</i> 2011)
APE1 inhibitors	Apoptosis induction	(Abbotts <i>et al.</i> 2014b)
TTK inhibitors	Cell death induction	(Brough <i>et al.</i> 2011)
NLK inhibitors	Cell death induction	(Mendes-Pereira <i>et al.</i> 2012)
PLK4 inhibitors	Cell death induction	(Brough <i>et al.</i> 2011, Mason <i>et al.</i> 2014)
Skp2 inhibitors	Senescence induction	(Lin <i>et al.</i> 2010, Chan <i>et al.</i> 2013)
Curcumin treatment	Cell death and cell cycle arrest induction	(Chen <i>et al.</i> 2013)
CID1340132	Apoptosis induction	(Li <i>et al.</i> 2011)
Irradiation	DNA damage-inducible cell size arrest	(Lee <i>et al.</i> 2004)

Some details on this discovery are going to be presented in this chapter, while the other synthetic lethality targets will be discussed in detail in Chapter 7. One of the first discoveries in the field of PTEN synthetic lethality was sensitivity PARP inhibitors reported by Mendes-Pereira *et al.* (Mendes-Pereira *et al.* 2009). This study showed that PTEN inactivation sensitises cancer cells to inhibitors of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP) both *in vitro* and *in vivo*. PARP inhibitors themselves are strong anti-tumour agents. It has also been shown that PARP inhibitors can be successfully used for treating cancer in patients with common breast cancer mutations in *BRCA1* or *BRCA2*. These inhibitors are currently undergoing clinical trials and the preliminary results are very promising (Bryant *et al.* 2005, Farmer *et al.* 2005, Ashworth 2008, Fong *et al.* 2009, Naipal, Van Gent 2015, Lord, Tutt & Ashworth 2015, Piha-Paul *et al.* 2015). Mendes-Pereira *et al.* suggests that these inhibitors could be used for all cancers involving PTEN mutations, and not simply those with BRCA mutations (Mendes-Pereira *et al.* 2009).

It has previously been demonstrated that the sensitivity of *BRCA* mutant cells to PARP inhibitors is due to a defect in RAD51-regulated DNA double strand break repair (DSBR). This in turn is due to homologous recombination, the exchange of nucleotide sequences between two homologous DNA molecules (Farmer *et al.* 2005, McCabe *et al.* 2006). Mendes-Pereira *et al.* determined that PTEN deficiency can also result in a homologous recombination defect in human cancer cells. These data have been supported experimentally by the fact that homozygous *PTEN*^{-/-} MEFs show genomic instability, which is due to defects in RAD51-mediated DNA double strand break repair (DSBR) (Shen *et al.* 2007). In this manner the PTEN deficiency-induced homologous recombination defect in cells make them a primary target for PARP activity. The process is known as PARP inhibitor sensitisation. It is striking that expression of the C124S PTEN mutant (a phosphatase inactive mutant) in a PTEN null cell line resulted in PARP inhibitor resistance *in vivo* mutations (Mendes-Pereira *et al.* 2009). This implies that the phosphatase activity of PTEN is not responsible for PARP inhibitor sensitisation implying that other PTEN domains/functions are responsible for this effect.

Consistent with these findings, PARP inhibitors exclusively target cells with malfunctioning PTEN without affecting normal cells (Mendes-Pereira *et al.* 2009). Therefore, these inhibitors can potentially be used to treat cancers with mutations with loss of PTEN expression and potentially some loss of function mutants as well.

Based on these reports, other synthetic lethality targets (TTK, NLK, Skp2 and curcumin) were analysed in details on the basis of cells with various PTEN status – similar to the experiments performed by (Mendes-Pereira *et al.* 2009) (See Chapter 7).

1.7. Aims and Objectives

This study aims to address the mechanisms underlying the synthetic lethality and PTEN-induced cellular senescence (PICS) approaches in cells with various *PTEN* genotypes and those expressing defined mutant forms of PTEN. Such understanding would enable greater development of PTEN targeted therapies and their validation prior to potential clinical utilisation.

Firstly, in Chapter 3, heterozygous $PTEN^{+/-}$ and homozygous $PTEN^{-/-}$ cells isogenic to PTEN wild type are generated using CRISPR/Cas9 technology. In Chapter 4, PTEN mutants that would allow analysis of the structure-function requirements for PTEN in PICS and synthetic lethality experiments are cloned and characterised. Next in Chapter 5, PTEN mutants and wild type PTEN as a control are introduced in $PTEN^{-/-}$ HCT116 cells following by detailed assessment of these lines in PICS (upon PTEN inhibition with VO-OHpic, Chapter 6) and synthetic lethality experiments (upon PARP, TTK and Skp2 inhibition and curcumin treatment, Chapter 7). Synthetic lethality upon NLK inhibition via chemical genetic approach is dissected in Chapter 8. Finally, Chapter 9 is focused on attempts to determine VO-OHpic specificity and intracellular targets in order to further characterise PTEN inhibition.

2. Materials and Methods

2.1. Biology

2.1.1. Reagents and Instruments

Plasmids

Mammalian expression vector pcDNA3 was purchased from ThermoFisher Scientific (UK); pIRES-Puro2 was from Clontech (USA); pcDNA5/FRT/TO and pFRT//acZeo plasmids (Invitrogen, UK) were kindly provided by Dr Lorna Fiedler (National Heart & Lung Institute, Imperial College London, UK); pFAST-BAC1-GST plasmid was a gift from Prof. Paul Freemont (Department of Medicine, Imperial College London); pcDNA3-FLAG was made by Dr David Mann (D. Mann *et al.*, unpublished) The following plasmids have been reported previously: pcDNA3-p21^{Cip1}, pBabePuro, pBabeHygro (Morgenstern, Land 1990). pOTB7-PTEN (clone ID 3937787) was purchased from ThermoFisher Scientific (UK). pEGFP-puro was a gift from Michael McVoy (Addgene plasmid # 45561); hCas9 (Addgene plasmid # 41815), hCas9_D10A (Addgene plasmid # 41816) and gRNA_AAVS1-T2 (Addgene # 41818) plasmids were kindly provided by George Church. pCMV(CAT)T7-SB100 was a gift from Zsuzsanna Izsvak (Addgene plasmid # 34879), pSBbi-GB was provided by Eric Kowarz (Addgene plasmid # 60520). pDONR223-NLK plasmid was constructed by William Hahn & David Root (Addgene plasmid # 23642).

Antibodies

Primary antibodies used in this study are listed in Table 2.1.

Table 2.1. The list of primary antibodies and the dilution they were used at within this project.

Antibody target	Dilution	Company	Catalogue number
p53	1:3000	Santa Cruz Biotechnology Inc (USA)	sc-126
p21 ^{CIP}	1:2000	Santa Cruz Biotechnology Inc (USA)	sc-397
p27 ^{KIP}	1:1000	Santa Cruz Biotechnology Inc (USA)	sc-538
Cdk2	1:1000	Santa Cruz Biotechnology Inc (USA)	sc-163
PCNA	1:5000	Santa Cruz Biotechnology Inc (USA)	sc-56
GFP	1:1000	Santa Cruz Biotechnology Inc (USA)	sc-9996
GST	1:1000	Santa Cruz Biotechnology Inc (USA)	sc-138
PTEN	1:1000	Santa Cruz Biotechnology Inc (USA)	sc-7974
PTEN	1:2000	Cell Signaling Technology, USA	138G6
NLK	1:1000	Santa Cruz Biotechnology Inc (USA)	sc-8210
NLK	1:5000	abcam	ab154199
Akt (total)	1:1000	Santa Cruz Biotechnology Inc (USA)	sc-81434
P-Akt(Ser473)	1:1000	NEB, UK	9271
P-Akt(Thr308)	1:1000	NEB, UK	4056
pRb (total)	1:500	BD Biosciences, UK	554136
P-pRb(Ser801/811)	1:1000	Cell Signaling Technology, USA	9308
FLAG	1:5000	Sigma-Aldrich Ltd, UK	F3165
α -tubulin	1:2000	Sigma-Aldrich Ltd, UK	00020911
CAT*		Invitrogen, UK	MA1-91137
Cyclin D1**	1:500	Cancer Research UK London	-

* Anti-CAT antiserum was kindly provided by Dr Lorna Fiedler (National Heart & Lung Institute, Imperial College London, UK)

** Anti-Cyclin D1 antibody was a kind gift from Dr Gordon Peters's laboratory, Cancer Research UK London Research Institute, London, UK

Secondary anti-species antibodies were purchased from Jackson ImmunoResearch (USA).

Other materials

pGEM-T-vector, GoTaq G2, GoTaq G2 Flexi and Phusion High-Fide polymerases were purchased from Promega (UK); *PfuUltra* II fusion HS DNA polymerase was purchased from Agilent (USA), Q5 High-Fidelity polymerase and Klenow Fragment - from New England Biolabs (NEB, UK). Lipofectamine 2000, FDG-C12, T4 DNA ligase, DH10Bac *E. coli*, Hygromycin and Zeocin solutions were purchased from Invitrogen (UK). X-Gal was purchased from Melford Laboratories Ltd (UK); Ampicillin, Tetracycline, Gentamycin, Puromycin, Neomycin (aka G418 or Geneticin), FDG, Insulin, RNase A, chloroquine, bafilomycin A1 and pyridostatin were purchased from Sigma-Aldrich Ltd (UK). All restriction enzymes were from ThermoFisher Scientific (UK) or NEB (UK). gBlocks were synthesised at IDT (Integrated DNA Technologies, USA). The oligonucleotides were synthesised by Sigma-Aldrich Ltd (UK). ECL substrate was from Merck Millipore, Germany. α -³²P-dCTP material was provided by Perkin Elmer Inc. (UK). PTPMT1 phosphatase was kindly provided by Dr Rudiger Woscholski's group. BpV(HOpic) was purchased from Santa Cruz (USA). PARP inhibitor AZD2281 and TTK inhibitor AZ3146 were purchased from Stratech Scientific Ltd. (UK), Skp2 inhibitor Skp2-25 was ordered from Chembridge Co (USA). Curcumin was kindly provided by Dr Ed Tate's laboratory.

All solvents and inorganic salts were purchased from Sigma-Aldrich Ltd (UK) and VWR (UK). All buffers and solutions were prepared in distilled water if not stated otherwise. Stock solution concentrations and solvents for the compounds used in this project are shown in Table 2.2. For each experiment stocks were diluted to the required concentration in media.

Table 2.2. Stock solution concentrations and solvents for the key reagents used within this project.

Compound	Solvent	Stock concentration
VO-OHpic	DMSO	10 mM
BpV(HOpic)	H ₂ O	10 mM
Pyridostatin	DMSO	10 mM
4-hydroxytamoxifen (4-OHT)	Methanol	12.9 mM
4-methylumbelliferyl- β -D- galactopyranoside (MUG)	DMSO	34 mM
5-dodecanoylamino fluorescein di- β -D-galactopyranoside (FDG-C12)	DMSO	20 mM
5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal)	DMF	122 mM
fluorescein- β -D-galactopyranoside (FDG)	DMSO	1.52 mM
Bafilomycin A1	DMSO	20 μ M
Chloroquine	H ₂ O	100 mM
Phenylmethylsulfonyl fluoride (PMSF)	Methanol	0.1 M
Benzamidine hydrochloride	H ₂ O	0.1 M
3-O-methylfluorescein phosphate (OMFP)*	DMSO	15 mM*
AZD2281 (PARP inhibitor)	DMSO	10 mM
AZ3146 (TTK inhibitor)	DMSO	20 mM
Curcumin	DMSO	40 mM
Skp2-25 (Skp2 inhibitor)*	DMSO	10 mM*
MBPP1	DMSO	100 mM

* these solutions required sonication

Instruments

Western blot chemiluminescence and DNA visualisation was performed on a FujiFilm LAS-3000. Bands area was quantified using ImageJ (National Institutes of Health, Bethesda, MD, USA) or ImageStudioLite (LI-COR Biosciences, Ltd., UK) software. PCR was conducted on an Eppendorf Mastercycler Gradient (Eppendorf Ltd, UK). Electroporation was performed on a Bio-Rad Gene Pulser, Optical density and fluorescence measurements from 96 well plates were recorded on a Spectra Max 340PC Spectrophotometer. UV crosslinking was performed on Stratalinker UV Crosslinker (Agilent, USA). Flow cytometry analyses were carried out on a Fortessa flow cytometer (BD Bioscience) at the Flow Cytometry Facility (Imperial College London, UK). DNA concentration was determined on a Jenway 7305 Spectrophotometer (Bibby Scientific Ltd, UK). DNA sequence determination was performed by Beckman Coulter Genomics (UK).

2.1.2. Plasmid preparation

2.1.2.1. DNA digestion and electrophoresis

DNA digestions were performed in appropriate buffer (ThermoFisher Scientific (UK) or NEB (UK)), using 0.5-4 µg of DNA and 10 units of each enzyme in a total volume of 20 µl or 50 µl. The samples were incubated at 37° C for 1 hour and mixed with 5x Tris-acetate-EDTA loading buffer (5x TAE LB: 200 mM Tris-HCl, 100 mM acetic acid, 5 mM ethylenediamine tetraacetic acid (EDTA, pH 8.0), 12.5% w/v Ficoll, 0.125 w/v bromophenol blue). If RNA-free DNA was required, Ribonuclease A (RNase A) was added to a final concentration of 200 µg/ml. Then the samples were run at 60 mA for 50 min on a 1% w/v agarose gel for DNA >800 bp (Agarose, Sigma-Aldrich Ltd, UK) or 3% w/v agarose gel for DNA <800 bp (SeaKem agarose, Lonza, UK) supplemented with 0.6 µg/ml ethidium bromide. DNA bands were visualised on a FujiFilm LAS-3000 and, if required, excised for DNA purification.

2.1.2.2. DNA ladder

30 µg of Lambda DNA (ThermoFisher Scientific (UK)) was digested with Eco91I (aka BstEII) in a total volume of 400 µl at 37° C overnight. After incubation the marker was mixed with 5x TAE LB and adjusted up to 1 ml total volume with water. This molecular weight marker was used as a DNA ladder for all DNA agarose gels unless stated otherwise (Appendix 12.1).

2.1.2.3. DNA purification (>500 bp) (gene cleaning)

DNA samples were melted in ~500µL 6 M NaI at 65° C for 5 min. A homemade silica suspension (Boyle, Lew 1995) was added to samples following by cooling on ice for 5 min. DNA bound to silica beads was centrifuged at 13,000 rpm for 1 sec and washed three times in gene cleaning wash solution (50 mM NaCl, 10 mM Tris-HCl pH 7.5, 2.5 mM EDTA, 50 % v/v ethanol). DNA was eluted from silica by resuspending in water and incubating at 65° C for 5 min followed by centrifugation at 13,000 rpm for 1 sec. The DNA supernatant was recovered and used further as required.

2.1.2.4. Short PCR products (<500 bp) purification for sequencing

PCR reaction mixtures were purified via homemade size-exclusion columns filled with G50 Sepharose resin (Amersham Pharmacia, UK). For this, columns were pre-incubated with STE buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM EDTA) by centrifugation at 1,000 rpm for 2 min at 4 ° C, then 50 µl of PCR reaction mixtures were loaded onto the columns and centrifuged again at 1,000 rpm for 2 min at 4 ° C collecting the eluates in fresh tubes. These purified samples would be free from the short oligonucleotides used as PCR primers and from old buffer components. The samples were then concentrated according to 2.1.3.5.

2.1.2.5. Short PCR products (<500 bp) concentration

50 µl of PCR reaction mixture was concentrated with 1 µl of Glycogen (ThermoFisher Scientific, UK), 2 µl of NaOAc (pH5.2) and 100 µl of ethanol at -80 ° C for 30 min, following by centrifugation at 13,000 rpm for 3 min at Room Temperature (RT). The pellet was washed with 200 µl of 70% ethanol at -20 ° C for 10 min and centrifuged again at 13,000 rpm for 1 min at RT. After drying at 37 ° C for 3 min, the pellet was resuspended in 10 µl of water by heating at 65 ° C for 5 min following by cooling to 4 ° C.

2.1.2.6. A-tailing

Purified DNA was mixed with 1x GoTaq G2 buffer, 20 mM dATP and 5 units of GoTaq G2 polymerase in a total volume of 30 µl. The mixture was incubated at 70 ° C for 30 min followed by gene cleaning.

2.1.2.7. Ligation

Purified DNA insert was mixed with 10 ng of appropriate digested vector and 2 units of T4 DNA ligase (Invitrogen) in a 1x ligase buffer (50 mM Tris-HCl pH 7.6, 10 mM MgCl₂, 1 mM ATP, 1 mM DTT, 5% w/v polyethylene glycol (PEG)-8000). The mixture was incubated at RT overnight following by gene cleaning.

For pGEM-T vector ligation, A-tailed DNA insert was mixed with 1 µl of linearised pGEM-T backbone and 3 units of T4 DNA ligase (Promega) in a 1x ligase buffer (30 mM Tris-HCl pH 7.8, 10 mM MgCl₂, 1 mM ATP, 10 mM DTT, 5% w/v PEG-8000). The mixture was incubated at 4 ° C overnight following by gene cleaning.

2.1.2.8. Annealing adaptors

50 mM NaCl was added to a 1:1 mixture of oligos (50 µM of each) and incubated in a tank with 1 L of boiling water for 5 min. The heat source was removed and the water bath and contents allowed to cool until they reached room temperature.

2.1.2.9. Phosphorylation of oligos

Purified DNA insert was mixed with 1 mM ATP, 10 units of T4 Polynucleotide Kinase (PNK, ThermoFisher Scientific, UK) in 1x ligation buffer (70 mM Tris-HCl pH 7.6, 10 mM MgCl₂, 5 mM DTT). The solution was incubated at 37 ° C for 30 min followed by 30 min incubation at 74 ° C for heat inactivation.

2.1.2.10. DNA dephosphorylation

Digested vector backbone was mixed with 1 unit of alkaline phosphatase (Roche, Germany) in 1x phosphatase buffer (50 mM Tris-HCl pH 8.5, 0.1 mM EDTA). The solution was incubated at 37 ° C for 1 hour following by DNA electrophoresis and gene cleaning.

2.1.2.11. Filling in 5' overhangs

DNA digested with restriction enzymes that generate 5'-overhangs (i.e. EcoRI: G'AATTC or XhoI: C'TCGAG) were filled with Klenow (NEB, UK). 0.5 µg of DNA was digested with appropriate restriction enzyme and mixed with Klenow fragment (1 unit) and 0.5 mM dNTP. The mixture was incubated for 30 min at 30° C and reaction was stopped by Klenow inactivation at 65° C for 30 min.

2.1.2.12. Transformation

Purified ligation products were mixed with electrocompetent *Escherichia coli* (*E. coli*) strain DH5α. Electrocompetent cells were obtained by washing exponentially growing bacteria with ice-cold water three times and concentrating with 10% glycerol; the cells were then stored at -80° C. Electroporation was performed at 25 µFD, 200 Ω, 2.2 kV. Sterile Luria Broth (LB) media (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl, pH 7.0) was then added and the bacterial solution was plated onto LB-Agar plates (1.5% w/v agar, 1.25% w/v tryptone, 0.625% w/v yeast extract, 1.25% w/v NaCl, pH 7.0) supplemented with 50 µg/ml ampicillin. The plates were incubated at 37° C overnight.

For pGEM-T plasmids 35 µl of 50 µg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) solution and 20 µl of 1M IPTG (isopropyl β-D-1-thiogalactopyranoside) solution were added onto LB-Agar plates together with bacterial solution.

2.1.2.13. Small scale plasmid preparation (mini-prep)

Individual bacterial colonies were grown in 2 ml LB supplemented with 50 µg/ml ampicillin at 37° C overnight with shaking. 1.5 ml aliquots from each sample were centrifuged at 13,000 rpm for 1 sec. The supernatant was removed and the cell pellets were resuspended in 100 µl of water, followed by sequential addition of 200 µl of Solution II (0.2 M NaOH, 1% w/v Sodium Dodecyl Sulphate (SDS)) and 150 µl of Solution III (3M potassium acetate, 11.5% v/v glacial acetic acid). After mixing, the samples were centrifuged at 13,000 rpm for 3 min and the supernatant was precipitated by addition of 2 volumes (v/v) 100% ethanol followed by centrifugation at 13,000 rpm for 3 min again. The supernatant was discarded and the DNA pellet was resuspended in water.

2.1.2.14. Large scale plasmid preparation (maxi-prep)

Transformed bacterial cultures were grown in 200 ml of LB supplemented with 50 µg/ml ampicillin at 37° C overnight with shaking. Next day the cells were centrifuged at 5,000 rpm for 5 min at 4° C and the supernatant was discarded. The pellets were resuspended in 10 ml of Solution I, followed by sequential addition of 20 ml of Solution II and 15 ml of Solution III. After mixing, samples were centrifuged at 7,500 rpm for 10 min and the supernatant was filtered. Nucleic acids were precipitated by incubation on ice for 5 min with 45 ml of iso-propanol and

the solution was centrifuged at 7,500 rpm for 10 min again. The supernatant was removed and the pellets were resuspended in 3 ml of water, followed by addition of 4 ml of -20° C 5M LiCl. After incubation on ice for 5 min, the solutions were centrifuged at 13,000 rpm for 5 min. The resulting supernatant was incubated on ice for 5 min with 14 ml of ethanol following by centrifugation at 5,000 rpm for 5 min at 4° C. The supernatant was discarded and the precipitates were resuspended in 500 µl of water supplemented with 100 µg/ml RNase A. The DNA was precipitated by incubating the solution with the precipitation solution (2.5 M NaCl, 20% w/v PEG-6000) on ice for 5 min. The solution was centrifuged at 13,000 rpm for 5 min and the supernatant was discarded. The DNA pellet was resuspended in 500 µl of water and purified from proteins via extraction with 500 µl of phenol/chloroform (1:1) solution, followed by second purification with 500 µl of chloroform. For this, the DNA solution was centrifuged at 13,000 rpm for 5 min after each extraction and the organic fraction was discarded. The aqueous fraction was then precipitated by addition of 0.1 volumes of 3M NaOAc pH 5.2 and 2.5 volumes of ethanol followed by centrifugation at 13,000 rpm for 1 min. The resulting DNA pellet was resuspended in water.

2.1.3. Genomic DNA preparation

2.1.3.1. Genomic DNA purification: short protocol

This express protocol was modified from (Strauss 2001). Near confluent HCT116 cells were harvested from a 24-well plate (~12,500 cells/well), transferred to an eppendorf tube and spun at 2000 rpm for 2 min. After removing the supernatant cells were washed with 500 µl PBS, resuspended in 100 µl of lysis buffer (0.2 mg/ml Proteinase K, 100 mM Tris-HCl pH 8.0, 200 mM NaCl, 5 mM EDTA, 0.2% SDS, 0.45% NP40, 0.45% Tween 20) and incubated for 2 h at 60-65° C. Then, RNase A (0.2 mg/ml) was added and the cellular lysate was incubated 15 min at 37° C, followed by precipitation with 10 µl of 3M NaOAc and 250 µl ethanol for 10 min at -20° C. Next, the sample was centrifuged at 13,000 rpm for 10 min at 4° C. The pellet was washed with 70% ethanol, dried at 37° C for 10 min and resuspended in 80 µl water.

2.1.3.2. Genomic DNA purification: long protocol

This protocol was modified from The Hedrick Lab protocols (The Hedrick Lab, 2016). Near confluent HCT116 cells were harvested from a 10 cm dish, transferred to a centrifuge tube and spin at 1000 rpm for 3 min. After removing the supernatant cells were washed with excess PBS, resuspended in 0.5 ml of lysis buffer (0.2 mg/ml Proteinase K, 10 mM Tris-HCl pH 7.5, 10 mM NaCl, 10 mM EDTA, 0.5% Sarcosyl) and incubated overnight at 55° C. Then, 0.5 ml of phenol:chloroform was added to the cellular lysate and the sample was mixed by gentle rocking for 1 h at RT. After spinning at 13,000 rpm for 5 min, the top aqueous layer was

transferred to a new tube and 0.5 ml of chloroform was added. After gentle rocking for 30 min at RT, the sample was centrifuged at 13,000 rpm for 5 min and the top aqueous layer was transferred to a new tube again. gDNA was precipitated with 20 µl of 5M NaCl and 1 ml ethanol, gently mixed and transferred to a new tube with 70% ethanol. Next, the sample was centrifuged at 13,000 rpm for 10 min at 4° C and the pellet was air-dried and resuspended in 300-400 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0).

2.1.3.3. Genomic DNA purification: ThermoFisher Scientific Kit

gDNA was isolated as described in the manufacturer's manual (ThermoFisher Scientific Genomic DNA Purification Kit (K0512)). Briefly, near confluent HCT116 cells were harvested from a 24-well plate, transferred to a microfuge tube and spin at 2000 rpm for 2 min. After removing the supernatant cells were resuspended in 200 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) followed by addition of 400 µl lysis solution and 10 min incubation at 65° C. Then, the sample was gently mixed with 600 µl chloroform, and spun at 13,000 rpm for 4 min. The upper aqueous layer was transferred to a new tube and mixed with 800 µl of precipitation solution (diluted from 10x with water). After centrifugation at 13,000 rpm for 2 min the supernatant was removed and the pellet was resuspended in 100 µl of NaCl solution. Then, RNase A (0.2 mg/ml) was added and the sample was incubated 10 min at 37° C, and then precipitated by 300 µl of ice-cold ethanol for 10 min at -20° C. The sample was then centrifuged at 13,000 rpm for 5 min at 4° C. The pellet was washed with 70% ethanol, dried at 37° C for 5 min and resuspended in 80 µl water.

2.1.4. Southern Blotting Protocols

2.1.4.1. DNA Gel Separation and Blotting

Genomic DNA (or plasmid) samples were digested to completion with a range of restriction enzymes (EcoRI, HindIII, SpeI, BglII, EcoRV and PstI) at 37° C overnight. In order to reduce gDNA volumes, samples were precipitated by adding 1 µl of glycogen, 1/10th volume of NaOAc, 2.5 volumes of ethanol and incubation at -80° C for 30 min. Following centrifugation at 14,000 rpm for 30 min at 4° C, DNA pellets were washed with 70% ethanol and dried at 37° C for 5 min. Then, gDNA was dissolved in 6 µl water and after addition of 1.5 µl loading dye, was resolved on a 0.8% agarose gel at 20-30 V for 3-5 hours using TAE buffer (40 mM Tris-HCl, 20 mM acetic acid, 1 mM EDTA pH 8.0) containing ethidium bromide (in order to visualise the DNA). Then the gel was subjected to: a) depurination for 7 min by shaking in depurination solution (0.125 M HCl); b) denaturation for 30 min by shaking in denaturation solution (1.5 M NaCl, 0.5 M NaOH); c) neutralisation for 30 min by shaking in neutralisation solution (1.5 M

NaCl, 0.5 M Tris-HCl 7.5); and d) equilibration in 20x SSC buffer (300 mM trisodium citrate, 3 M NaCl, pH 7.0). The gel was rinsed with water between each steps. Then, the samples were blotted by capillary transfer onto positively charged nylon membrane, Hybond-N+ (GE Healthcare Life Sciences, UK) overnight, and fixed by UV crosslinking (at 120 mJ/cm²).

2.1.4.2. Labelling DNA probe with biotin

p21^{Cip} (600 bp) probe was prepared by excising the p21^{Cip} cDNA from pcDNA3-p21^{Cip} (by BamHI and EcoRI) and purifying p21^{Cip} fragment by gel electrophoresis (on 2% agarose gel). Purified p21^{Cip} fragment and DNA ladder were labelled with biotin-11-dUTP by a random-priming method using Biotin DecaLabel DNA Labelling Kit (K065, ThermoFisher Scientific, UK) according to manufacturer instructions. Briefly, 1 µg of p21^{Cip} fragment or DNA ladder were mixed with 10 µl of Decanucleotide in 5x Reaction buffer to make 44 µl total volume. The samples were denatured, cooled down and mixed with 1 µl Klenow-exo⁻ fragment (5 u/µl, engineered to remove exonuclease activity to reduce degradation of a labelled probe during reaction) and 5 µl of Biotin Labelling mix (containing dNTPs and biotin-11-dUTP). The mixtures were incubated at 37° C overnight and the reaction was stopped by adding 2 µl 0.2 M EDTA (pH 8.0).

2.1.4.3. Membrane hybridisation with biotinylated probes and colourimetric detection

The following steps were carried out according to the manufacturer's protocol (Biotin Chromogenic kit K066, ThermoFisher Scientific, UK). The membrane was placed in a hybridisation bag and incubated for 2 h at 42° C in pre-hybridisation solution (0.1% BSA, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 6x SSC, 50% formamide, 0.5% SDS and 50 µg/ml denatured sonicated salmon sperm DNA as a blocking reagent). After that, denatured biotin-labelled DNA probe was added to the pre-hybridisation solution (50 ng/ml). Lambda DNA/HindIII (Appendix 12.1) provided within the kit was added as well as a biotin-labelled DNA ladder (3 ng/ml). The membrane was hybridised at 42° C overnight, following by two 10 min washes in 2x SSC, 0.1% SDS solution at RT, then two 10 min washes in 0.1x SSC, 0.1% SDS solution at 65° C. Next, the membrane was briefly rinsed with 1x blocking/washing buffer (from 10x Blocking/Washing stock diluted in water), incubated in blocking solution (1% of Blocking Reagent in 1x blocking/washing buffer) for 30 min at RT, following by incubation with 1:5000 Streptavidin-Alkaline Phosphatase (AP) conjugate in blocking solution for 30 min at RT. After two 15 min washes in 1x blocking/washing buffer, the membrane was equilibrated in 1x detection buffer (from 10x Detection stock diluted in water) and placed in a hybridisation bag. After 30-60 min

incubation with BCIP/NBT (colourimetric substrate for AP diluted from 50x stock in detection buffer) at 37° C, the blue precipitate was visualised and documented by camera.

2.1.4.4. Membrane hybridisation with biotinylated probes and chemiluminescence detection

This protocol was identical to the previous one except the last steps. After the last 0.1x SSC washing step, the membrane was washed in PBST buffer (10 mM Na₂HPO₄, 1.9 mM KH₂PO₄, 137 mM NaCl, 3.4 mM KCl, 0.05% Tween 20, pH 7.4) for 15 min at RT, then incubated in blocking solution (1% bovine serum albumin (BSA) in PBST) for 30 min at RT, following by incubation with 1:2000 Neutravidin-horse radish peroxidase (HRP) conjugate in blocking solution for 1 h at RT. Then membrane was washed 3 times with PBST for 10 min. Chemiluminescence was detected after adding ECL substrate (Merck Millipore, Germany) to the membrane and detected via the FujiFilm LAS-3000.

2.1.4.5. Labelling DNA probe with DIG

p21^{Cip} (600 bp) and PTEN (2250 bp) probes were prepared by excising insert DNA from the following plasmids: pcDNA3-p21^{Cip} (by digestion with BamHI and EcoRI) and pGEM-T_PTEN^{extra-long} ‡‡‡ (by digestion with BglII and EcoRI) and purifying these DNA fragments by gel electrophoresis (1-2% agarose gels). Probes were labelled with digoxigenin by the random-priming method with DIG High Prime DNA Labelling and Detection Starter Kit II (11585614910, Roche Diagnostics, UK) according to the manufacturer's recommendations. Briefly, 100-300 ng of each probe was diluted in 16 µl of water, denatured, cooled down and mixed with 4 µl of 5x DIG-High Prime solution containing random primers, dNTP, DIG-dUTP and Klenow enzyme. The samples were incubated at 37° C overnight and the reaction was stopped by adding 2 µl of 0.2 M EDTA (pH 8.0). Lambda DNA digested with Eco91I was also labelled with DIG in order to be used as a DNA ladder.

2.1.4.6. Membrane hybridisation with DIGilated probes and chemiluminescence detection

The following steps were carried out according to the manufacturer's protocol (DIG High Prime DNA Labelling and Detection Starter Kit II). The membrane was placed in a hybridisation bag and pre-hybridised in DIG Easy Hyb buffer at 42° C for 30 min. After that, denatured DIG – labelled DNA probe was added to the pre-hybridisation solution. DIG-labelled lambda DNA/Eco61I was added to hybridise to the DNA ladder. The membrane was incubated at 42° C overnight, followed by two 5 min washes in 2x SSC, 0.1% SDS solution at RT, then two 15 min

‡‡‡ pGEM-T_PTEN^{extra-long} has longer promoter region compared pGEM-T_PTEN^{long} (2250 bp instead of 1410 bp). This region had a 840 bp overlap with cDNA region that is why the new pGEM-T_PTEN^{extra-long} plasmid was synthesised.

washes in 0.5x SSC, 0.1% SDS solution at 65° C. Next, the membrane was briefly rinsed with washing buffer (0.1 M Maleic acid, 0.15 M NaCl, 0.3% Tween 20, pH 7.5), incubated in 1x blocking solution (from 10x Blocking stock diluted in 0.1 M Maleic acid, 0.15 M NaCl, pH 7.5) for 30 min at RT, followed by incubation in antibody solution (1:10,000 (75 mU/ml) Anti-Digoxigenin-Alkaline Phosphatase (anti-DIG-AP) conjugate in blocking solution) for 30 min at RT. After two 15 min washes in washing buffer, the membrane was equilibrated in detection buffer (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl) and placed in a hybridisation bag. After 15-18 h incubation with CSPD (chemiluminescent substrate for AP) at 37° C, the chemiluminescence was detected on a FujiFilm LAS-3000 (1 h exposure).

2.1.4.7. Labelling DNA probe with α -³²P-dCTP

p21^{Cip} (600 bp), PTEN (1410 bp) and EGFP (728 bp) probes were prepared by excising insert DNA from the appropriate plasmids: pcDNA3-p21^{Cip} (by digestion with BamHI and EcoRI), pGEM-T_PTEN^{long} (by digestion with BglII and EcoRI) and pEGFP-Puro (by digestion with BglII and NcoI) respectively and purifying this DNA fragments by gel electrophoresis (1-2% agarose gels). Probes and Lambda DNA/Eco91I were labelled with α -³²P-dCTP by the random-priming method using DecaLabel DNA Labeling Kit (K062, ThermoFisher Scientific, UK) according to the manufacturer's instructions. Briefly, 100-300 ng of each DNA fragment was mixed with 10 μ l of Decanucleotide in 5x Reaction buffer up to 43.5 μ l total volume (in water). Samples were denatured, cooled down and mixed with 3 μ l of dNTP (without dCTP), 1 μ l Klenow-exo⁻ fragment (5 u/ μ l) and 2.5 μ l α -³²P-dCTP (approximately 1 MBq). The mixture was incubated at 37° C for 15 min, then 4 μ l of complete dNTP mix was added and incubated for further 30 min at 37° C. The unincorporated dNTP was removed by spinning the probe through the Illustra MicroSpin G-50 columns (GE Healthcare Life Sciences, UK).

2.1.4.8. Membrane hybridisation with ³²P – labelled probes and radioactive detection

The membrane was incubated in pre-hybridisation solution (modified Church and Gilbert buffer: 0.25 M Na₂HPO₄, 7% SDS, pH 7.0 and 50 μ g/ml denatured sonicated salmon sperm DNA) at 68° C for 1 h. After that, the denatured ³²P – labelled DNA probe was added to the pre-hybridisation solution and membrane was incubated at 68° C overnight, following by 20 min washing in 2x SSC, 0.1% SDS solution, 20 min in 1x SSC, 0.1% SDS solution and 5 min in 0.1x SSC, 0.1% SDS solution (all at 68° C). Finally, the membrane was exposed to a intensifying Phosphor Screen overnight and developed on the FujiFilm FLA 5000 PhosphorImager.

2.1.5. PTEN Mutant Construction

2.1.5.1. QuikChange mutagenesis

A 20 µl PCR reaction was carried out with 15 - 75 ng template DNA, 0.2 µM 5' primer, 0.2 µM 3' primer, 2 µl 10x *PfuUltra* II Reaction Buffer, 250 µM dNTP and 0.4 µl *PfuUltra* II fusion HS DNA polymerase according to the manufacturer's manual using several thermocycling conditions and primers (Table 2.3(A), Chapter 4: Table 4.2). 10 units DpnI was added to the reaction mixture with the PCR product and incubated at 37° C for 1 h. Digestion was followed by DNA purification by adding 2 µl 3M NaOAc (pH 5.2) and 100 µl 100% ethanol and precipitated at -80° C for 30 min. The reaction mixture was centrifuged (16,000 g, 3 min) and the pellet was resuspended in 10 µl H₂O. 1 µl of the mixture was used for *E. coli DH5α* transformation. Purified DNA plasmids were tested by digestion with mutant-specific restriction enzymes.

2.1.5.2. Two-step site-directed mutagenesis

Two-step PCR reactions were carried out in 25 µl and 50 µl volume respectively using various polymerases following manufacturers protocols and various thermocycling conditions (Table 2.3(B)). A range of oligonucleotide primers specific to the desired amplification product were used in a two-step amplification: the primers 5' (specific for vector) and 3' (mutated PTEN), and the primers 5' (mutated PTEN) and 3' (specific for vector) were initially used to produce two distinct PCR products which then used as templates for the next step PCR reaction using 5' and 3' primers (specific for vector). The complete list of primers used for PTEN mutagenesis is presented in Table 4.2 (Chapter 4). PCR products were separated by gel electrophoresis on a 1.5% agarose gel (60V, 50 min), visualised under UV light, excised, purified by silica matrix (Boyle, Lew 1995) and 0.5 µl of each product was used as a template for the second step PCR. Full-length constructs generated in a second round PCR were ligated into pcDNA3 or pGEM-T vector and then transformed into competent *E. coli DH5α* using electroporation. Purified DNA constructs were digested by the appropriate restriction enzymes (HindIII & XhoI) for further cloning into destination vectors. All constructs obtained were confirmed by restriction digestion and sequencing.

Table 2.3. Thermocycling conditions. **(A)** QuikChange mutagenesis conditions to generate the C124S_PTEN mutant using *PfuUltra* II Fusion HS DNA polymerase. **(B)** Two-step PCR mutagenesis conditions to generate all mutants using *PfuUltra* II Fusion HS, GoTaq G2 and GoTaq G2 Flexi, Q5 High-Fidelity and Phusion High-Fidelity polymerases. Annealing temperatures specific for each primer set were chosen according to manufacturer recommendations.

(A) Quikchange mutagenesis

Polymerase	PfuUltra II Fusion HS			
	Temperature, °C	Duration	No of cycles	PCR composition (20 µl)
Initial denaturation	95	2 min	1	15-75 ng DNA, 1x Reaction Buffer, 40 ng each primer, 200 µM dNTP, 0.4 µl polymerase
Denaturation	95	20 s	30	
Annealing	68	20 s		
Extension	72	1 min 40 s		
Final extension	72	3 min	1	
Hold	4	Indefinite	-	

(B) 2-step mutagenesis

Polymerase	<i>PfuUltra II Fusion HS</i>			
	Temperature, °C	Duration	No of cycles	PCR composition (20 µl)
Initial denaturation	95	2 min	1	15 ng DNA, 1x Reaction Buffer, 40 ng each primer, 250 µM each dNTP, 0.4 µl polymerase
Denaturation	95	20 s	30	
Annealing	60-70*	20 s		
Extension	72	15/22 s**		
Final extension	72	3 min	1	
Hold	4	Indefinite	-	

* 60-70°C annealing temperature for 1st round PCR reactions; 70°C annealing temperature - for 2nd

** 15 sec extension time for first round PCR; 22 sec extension time for second round PCR

Polymerase	GoTaq G2 and GoTaq G2 Flexi			
	Temperature, °C	Duration	No of cycles	PCR composition (20 µl)
Initial denaturation	95	2 min	1	15 ng DNA, 1x Reaction Buffer, 80 ng each primer, 200 µM each dNTP, 0-5 % DMSO, 0-1 M betaine 0.1 µl polymerase
Denaturation	95	30 s	25	
Annealing	66-70*	1 min		
Extension	72	60/90 s**		
Hold	4	Indefinite	-	

* 66-70°C annealing temperature for 1st round PCR reactions; 70°C annealing temperature - for 2nd

** 60 sec extension time for first round PCR; 90 sec extension time for second round PCR

Polymerase	Q5 High-Fidelity			
	Temperature, °C	Duration	No of cycles	PCR composition (25 µl)
Initial denaturation	98	30 s	1	0.5 ng DNA, 1x Reaction Buffer, 125 ng each primer, 200 µM each dNTP, 0.25 µl polymerase
Denaturation	98	8 s	30-35	
Annealing	72	35/50 s**		
Extension				
Final extension	72	2 min	1	
Hold	4	Indefinite	-	

** 35 sec extension time for first round PCR; 50 sec extension time for second round PCR

Polymerase	Phusion High-Fidelity			
	Temperature, °C	Duration	No of cycles	PCR composition (25 µl)
Initial denaturation	98	30 s	1	5 ng DNA, 1x Reaction Buffer, 125 ng each primer, 200 µM each dNTP, 0.25 µl polymerase
Denaturation	98	8 s	30	
Annealing	72	30-35 s*		
Extension				
Final extension	72	5 min	1	
Hold	4	Indefinite	-	

* 30 sec extension time for first round PCR; 35 sec extension time for second round PCR

2.1.6. Cell culture

2.1.6.1. Growth and maintenance of cell cultures

HCT116 WT cells were supplied by Bert Vogelstein's laboratory; original PTEN^{+/+}, PTEN^{+/-} and PTEN^{-/-} HCT116 derived cell lines were kindly provided by Dr Todd Waldman (Georgetown University School of Medicine, USA). These cells were grown in McCoy's 5A modified medium (Sigma-Aldrich Ltd, UK) containing 10% FBS (PAA Laboratories, USA) and supplemented with 1.5 mM L-Glutamine (Sigma-Aldrich Ltd, UK) and 1% penicillin/streptomycin (p/s, PAA Laboratories, USA).

MRC-5 cells (ECACC), A549 cells (Cancer Research UK London Research Institute, London, UK) and U2OS^{D1-GFP} (generated by Dr Natsuko Suwaki) were grown in high-glucose DMEM (Sigma-Aldrich Ltd, UK) supplemented with 10% FBS. NIH 3T3, PNT1A and PC3 cells (a kind gift from Dr Rudiger Woscholski's laboratory) were cultured in RPMI and Hem/F12-K (Sigma-Aldrich Ltd, UK) media respectively supplemented with 10% FBS (or 10% NCS for NIH 3T3) and 2 mM L-Glutamine.

U87 cells (kindly provided by Dr Ioanna Stamati) were grown in MEM media (Invitrogen, UK) containing 10% FBS and 1% non-essential amino acids (Sigma-Aldrich Ltd, UK).

T-Rex-293 cells (ThermoFisher Scientific, UK) were kindly provided by Dr Lorna Fiedler (National Heart & Lung Institute, Imperial College London, UK) and cultured in DMEM media supplemented with 10% FBS, 1% p/s, 150 µg/ml zeocin and 15 µg/ml blasticidin (Sigma-Aldrich Ltd, UK).

All mammalian cells were grown in a humidified atmosphere containing 5% CO₂ at 37° C.

Insect Sf9 cells (Cancer Research UK London Research Institute, London, UK) were grown in Graces insect media (Lonza, UK) supplemented with 10% FBS in a humidified atmosphere at 27° C.

2.1.6.2. Transfection

The plasmids were transfected into cells using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). After 24 hours the cells were trypsinised and plated into the fresh medium in a various density. 24 hours later selection drug was added to select for clones carrying stably integrated plasmid. The concentration of drugs used was based on a previously established kill curve assay. Media was replaced every three days. After 3-4 weeks individual colonies were transferred into 24 well plates for further analysis.

2.1.6.3. Electroporation

The cells were cultured up to 50-70% confluence (in a 10 cm dish) and the day before electroporation the media was changed to RPMI media supplemented with 0.1 mM DTT. The

following day the cells were trypsinised and centrifuged at 1,000 rpm for 5 min. The pellet was resuspended in 400 µl of RPMI media supplemented with 0.1 mM DTT, followed by addition of 10µg of plasmid DNA. After 10 min incubation with occasional mixing the cells were electroporated at 500 µFD, 200 Ω, 0.3 kV and plated into the fresh media at various densities. 24 hours later the selection drug was added and the selection process was carried out as described above.

2.1.6.4. SDS-Polyacrylamide Gel Electrophoresis (PAGE)

Cells were lysed with 2x SDS-PAGE loading buffer (64 mM Tris-HCl pH 6.8, 9.6% w/v Glycerol, 2% w/v SDS, 5% v/v β-mercaptoethanol and 0.1% w/v bromophenol blue) and boiled for 10 minutes. Proteins were separated on 10% or 12% polyacrylamide gels (10 or 12% w/v acrylamide, 0.4 M Tris-HCl pH8.8, 0.1% w/v SDS, 0.1% w/v ammonium persulphate (APS), 0.1% v/v TEMED) and 5% stacking gels (5% w/v acrylamide, 0.1 M Tris-HCl pH6.8, 0.1% w/v SDS, 0.1% w/v APS, 0.1% v/v TEMED). The gels were run at 200 V for 1 hour in running buffer (25mM Tris-HCl pH8.3, 250 mM glycine, 0.1% w/v SDS,) and then stained with Coomassie blue staining solution (0.25% w/v Coomassie blue dye, 10% v/v methanol, 10% v/v acetic acid) or electrotransferred for Western Blotting detection.

2.1.6.5. Western Blotting

The proteins from each sample were electrotransferred onto nitrocellulose membranes by immunoblotting in electrotransfer buffer (25mM Tris-HCl, 192mM glycine, 20% v/v methanol) at 400mA for 1h. The membranes were stained with 1:1000 (unless stated otherwise) primary antibody (overnight, at 4° C) and 1:2000 HRP-conjugated anti-species IgG (1 hour, RT) in 5% skimmed milk in TBST buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween 20). The same membrane was either (i) re-stained with 1:4000 mouse anti-α-Tubulin or 1:1000 mouse anti-total Akt antibodies, or (ii) split and stain with 1:1000 rabbit anti-Cdk2 antibody for the loading control. Chemiluminescence was detected after adding the ECL substrate to the membrane.

For most samples tubulin (50 kDa) or total Akt (60 kDa) signals were measured as total protein level controls. However, for several western blots Cdk2 (33 kDa) was utilised as a loading control due to its distinctive size from PTEN (47 kDa). Thus, the same blot could be split and immunostained for both proteins rather than run separate blots or reprobe to determine the tubulin or Akt signals. Nevertheless, the tubulin and Akt controls were used more widely within this study as they are more recognised loading controls than Cdk2.

2.1.6.6. Bio-Rad Detergent-Compatible (DC) protein assay

When total protein levels had to be determined in cell samples, cells were washed with PBS, lysed with RIPA buffer (50 mM Tris-HCl pH 7.5), 150 mM NaCl, 0.1% w/v SDS, 1% w/v sodium

deoxycholate, 1% v/v NP40, 1 mM PMSF (phenylmethylsulfonyl fluoride), 1 mM benzamidine) and incubated on ice for 10 min. The samples were afterwards centrifuged at 14,000 rpm for 20 min at 4 ° C and the pellet was discarded. A small fraction of the supernatant was analysed with DC protein assay (Bio-Rad Laboratories Ltd, UK): using a transparent 96-well plate (Greiner Bio-One, UK) the following components were assembled: protein sample solution (5 µl/well), Solution A (25 µl/well) and Solution S (0.5 µl/well), followed by sequential addition of Solution B (200 µl/well). The plate was gently mixed and incubated for 15-45 min at RT. Optical density was measured at 750 nm. The remainder of the lysate was mixed with 2x SDS-PAGE loading buffer, boiled and used for protein electrophoresis.

2.1.6.7. Measuring β -Galactosidase activity in pFRT-LacZeo transfected cells

Cultured cells were fixed with 4% formaldehyde for 10 min at RT, washed three times with PBS and then incubated with 1 mg/ml X-Gal solution in PBS at 37° C overnight. The cells containing blue precipitate were observed on a bright field or phase contrast microscope.

2.1.6.8. Flow cytometry

Cells were washed with PBS, trypsinised and fixed if required. Live cells were treated with 25 µg/ml propidium iodide (PI, Sigma-Aldrich Ltd, UK); fixed cells were treated with 200 µg/ml RNase A and 50 µg/ml PI for 10 min at room temperature to stain DNA. Subsequently, cells were filtered through 38 µm nylon filter mesh (Baldwin Filters Ltd, UK) and analysed on a Fortessa flow cytometer using the laser excitation at 488 nm with 530/30 filter (for GFP/BDG signal) and at 561 nm with 610/20 filter (for DNA profiles). 10,000 events per sample were acquired. Cell aggregates and debris were excluded by gating on dot plots of side scatter pulse area (SSC-A) vs. forward scatter pulse area (FSC-A) and forward scatter pulse area (FSC-A) vs. forward scatter pulse height (FSC-H). Single cells were also gated on PI-Width and PI-Area plots (Figure 2.1). Dead cells were excluded by selecting PI-negative population. Profiles were analysed by plotting UV-fluorescence intensity using FlowJo software (Tree Star Inc, USA). Non-stained samples were used as a control for autofluorescence.

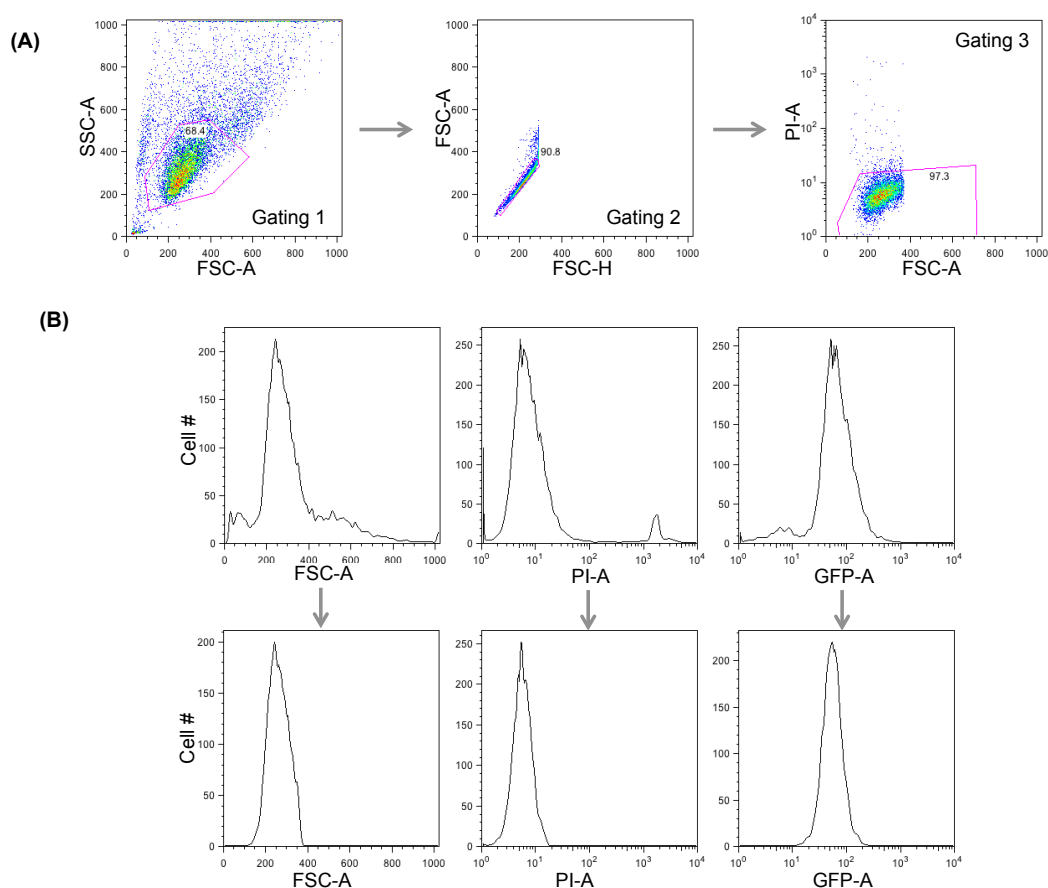


Figure 2.1. Population gating strategy. (A) SSC-A vs. FSC-A dot plot was used for gating out cell debris and aggregates (Gating 1); FSC-A vs. FSC-H plot allowed exclusion of cell doublets (Gating 2); live cells were selected as the PI-negative population on PI-A vs. FSC-A plot (Gating 3). (B) Histograms represent the effect of combined gating strategy (after Gating 1 to 3 were performed) on FSC-A, PI-A or GFP-A channels. Representative data for the gating strategy were taken from pSBbi-GB-PTEN_K13E&K289E knock-in Clone 6 on the basis of HCT116 PTEN^{-/-} cells developed within this project.

2.1.6.9. Cell viability MTS assay

Cytotoxicity from all compounds was measured by MTS assay (G5421, Promega). Cells were seeded in 96-well plate at such concentration that they would reach a near confluent state on the day of reading (in 50 μ l per well). 6-24 hours after plating cells were treated with compounds at a range of concentrations for three and six days. Cell media was changed every three days. MTS/PMS reagents were added according to manufacturer's recommendations and cell viability was calculated after measuring the absorption at 490 nm (as % of the control, i.e. cells treated with MTS/PMS but without added compounds). Assays were performed in triplicate and data analysed using GraphPad Prism 7 software.

2.1.6.10. Senescence-associated β -galactosidase (SA β -gal) staining with FDG

This method was adopted from (Kikuchi et al. 2010) Cells were treated with the compounds or vehicle (DMSO/H₂O) 18 hours after plating in concentrations indicated in the Results and

Discussion. Cells were split into fresh compound-containing media every 3 days. 3-15 days after compound addition the cells were trypsinised, resuspended in PBS, fixed with 4% formaldehyde for 15 minutes, washed with PBS and then incubated for 2 hours at 37°C with 20 µM FDG. Each sample was filtered through 38 µm nylon filter mesh (Baldwin Filters Ltd, UK) and diluted to get final concentration 10⁶ cells/ml following by analysis by flow cytometry.

2.1.6.11. Senescence-associated β -galactosidase (SA β -gal) staining with FDG-C12

This protocol was taken from (Debacq-Chainiaux et al. 2009, Noppe et al. 2009) Adherent cells were incubated with the compounds or vehicle for 3-18 days. Cells were diluted in fresh media supplemented with compounds every 3 days. On the day of analysis, the media was changed to fresh media containing 100 nM bafilomycin A1 or 300 µM chloroquine and cells were incubated for 1 hour (with bafilomycin A1) or 2 hours (with chloroquine) following by addition of FDG-C12 substrate to the final concentration of 33 µM. After 1-2 hours cells were trypsinised, washed and analysed via flow cytometry.

2.1.6.12. SA β -gal staining with 5-bromo-4-chloro-3-indolyl β -D-galactoside (X-Gal)

The protocol for this method was modified from (Dimri et al. 1995) PTEN^{+/-} HCT116 cells were treated with 500 nM VO-OHpic or vehicle (DMSO) 18 hours after plating. 3 days later the cells were washed in PBS, fixed for 15 min (RT) in 4% formaldehyde, washed and incubated at 37°C with fresh SA β -gal staining solution: 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 40 mM citric acid/sodium phosphate, pH 6.0, 150 mM NaCl, 2 mM MgCl₂, 1 mg/ml X-Gal. The cells were observed on a bright field or phase contrast microscope.

2.1.6.13. SA β -gal staining with 4-methylumbelliferyl- β -D-galactopyranoside (MUG)

The protocol was adopted from (Gary, Kindell 2005) Cells were treated with the compounds or vehicle 18 hours after plating. 3-15 days later the cells were washed six times with PBS and lysed with 5 mM CHAPS, 40 mM citric acid, 40 mM sodium phosphate, 0.5 mM benzamidine, 0.25 mM PMSF, pH 6.0 buffer. The cellular lysate was vortexed and centrifuged for 5 min at 12,000 g. The clarified supernatant was mixed with an equal volume of 40 mM citric acid, 40mM sodium phosphate, 300 mM NaCl, 10 mM β - mercaptoethanol, 4 mM MgCl₂, pH 6.0 buffer with 1.7 mM of MUG. After incubation at 37° C for 1 hour, 50 ul of the reaction mixture was added to 500 ul of 400 mM sodium carbonate stop solution. Fluorescence was measured in a 96-well plate (150 ul per well) with excitation at 360 nm, emission at 465 nm. The resulting signal was normalised by the total protein levels (measured by Bio-Rad Detergent-Compatible (DC) protein assay).

2.1.7. Phosphatase assay

Recombinant GST-free PTPMT1 (kindly provided by Dr Rudiger Woscholski's group) was prepared in the reaction buffer (100 mM Tris-HCl pH 7.5, 4 mM MgCl₂, 2 mM DTT) to a final concentration 2.5 ng/μl (0.11 μM) per assay point. VO-OHpic was dissolved in DMSO to a concentration 1 mM. Further dilutions were made to final compound concentrations 10 - 1000 nM in the assay buffer with 1% DMSO. Samples were pre-incubated with PTPMT1 for 15 min at RT before adding 15 μM of 3-O-methylfluorescein phosphate (OMFP, Sigma-Aldrich Ltd) to initialise the reaction (total volume, 100 μl per assay point). OMFP was dissolved in DMSO to a concentration of 15 mM by sonication for 12 minutes. Further dilution was made to a final concentration 150 μM in 1% DMSO. The reaction was performed in a 96-well microplate. PTPMT1 activity was detected by measuring hydrolysis of OMFP to OMF over time at RT, which is accompanied by the changes in fluorescence intensity (excitation at 485 nm and emission at 525 nm). Background measurements were carried out in the absence of PTPMT1 and subtracted during the data analysis. The slope for the reaction rate was calculated for each VO-OHpic concentration and then plotted in a logarithmic scale graph (using GraphPad Prism 7).

2.1.8. UV-VIS analysis of VO-OHpic stability

UV-VIS spectra of VO-OHpic were obtained on a Perkin Elmer Lambda 25 spectrophotometer. HCT116 WT cells were incubated with VO-OHpic in several concentrations (1, 5, 10 and 50 μM) for 3 days. Absorbance spectra were taken on day 0 and day 3 and changes in VO-OHpic characteristic peak at 305 nm was analysed.

2.1.9. Statistical analysis

Statistical analysis of the means (using GraphPad Prism 7) was performed by two-way ANOVA test (with a 5% cut-off for significance). The degree of significance is highlighted by stars (i.e. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.001) within the corresponding figures.

Cell survival sigmoidal curves were calculated via GraphPad Prism 7, GF metrics was computed via online calculator at <http://www.grcalculator.org> (Hafner et al. 2016, Clark et al. 2016).

2.1.10. CEL1 mismatch cleavage assay

The CEL1 mismatch cleavage assay was performed according to the protocol in (Till et al. 2006). Re-annealed PCR DNA (10 μl) in GoTaq G2 buffer was added to 10 μl CEL1 reaction mixture (1 unit CEL1, 10 mM HEPES pH 7.5, 0.002% v/v Triton X-100, 0.1 M KCl, 0.1 M MgSO₄), followed by incubation for 30-45 min at 37° C. Then 5x Tris-acetate-EDTA loading

buffer (5x TAE LB: 200 mM Tris-HCl, 100 mM acetic acid, 5 mM ethylenediamine tetraacetic acid (EDTA, pH 8.0), 12.5% w/v Ficoll, 0.125 w/v bromophenol blue) was added to stop reaction.

2.1.11. T7E1 mismatch cleavage assay

The T7 endonuclease I (T7E1) mismatch cleavage assay was performed according to the manufacture's recommendations (M0302S, NEB, UK). The digestion reactions contained 10 µl re-annealed PCR DNA in GoTaq G2 buffer mixed with 10 µl T7E1 (1 unit) in 1x buffer 2 (10 mM Tris-HCl pH 7.9, 50 mM NaCl, 10 mM MgCl₂, 1 mM DTT) and extra 10 mM MgCl₂. Then the reactions were incubated for 1 hour at 37° C and mixed with 5x Tris-acetate-EDTA loading buffer.

2.1.12. Bac-toBac Baculovirus Expression System

2.1.12.1. Transposition

pFast-Bac1-GST-NLK plasmids (2-3 µg) were mixed with electrocompetent *E. coli* strain DH10Bac (Invitrogen) and incubated for 2-4 hours at 37° C with shaking. Electroporation was performed at 25 µFD, 200 Ω, 2.2 kV. Sterile Luria Broth (LB) media (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl, pH 7.0) was then added and the bacteria solution was plated onto LB-Agar plates (1.5% w/v agar, 1.25% w/v tryptone, 0.625% w/v yeast extract, 1.25% w/v NaCl, pH 7.0) supplemented with 50 µg/ml kanamycin, 7 µg/ml gentamycin, 10 µg/ml tetracycline, 100 µg/ml X-Gal and 40 µg/ml IPTG. The plates were incubated at 37° C for 24-36 hours. The white colonies were inoculated and amplified in mini-cultures.

2.1.12.2. Bacmid mini-prep

Individual DH10Bac colonies were grown in 2 ml LB supplemented with 50 µg/ml kanamycin, 7 µg/ml gentamycin and 10 µg/ml tetracycline at 37° C overnight with shaking. 1.5 ml aliquots from each sample were centrifuged at 13,000 rpm for 1 sec. The supernatant was removed and the cell pellets were resuspended in 300 µl of Solution I (15 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 µg/ml RNase A), followed by addition of 300 µl of Solution II (0.2 M NaOH, 1% w/v SDS). After gentle mixing and incubation at RT for 5 min, 300 µl of 3M NaOAc (pH 5.5) was slowly added and the solution was gently mixed again and incubated for 5-10 min on ice. Then the samples were centrifuged at 13,000 rpm for 10 min and the supernatant was precipitated by addition of 800 µl of absolute iso-propanol followed by incubation for 5-10 min on ice and centrifugation at 13,000 rpm for 15 min. The supernatant was discarded and the DNA pellet was resuspended in 40 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA).

2.1.12.3. Transfection of Sf9 cells with bacmids

The bacmids were transfected into 80-90% confluent Sf9 cells using Lipofectamine 2000 according to the manufacture protocol (Invitrogen). 1 hour after transfection, the FBS-free media was supplemented with 10% FBS, 24 hours after transfection media was refreshed. After 7-8 days the baculovirus-containing supernatant was collected and centrifuged at 1000 rpm for 5 min at 4° C.

2.1.12.4. Infection of Sf9 cells with baculovirus

Near 80% confluent Sf9 cells were incubated with 0.5 ml of baculovirus for 5-7 days following by centrifugation of supernatant at 1000 rpm for 5 min at 4° C. To amplify baculovirus titer, baculovirus from the new supernatant was infected again to the fresh Sf9 cells. 5-7 days later the supernatant with amplified baculovirus was collected and ready for the use in protein expression and purification protocol.

2.1.12.5. Expression and isolation of GST-NLK proteins from Sf9 cells

GST tagged NLK_WT and NLK_T219G baculoviruses (0.5 ml each) isolated from Sf9 cells according to the protocol above, were incubated with fresh Sf9 cells (confluent 10 cm dishes) for 72 hours. Cells were re-suspended in NETN buffer (2 ml of 150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl pH 8.0, 1% NP-40) and lysed by passing through a 21 guage needle. Lysates were centrifuged at 5,000 rpm for 10 min at 4° C and supernatants were bound to 200 µl of glutathione Sepharose beads (Amersham Pharmacia, UK) for 1 hour at 4° C before being washed (3x 2 ml NETN) and resuspended in storage buffer (NETN, 50% glycerol).

2.1.13. Kinase assay

Kinase Assay was performed in a 16 µl total volume, which contained GST-purified NLK-WT, NLK_T219G or MAPK (as a positive control) bound to Sepharose beads; 4 µg MBP substrate (Sigma-Aldrich Ltd, UK); 1 mM ATP or N⁶-(cyclohexyl)ATP (CxATP, synthesised by Dr Sarah Lee, Oxford University); 1x Kinase Buffer in the presence or absence of 3-MBPP1 inhibitor (150 nM, synthesised by Dr Karen McCague, Imperial College London). Immobilised kinase suspensions were washed in 1x Kinase Buffer prior to addition. 1x Kinase buffer composition was either Tris-HCl (25 mM Tris-HCl pH8.0, 5 mM MgCl₂, 0.1 mM EGTA) or MOPS-based (5 mM MOPS-NaOH pH 7.2, 2.5 mM glycerol-2-phosphate, 5 mM MgCl₂, 1 mM EGTA, 0.4 mM EDTA, 0.5 mM DTT, 2% w/v BSA). The samples were incubated at 30° C for 30 min and the reactions were stopped through the addition of 4 µl of 5x SDS-PAGE loading buffer (160 mM Tris, 24% w/v Glycerol, 5% SDS, 12.5% v/v 2-Mercaptoethanol, 0.25% w/v bromophenol blue). The products were separated on Phos-Tag SDS-PAGE.

2.1.14. Phos-tag SDS-PAGE

Proteins were separated on 12.5% Mn-Phos-Tag SDS-PAGE (100 μ M Phos-Tag acrylamide (synthesised by Dr Chirag Patel, Imperial College London), 200 μ M MnCl_2 , 10% w/v polyacrylamide, 0.4 M Tris-HCl pH 8.8, 0.1% w/v SDS, 0.1% w/v ammonium persulphate (APS), 0.1% v/v TEMED) and 5% stacking gel (5% w/v acrylamide, 0.1 M Tris-HCl pH6.8, 0.1% w/v SDS, 0.1% w/v APS, 0.1% v/v TEMED). The gels were run at 200 V for 1 hour in running buffer (25mM Tris-HCl pH8.3, 250 mM glycine, 0.1% w/v SDS) and then stained with InstantBlue staining solution (Sigma-Aldrich Ltd, UK) for 30 min.

2.2. Chemistry

2.2.1. General Chemistry

Vanadyl acetylacetonate ($\text{VO}(\text{AcAc})_2$), 3-hydroxypicolinic (OH-pic) and 2-picolinic (H-pic) acids were purchased from Sigma, vanadyl sulphate hydrate ($\text{VOSO}_4 \cdot 5\text{H}_2\text{O}$) was purchased from BDH Prolabo Chemicals (VWR, UK). All solvents, inorganic salts and silica gel were purchased from Sigma-Aldrich Ltd (UK) and VWR (UK). All chemicals and solvents were used as purchased without further purification.

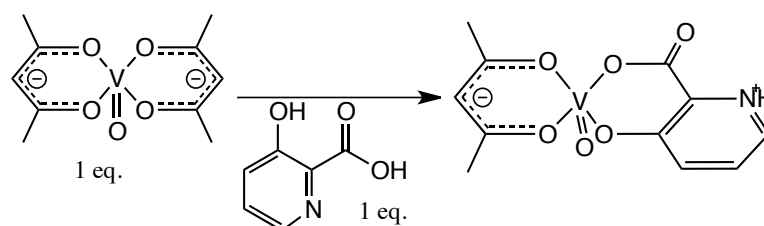
Elemental analyses were carried out by Alan Dickerson at Cambridge University. ESI+ mass spectra were obtained on a Brüker Daltronics Esquire 3000 spectrometer by Dr Lisa D. Haigh at the Chemistry Department Mass Spectrometry Service (Imperial College London). Infrared spectra were performed on a Perkin-Elmer FT-IR spectrometer. UV-VIS spectra were obtained on a Perkin Elmer Lambda 25 spectrophotometer.

2.2.2. Synthesis of 3-hydroxypicolinate V(IV) (VO-OHPic)

VO-OHPic was synthesised as previously described in the MRes part of this project (Fedorova et al. 2012) according to the reported procedure (Yano et al. 2002, Nakai et al. 2004). Briefly, 10% NaHCO_3 (2.06 ml, 2.46 mmol) was added to a solution of hydroxypicolinic acid (0.44 g, 3.17 mmol, 2 eq.) and $\text{VOSO}_4 \cdot 5\text{H}_2\text{O}$ (0.4 g, 1.57 mmol, 1 eq.) in water (13.7 ml). The mixture was stirred for 4 hours at RT and the precipitate was passed through a filter membrane and washed with water, giving the desired compound as a light-green powder.

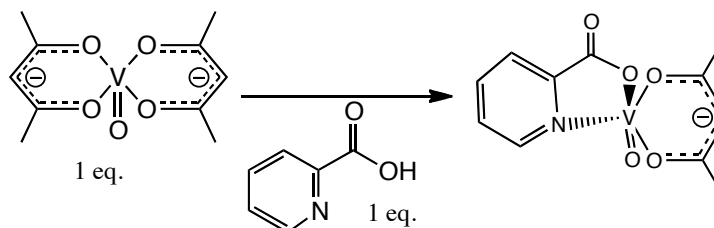
(0.6 g, 95%); ESI⁺-MS (m/z): 344 (calc. for $[\text{M}+\text{H}]^+ = 344$); Elem. Anal. Calc. for $\text{C}_{12}\text{H}_{16}\text{N}_2\text{O}_{11}\text{V}$ (aka VO-OHPic $\cdot$ 3 H_2O): C 34.71, H 3.88, N 6.75, Found: C 34.71, H 3.72, N 6.72; IR: $\nu(\text{cm}^{-1}) = 3500\text{--}2800$ (ν_{OH}), 1640 ($\nu_{\text{C}=\text{N}}$), 1567 ($\nu_{\text{C}=\text{C}}$), 956 ($\nu_{\text{V}=\text{O}}$); UV-VIS (MeOH): λ/nm ($\epsilon/\text{M}^{-1} \times \text{cm}^{-1}$) = 217 (2.1×10^4), 308 (1.09×10^4), 736 (38.5). Data is consistent with literature (Yano et al. 2002, Nakai et al. 2004).

2.2.3. Synthesis of [VO(OH-pic)(AcAc)] complex



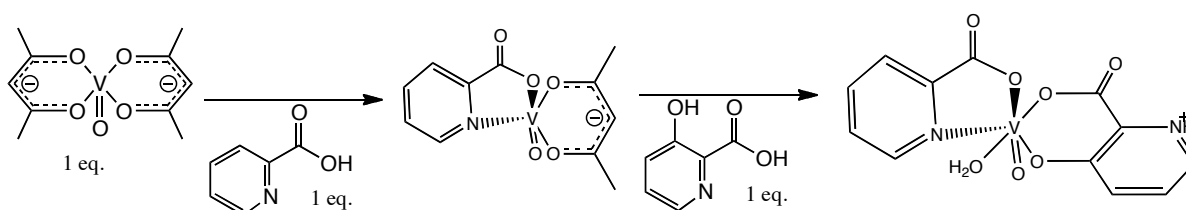
A solution of 3-hydroxypicolinic acid (0.139 g, 1 mmol, 1 eq.) in 10 ml methanol was added to a solution of VO(AcAc)₂ (0.265 g, 1 mmol, 1 eq.) in 10 ml methanol. The mixture was neutralised with a 2 M NaOH solution to pH 7.0 and stirred for 1 hour under reflux, then cooled down to room temperature. Several different experimental conditions were investigated for this synthesis; these are discussed in Chapter 9. For each experimental condition, the resulting precipitates were filtered, re-crystallised (where possible) and analysed by ESI⁺-MS and FT-IR spectroscopy. None of the precipitates gave a pure desired product. Spectra results suggested a mixture of complexes or decomposition in all cases.

2.2.4. Synthesis of [VO(H-pic)(AcAc)] complex



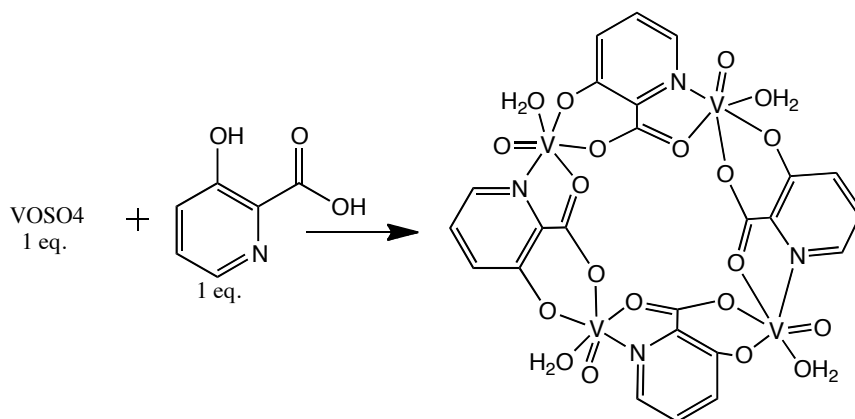
A solution of picolinic acid (0.092 g, 0.75 mmol, 1 eq.) in 8 ml water was added to a solution of VO(AcAc)₂ (0.199 g, 0.75 mmol, 1 eq.) in 16 ml 50% methanol. After stirring at room temperature for 1 hour the mixture became purple. Stirring continued for 6 days in air resulting in evaporating 2/3 of the liquid and formation of a light-blue precipitate. The mixture was passed through a filter membrane, giving a light-green filtrate and a light-blue powder that was washed with water, diethyl ether and THF. Powder and filtrate were analysed by ESI⁺-MS and FT-IR. Spectra results suggested a mixture of complexes. Crystallisation attempts did not result in product formation.

2.2.5. Synthesis of [VO(OH-pic)(H-pic)] complex from VO(AcAc)₂



A solution of picolinic acid (0.097 g, 0.79 mmol, 1.05 eq.) in 4 ml water was added to a solution of VO(AcAc)₂ (0.199 g, 0.75 mmol, 1 eq.) in 8 ml methanol. After stirring at room temperature for 1.2 hours a solution of 3-hydroxypicolinic acid (0.11 g, 0.79 mmol, 1.05 eq.) was added dropwise. The mixture was stirred overnight at room temperature and left until completely evaporated. The dry residue was dissolved in methanol that gave a light-green precipitate after 8 hours evaporation in air. The solid was separated and characterised by ESI⁺-MS and IR. The spectra suggested the presence of several complexes in the product. The filtrate was left for further crystallisation (at both +4 °C and room temperature). No crystals were observed.

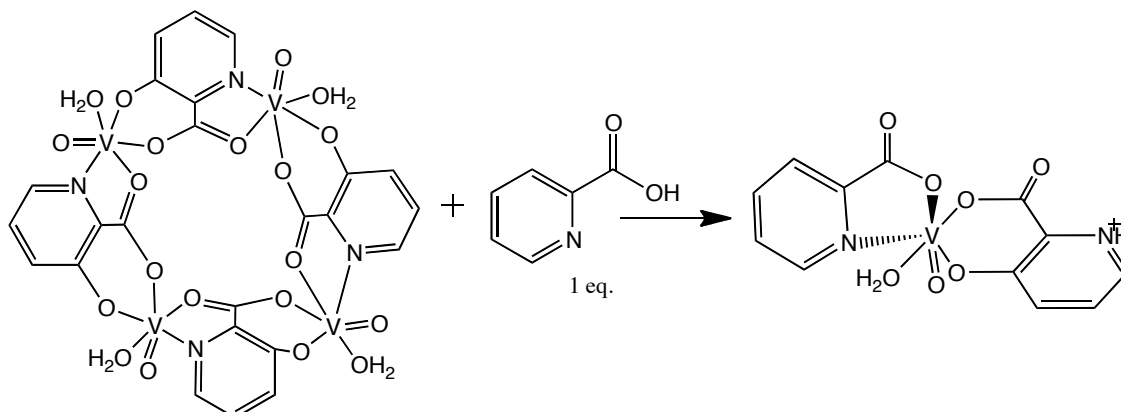
2.2.6. Synthesis of [VO(OH-pic)(H₂O)]₄ complex



This reaction was carried out according to the previously reported procedure (Yano et al. 2002, Nakai et al. 2004). To a solution of VOSO₄·5H₂O (0.55 g, 2.16 mmols, 1 eq.) and 3-hydroxypicolinic acid (0.3 g, 2.16 mmols, 1 eq.) in water (12.6 ml) 10% NaHCO₃ (2.37 ml, 2.83 mmols, 1.31 eq.) was added. The mixture was stirred for 3.5 hours at room temperature resulting in light-brown precipitate formation. The precipitate was passed through a filter membrane and washed with water, giving the desired product as a light-brown powder.

0.46 g, 82%; ESI⁺-MS (*m/z*): 857.8 (calc. for [M+H]⁺ + CH₃CN = 857.8, where M is [VO(OH-pic)₄]; Elem. Anal. Calc. for C₂₄H₃₂N₄O₂₆V₄ (i.e. for [VO(OHpic)(H₂O)]₄·6H₂O) C 28.93, H 3.24, N 5.62, Found: C 28.53, H 2.75, N 5.55; UV-VIS (MeOH): λ/nm (ε/M⁻¹ × cm⁻¹) = 209 (5.45×10⁴), 334 (1.56×10⁴), 543 (5.2), 774 (114); IR: ν(cm⁻¹)=3500-2800 (ν_{OH}), 1600 (ν_{C=N}), 1567 (ν_{C=C}), 945 (ν_{V=O}). X-Ray diffraction data indicated same as GUWTAT, [V(=O)(H₂O)L]₄; L = 3-hydroxypicolinate. Selected crystallographic data: *a* = 11.139(9) Å, *b* = 11.83(2) Å, *c* = 14.956 (11) Å, α = 98.86(10)°, β = 92.94(6)°, γ = 94.48(10)°, *V* = 1934(4) Å³. Data is consistent with literature (Yano et al. 2002, Nakai et al. 2004).

2.2.7. Synthesis of [VO(OH-pic)(H-pic)] complex from [VO(OH-pic)(H₂O)]₄



A solution of picolinic acid (0.048 g, 0.39 mmols, 4 eq.) in 5 ml methanol was added to a cloudy light-brown mixture of [VO(OHpic)(H₂O)]₄·8H₂O (0.1 g, 0.097 mmols, 1 eq.) in 20 ml methanol with stirring at room temperature. The pH was adjusted to 2 by 10% H₂SO₄. After 1 hour stirring the solution became clear and changed colour to green. The mixture was stirred for 20 more hours with no sign of precipitate formation. The pH was adjusted to 4 by addition of 10% NaHCO₃ and the filtered solution was left for crystallisation from various solvent mixtures.

ESI⁺-MS (m/z): 328.0 (calc. for [M+H]⁺ = 328.0, where M is [VO(OH-pic)(H-pic)]); Elem. Anal. Calc. for C₁₂H₁₃N₂O_{8.5}V (i.e. for [VO(OHpic)(H₂O)]₄·1.5H₂O) C 38.73, H 3.52, N 7.53, Found: C 38.30, H 3.02, N 7.40.

2.2.8. Native gels

2.2.8.1. PAGE native gels: Ornstein and Davis formulation

The protocol was adopted from (Ornstein 1964, Institute of Molecular Development LLC 2001). 4-10% resolving gels and 2.5/4% stacking gels were prepared as shown in Table 2.4, polymerised for 1 hour and equilibrated in running buffer (0.192 M glycine, 25 mM Tris-HCl pH 8.3).

Table 2.4. Native PAGE gel composition.

	Resolving gels					Stacking gels	
	4%	5%	6%	8%	10%	4%	2.5%
40% Acrylamide:Bis solution (37.5:1)	1 ml	1.25 ml	1.5 ml	2 ml	2.5 ml	0.4 ml	0.25 ml
1.5 M TRIS, pH 8.8	2.5 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml	1 ml	1 ml
50% glycerol	2.5 ml	-	-	-	-	-	-
ddH ₂ O	4 ml	6.25 ml	6 ml	5.5 ml	5 ml	2.6 ml	2.75 ml
APS, 10% (w/v)	50 µl	50 µl	50 µl	50 µl	50 µl	20 µl	20 µl
TEMED	10 µl	10 µl	10 µl	10 µl	10 µl	5 µl	5 µl

The protein samples containing 5 µl of each protein (ug amounts as stated below) were dissolved in the same volume of 2x sample buffer (0.625 mM Tris-HCl pH 6.8; 4% glycerol, 0.02% bromophenol blue) and loaded onto gels. The PAGE was run at 200 V until the bromophenol blue dye front reached the bottom of the gel (approximately 1.5-2.5 hours). Then the gels were stained in the InstantBlue (Sigma) staining solution for 1 hour and washed with water before visualisation on a FujiFilm LAS-3000.

Proteins tested: recombinant PTEN (2 µg, 47 kDa, kindly provided by Dr Rudiger Woscholski's lab), bovine serum albumin (1 µg, BSA, 66 kDa, Sigma), ovalbumin (1 µg, OVA, 42.7 kDa, Sigma), carbonic anhydrase (1 µg, 30 kDa, Sigma), lysozyme (1 µg, 14.3 kDa, Sigma).

2.2.8.2. PAGE native gels: no stacking gel

The composition for the full length 6% separating gel is given in Table 2.5. Running buffer was prepared from 25 mM Tris-HCl pH 8.6 and 0.2 M Glycine.

Table 2.5. Native PAGE gel composition (without stacking gel).

	6% resolving gel
30% Acrylamide:Bis solution (30:0.8)	2 ml
0.1 M TRIS, pH 8.6 + 0.8 M glycine	2.5 ml
ddH ₂ O	5.4 ml
APS, 10% (w/v)	100 µl
TEMED	8 µl

Samples were dissolved in 2x loading buffer (20 mM Tris-HCl pH 6.8, 10% glycerol, 0.004% bromophenol blue) with pH 6.8 or pH 8.6. The rest of the protocol is the same as for 2.2.8.1.

Proteins tested: recombinant PTEN (2 µg), BSA (1 µg), carbonic anhydrase (1 µg).

2.2.8.3. PAGE native gels: EMSA

Full length 4-4.95 % resolving gels were prepared according to the directions in Table 2.6 and pre-run at 200 V for 30 min in running buffer (0.5x TBE, 5% glycerol). The rest of the protocol is the same as for 2.2.8.1.

Table 2.6. Native PAGE gel composition: EMSA composition.

	Resolving gels		
	4%	4.2%	4.95%
40% Acrylamide:Bis solution (29:1)	1 ml		
30% Acrylamide:Bis solution (29:1)		1.4 ml	
55% Acrylamide:Bis solution (44:0.8)			0.9 ml
10x TBE	0.5 ml	0.5 ml	0.5 ml
80% glycerol	0.64 ml	0.64 ml	-
ddH ₂ O	7.71 ml	7.3 ml	8.5 ml
APS, 10% (w/v)	140 µl	140 µl	90 µl
TEMED	10 µl	10 µl	10 µl

10x TBE: 890 mM TRIS, 890 mM boric acid, 20 mM EDTA, pH 8.3

Proteins tested: recombinant PTEN (2 µg), BSA (1 µg), carbonic anhydrase (1 µg).

2.2.8.4. Agarose native gels

A horizontal native agarose gel was prepared from 0.8% agarose (SeaKem, Lonza) dissolved in buffer A (25 mM Tris-HCl pH 8.5, 19.2 mM glycine). Samples were dissolved in 2x loading buffer (50 mM Tris-HCl, 150 mM NaCl, 20% glycerol, 5% Ficoll (type 400), 0.05% bromophenol blue) with pH 6.8 or pH 8.6. The gel was run at 50 V for 1 hour and then stained with InstantBlue (Sigma) for 30 min.

Proteins tested: recombinant PTEN (4 µg, 47 kDa), recombinant PTPMT1 (4 µg, 23 kDa, kindly provided by Dr Rudiger Woscholski's lab), BSA (5 µg, 66 kDa), OVA (10 µg, 42.7 kDa), carbonic anhydrase (10 µg, 30 kDa), lysozyme (10 µg, 14.3 kDa), RNase A (10 µg, 13.7 kDa).

2.2.9. Metalloproteomics (ICP-MS)

Samples for ICP-MS were prepared according to the protocols described in (Abdelrahman et al. 2010, Sellner et al. 2003, Perring et al. 2001) with small modifications. Gel fragment samples were digested and mixed with concentrated nitric acid (15.6 M) for 15 min at 37° C. The acid supernatant was then evaporated at 65 °C until dry (about 43 hours) and the residue was dissolved in 3 ml (by weight) of 2% nitric acid prior analysis. ICP-MS characterisation was determined using an Agilent 7700x by Dr S. Strekopytov at the Imaging and Analysis Centre (Natural History Museum).

3. Results and Discussion: PTEN Cell Line Generation (PTEN Knock-Out (KO))

3.1. Introduction

In the beginning of this study PTEN knock-out (KO) cell lines were generated to recreate *PTEN* homozygous (*PTEN*^{-/-}) and heterozygous (*PTEN*^{+/-}) genotypes. There are several genome editing techniques available for performing gene KO such as the homology recombination methods (Cre-Lox or FRT/Flp) or site-specific endonuclease technologies (Zinc-finger nucleases (ZFNs) and Transcription Activator-like Effector (TALEN) nucleases).

Homology recombination methods consist of a recombinase enzyme (Cre (Kohli et al. 2004, Rago, Vogelstein & Bunz 2007) or Flp (Schlake, Bode 1994, Thomas et al. 2004, Adamson, Jackson & Davis 2011)) recognising a specific target sequence within the genome (LoxP or FRT respectfully). These techniques are quite time-consuming and rely on integration of the target site into the host genome, often a low efficiency process.

ZFNs utilise specific DNA-binding zinc-finger proteins and the FokI endonuclease (Kim, Cha & Chandrasegaran 1996, Smith, Berg & Chandrasegaran 1999, Bibikova et al. 2001). FokI cleavage requires dimerisation of the protein, thus binding to a pair of zinc-finger proteins allows enhanced target specificity and generates DSB at the target DNA locus. TALENs utilise the DNA-binding domain TALE repeats fused to the cleavage domain of the FokI endonuclease (Boch et al. 2009, Moscou, Bogdanove 2009). Each TALE repeat binds to single base pair of the genome. The last two endonuclease-based methods require the design construction of complicated plasmids for expression of DNA-binding proteins. This can be time-consuming and laborious (Horii, Hatada 2014).

In order to KO PTEN in cells, a novel genome-engineering technology, Clustered Regularly at Interspaced Short Palindromic Repeats (CRISPR) and CRISPR Associated 9 (Cas9) nuclease (Charpentier, Doudna 2013, van der Oost 2013), was chosen for this study. This technique allows site-specific genome engineering at a guide RNA (gRNA) directed chromosomal locus of interest and has the advantages of simple-to design target-specific constructs and high target mutation rate, compared to ZFN and TALEN systems (Horii, Hatada 2014).

CRISPR/Cas mechanisms are based on bacteria/archaea adaptive immune systems that use short RNAs to guide degradation of foreign nucleic acids (Barrangou et al. 2007, Terns, Terns 2011, Bhaya, Davison & Barrangou 2011, Wiedenheft, Sternberg & Doudna 2012, Barrangou, Horvath 2012). CRISPR defence response involves (i) integration of new targeting sequences ("spacers") from invading virus or plasmid DNA into the CRISPR locus, (ii) expression of short

guiding CRISPR RNAs (crRNAs) consisting of targeting sequences, and (iii) cleavage of nucleic acids complementary to the targeting sequence (Bhaya, Davison & Barrangou 2011). There are three classes of CRISPR defence that are known so far: Types I, II and III. Type II CRISPR system requires only one Cas9 endonuclease to cleave dsDNA, while Types I and III systems utilise multiple distinct enzymes acting as a complex (Makarova et al. 2011). As a result, the Type II CRISPR/Cas9 system is most amenable for use in alternative contexts such as eukaryotic cells and is becoming widely used (Chylinski et al. 2014). The Type II system utilises three components: (i) the Cas9 endonuclease; (ii) a long pre-crRNA transcribed from the targeting sequence-containing CRISPR locus, which defines this genomic target for Cas9; and (iii) a trans-encoded crRNA (tracrRNA) encoding RNA scaffold that binds to Cas9 and links it to pre-crRNA, thus processing mature crRNA. In the majority of CRISPR/Cas9-mediated genome editing methods, these two RNAs can be fused into single dual tracrRNA:crRNA sequence named the guide RNA (gRNA) (Jinek et al. 2012) (Figure 3.1).

In Type II CRISPR system, Cas9 unwinds the DNA duplex and searches for sequences matching the crRNA to cleave DNA. Recognition of the target site occurs upon detection of a “protospacer” sequence in the target DNA followed by a remaining target (spacer) sequence complementary to a crRNA. Importantly, Cas9 cleaves the DNA only if a correct Protospacer-Adjacent Motif (PAM) is located at the 3' end of the target DNA. Different Type II systems have distinct PAM requirements. The *S. pyogenes* Type II system used in this study requires an NGG sequence, in which N can be any nucleotide. In this CRISPR system, Cas9 generates a double-stranded break (DSB) 3 bp upstream of the PAM. This process is mediated by the two Cas9 catalytic domains: an HNH domain that cuts the complementary DNA strand and a RuvC-like domain that cuts the non-complementary strand (Mali et al. 2013b). If one of the two nuclease domains is disrupted, Cas9 can function as a nickase cleaving only one strand of the target DNA (Jinek et al. 2012, Davis, Maizels 2014).

One of the issues that is limiting CRISPR/Cas9 technology is its specificity. The off-target activity of Cas9 could cause unwanted DSB at other regions of the genome, potentially causing toxicity, unwanted mutations and even oncogenesis in gene therapy applications. The *S. pyogenes* system can tolerate mismatches in the first six bases out of the 20 bp of target sequence *in vitro* (Mali et al. 2013b). However, a greater stringency was required *in vivo* in a number of reports (Cong et al. 2013, Hsu et al. 2013, Cradick et al. 2013). There are several bioinformatics software available for gRNA design and optimisation to ensure gRNAs chosen have absolute specificity and are likely to minimise off-target effects (see below).

The repair of a Cas9-mediated DSB is performed using non-homologous end joining (NHEJ) or homology-directed repair (HDR) pathways (Horii, Hatada 2014). NHEJ is an error prone mechanism of repair that often leads to small insertions or deletions (indels) at the breakage site, it also can cause resection of breaks and subsequently loss of material distal and proximal

to the break point. Whereas HDR utilises homologous donor DNA sequences supplied exogenously to produce a desired recombination at a DSB site (Figure 3.1(A)). For the purposes of gene disruption, NHEJ was chosen as it can cause disruptions in open reading frames and so no donor DNA was added in this part of the project.

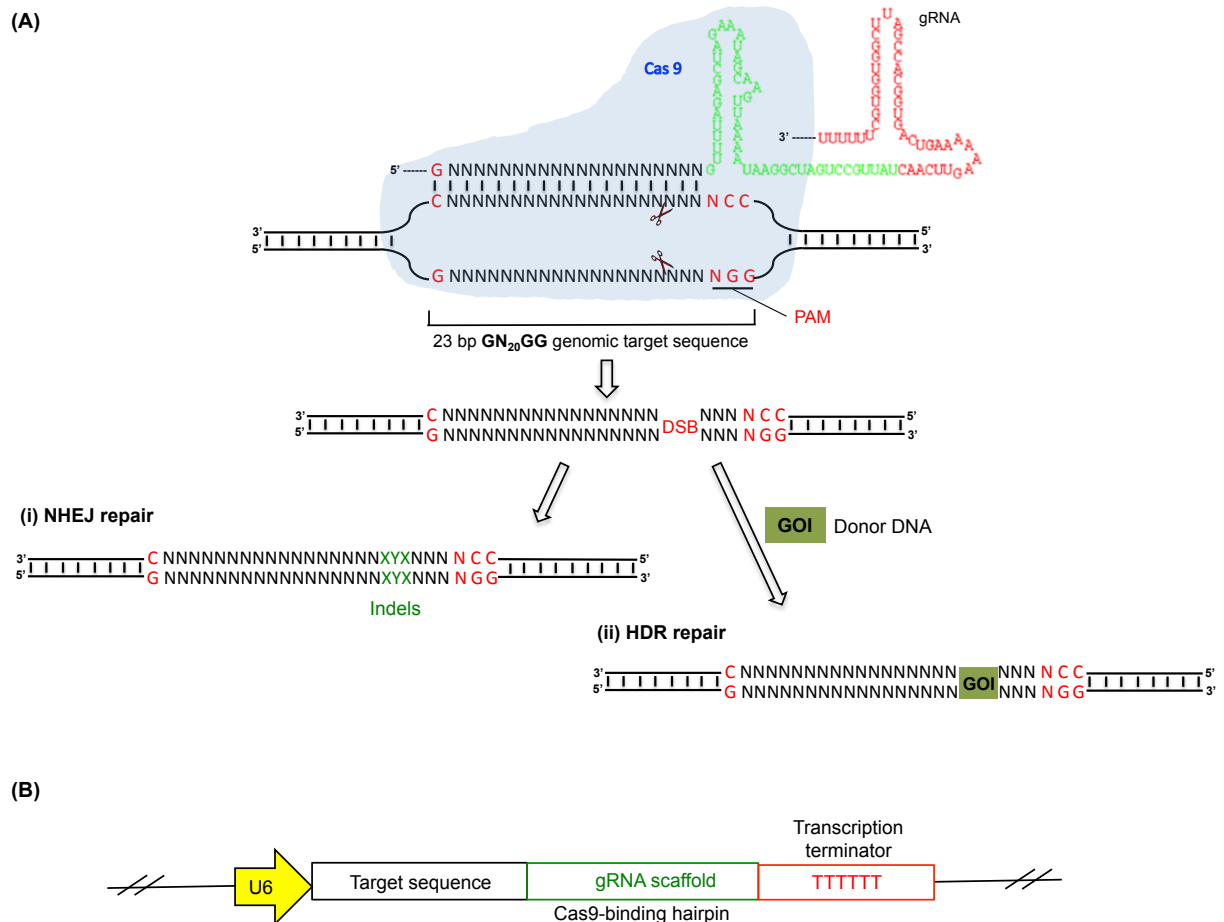


Figure 3.1. Schematic of the CRISPR/Cas9 and schematic of DNA construct **(A)** Schematic of the CRISPR/Cas9 mediated genome editing principles. Cas9 generates a sequence-specific double strand break (DSB) in target genomic DNA directed by a guide RNA (gRNA). The binding site of a target DNA contains a trinucleotide Protospacer-Adjacent Motif (PAM) (the sequence NGG in red) at the end of the 20 nucleotide specific sequence in the genomic DNA. The DSB generated by Cas9 is repaired by non-homologous end joining (NHEJ) or homology-directed repair (HDR). **(B)** Schematic map of DNA construct used for gRNA expression. The target site is incorporated directly between human U6 RNA polymerase III promoter and the gRNA scaffold, followed by a transcription terminator sequence (TTTTTT). The U6 promoter constrains the first position in the RNA transcript to be a “G” and therefore any genomic sites with the sequence GN₂₀GG can be targeted using this method.

3.2. PTEN KO via CRISPR/Cas9

The Cas9-encoding plasmid was obtained from Addgene (Mali et al. 2013b). The plasmid used for gRNA expression (Figure 3.1(B)) was engineered as described below.

3.2.1. gRNA design

Two suitable gRNA target sequences were selected within the *PTEN* gene according to the following criteria: (i) would have 5'→3' GN₂₀GG sequence, i.e. contain PAM Cas9 binding motif (NGG) and transcription initiating first G residue for U6 promoter; (ii) would be the part of the cDNA sequence (i.e. within exons and ideally in close proximity to the PTEN phosphatase signature motif at position 124-130 in exon 5) to disrupt the coding sequence; (iii) would contain 40 to 70% of GC-content for maximal efficiency (Wang et al. 2014, Tsai et al. 2015)); (iv) would not be self-complementary (self-complementarity within the target sequence or between the target sequence and gRNA scaffold can reduce gRNA efficiency (Labun et al. 2016)); and (v) would have minimal off-target sites in the genome. These criteria were incorporated within the online software <http://chopchop.cbu.uib.no/index.php> (Montague et al. 2014, Labun et al. 2016). This software takes into account current information on reducing off target effects, predicting off-target sites with pre-defined numbers of mismatches.

The list of possible GN₂₀GG gRNA target sequences within *PTEN* (Gene ID used was Ensembl ENST00000371953, PTEN-001 transcript) alongside the list of potential off-target sites outside of *PTEN* gene, were generated on <http://chopchop.cbu.uib.no/index.php>. The hits were organised by a score based on the criteria listed above. The two top hits closest to the PTEN phosphatase motif and with no predicted off-target sites, were chosen within exon 6 and 7 (PAM sequence in bold text, transcription initiating first G residue underlined):

- 1) 5'-GACTGGGAATAGTTACTCCCT**TGG**-3' (exon 6, TS1^{PTEN})
- 2) 5'-GAACTTGTCTTCCCGTCGTG**TGG**-3' (exon 7, TS2^{PTEN})

The TS1^{PTEN} target sequence was located within exon 6, contained 52% of GC-content and no self-complementary regions longer than three nucleotides. It targeted the sequence encoding amino acids 164-170 of PTEN protein, located within the phosphatase domain (aa 15-186). The off-target analysis showed that TS1^{PTEN} was unique in the human genome with no off-target sites predicted (with ≤3 nucleotide mismatches).

The TS2^{PTEN} target sequence consisted of 53% of GC-content and one self-complementary region longer than three nucleotides. It targeted the PTRREDKF sequence of PTEN protein (231-238 amino acids) in the C2 domain (186-351 aa). Mutations within this target sequence can be associated with cancer progression indicating that they are effectively KO mutations. A termination mutation at 1st R is frequently observed in human cancer, especially in the endometrium (233*, mutation ID COSM5154). The R234Q PTEN mutant found in glioblastoma fails to induce apoptosis, increases cell proliferation and leads to constitutively active Akt1 (Staal et al. 2002)). TS2^{PTEN} also was characterised as a unique sequence in the human

genome and the closest off-target site had three mismatches: CCAcGGAGTAACTcTTtCCAGTC (mismatches are shown in low case); this site is located on chromosome 10 and is 4566 bp away from the closest known coding sequence in the 5' direction, *SLC38A5* gene (encodes sodium-coupled neutral amino acid transporter 5). This protein acts as a sodium-dependent amino acid transporter which counter transports protons and mediates the pH-sensitive and electrogenic cotransport of several neutral amino acids with sodium (Nakanishi et al. 2001) and is 4436 bp away from 3' end of the gene *FTSJ1* (encoding a putative tRNA (cytidine(32)/guanosine(34)-2'-O)-methyltransferase that methylates the 2'-O-ribose of nucleotides at positions 32 and 34 of the tRNA anticodon loop of tRNAs (Ramser et al. 2004, Freude et al. 2004)).

These gRNA targets were ordered as a part of gBlocks bearing all components necessary for gRNA expression: (i) U6 promoter, (ii) the appropriate target sequence, (iii) Cas9-binding gRNA scaffold and (iv) transcription termination signal (Figure 3.1(B)) as described in (Mali et al. 2013b). gBlocks were A-tailed and cloned into an empty pGEM-T vector.

3.2.2. CRISPR/Cas9 transfection

In order to generate PTEN KO cell lines, it was important to utilise appropriate target cells. As a minimum, the cells employed should be human derived, as near normal as possible, immortalised and diploid (or near diploid) and importantly harbour two WT alleles of PTEN (PTEN^{+/+}). The majority of immortalised human cells are derived from cancer patients and are aneuploid. There are however, a small number of cells that meet the above criteria. HCT116 (human near diploid colorectal carcinoma epithelial immortalised cells (ATCC)), MRC-5 (human normal diploid lung fibroblasts, immortalised with SV40 (ATCC)) and PNT1A (human diploid post pubertal normal adult prostatic epithelial cells, immortalised with SV40 (Degeorges et al. 1995)) were all suitable and chosen for genetic engineering (Waldman et al. 2003, Bardelli, Di Nicolantonio & Arena 2012). HCT116 cells were used previously to create PTEN heterozygous and homozygous KO via Cre-Lox homologous recombination (Lee, Kim & Waldman 2004). HCT116 cells are known to be very robust in cell culture, suitable for somatic cell gene targeting and to have intact checkpoint responses (Waldman et al. 2003, Waldman 2016). However, HCT116 cells carry mutations in p110 α and p110 δ isoforms of PI3K catalytic subunit encoded by PIK3CA and PIK3CD (Dan et al. 2010). These mutations seem not to change PI3K activity, but increase cellular resistance to Growth Factor Deprivation Stress (GFDS) (Wang et al. 2007), reduce apoptosis and cellular dependence on growth factors, facilitate tumour invasion (Samuels et al. 2005) and raise resistance to the epidermal growth factor receptor inhibitor Cetuximab (Jhawer et al. 2008). According to these data, one can conclude that these mutations could affect the Akt pathway that could affect the PICS and synthetic lethality results. Keeping this in mind, HCT116 cells were still chosen for this study because they have

been shown to work efficiently for CRIPR/Cas9 gene targeting and a few published reports (targeting HCT116s with Cas9) were available at the beginning of this project (Smurnyy et al. 2014, Kim et al. 2014). Considering that other human diploid lines had not been so well studied in this context and we were one of the first applying CRISPR/Cas9 published protocols on human cells, we needed precedent of well-characterised model cell line. Consequently other two immortalised cell lines (MRC-5 and PNT1A) were selected for comparison and as non-tumourigenic models.

Cas9 and gRNA encoding plasmids were co-transfected into HCT116, MRC-5 and PNT1A cells using Lipofectamine 2000 transfection reagent according to the manufacturer's instructions. Both TS1^{PTEN} and TS2^{PTEN} gRNA plasmids were transfected simultaneously in ratio 1:1 in order to increase efficiency of targeting (earlier data generated in the laboratory indicated that only about 50% of guides tested actually worked (D. Mann, unpublished) so to maximise chances of making mutants and reduce screening, both gRNA were used together in this experiment). pBabePuro was co-transfected as a selectable marker to enable selection of transfected cells by the addition of puromycin. DNA:Lipofectamine 2000 ratio was optimised as 1:3 for HCT116, 1:2 or 1:5 for MRC-5 and 1:5 for PNT1A cells (data not shown). The composition of transfection mixture is shown in Table 3.1.

Table 3.1. Composition of Lipofectamine 2000 based transfection mixtures for CRISPR/Cas9 PTEN KO. Amounts are shown for cells cultured in 10 cm dishes.

	HCT116	MRC-5	PNT1A
DNA:Lipofectamine 2000 ratio	1:3	1:2 or 1:5	1:5
Total DNA, µg	24	48 or 24	12 or 24
DNA mix			
hCas9	5 parts	5 parts	5 parts
gRNA (TS1 ^{PTEN} +TS2 ^{PTEN})	5 parts	5 parts	5 parts
pBabePuro	1 part	1 part	1 part

On the day following transfection, cells were split at varying density and 0.4 µg/ml puromycin (puro) was added to select for resistant clones. The concentration of puromycin used was pre-determined via kill curve assay. Media was replaced every three days and after all non-transfected cells on a control plate were dead (~7-10 days), the selection was removed. Continued selection was not necessary as by this point the genomic engineering should have been complete and it was advantageous to loose the plasmids to help reduce off-target effects. Cells were then cultured until individual colonies could be identified and isolated. Colonies were collected on the 18th (for HCT116) – 40th (for MRC-5 and PNT1A) days following transfection and subsequently targeted genomic changes assessed via Western Blotting and PCR assays.

3.2.2.1. Western Blot - based analysis of KO clones

Individual transfectant colonies that were resistant to puromycin were assessed for PTEN status by Western Blotting. PTEN expression levels were compared to PTEN^{+/+}, PTEN^{+/-} and PTEN^{-/-} HCT116 cells obtained from Todd Waldman's group (Lee, Kim & Waldman 2004) ^{§§§}. Screening by Western Blotting would provide a qualitative evaluation of the levels of PTEN in the individual clones, allowing tentative assignment of each clone to homozygous wild type, heterozygous or homozygous null classes.

For each cell line (HCT116, MRC-5 and PNT1A), 20-30 individual transfectant clones that were resistant to puromycin were isolated and assessed for PTEN expression by Western Blotting. The cellular clones were lysed with by RIPA buffer from 80-90% confluent 24-well plate and loaded onto SDS-PAGE according to the protein amount in each sample (calculated with Bio-Rad DC Protein Assay). Separated proteins were subject to immunoblotting and probed with mouse anti-PTEN antibody (Cell Signalling Technology).

Intensities of the PTEN band in each sample were compared to the Todd Waldman's samples and partial decrease in signal was attributed to potential PTEN^{+/-} genotype, while complete loss of the band was characterised as a putative PTEN^{-/-} sample. As a result several clones in each KO condition (with very clear ~50% decrease in band intensities or complete loss of a signal) were characterised on the basis of HCT116 and MRC-5 cell lines, while only heterozygous KO was observed in PNT1A-based clones (Figure 3.2(A)). PTEN expression levels indicated that putative homozygous KO dominated over heterozygous within HCT116 (five versus two) and MRC-5 lines (eight versus one). However, only potential heterozygous PTEN^{+/-} PNT1A.P clones (five out of 22 tested) were identified by Western Blotting but no clones with homozygous KO were observed.

Then, in order to make a full PTEN KO in PNT1A cells, the putative heterozygous clone PNT1A.P was used for the second round of CRISPR/Cas9-directed genome editing. For this the cellular clone was co-transfected with Cas9 plasmid, both TS1^{PTEN} and TS2^{PTEN} gDNA plasmids and pBabeHygro expressing hygromycin (hygro) resistance gene (alternative resistance was required as PNT1A.P cells were puromycin-resistant after the first round of genome editing). The transfected cells were selected under 600 µg/ml hygromycin and analysed via Western Blotting. The second attempt appeared successful and resulted in a few homozygous PTEN^{-/-} clones (Figure 3.2(B)). The number of all PTEN KO clones in each cell line is given on Figure 3.2(C).

^{§§§} HCT116 cells with heterozygous and homozygous PTEN KO obtained via Cre-Lox homologous recombination (Lee, Kim & Waldman 2004), were kindly provided by Todd Waldman's group and were used as a positive control for PTEN KO cell lines created within this project and for their characterisation.

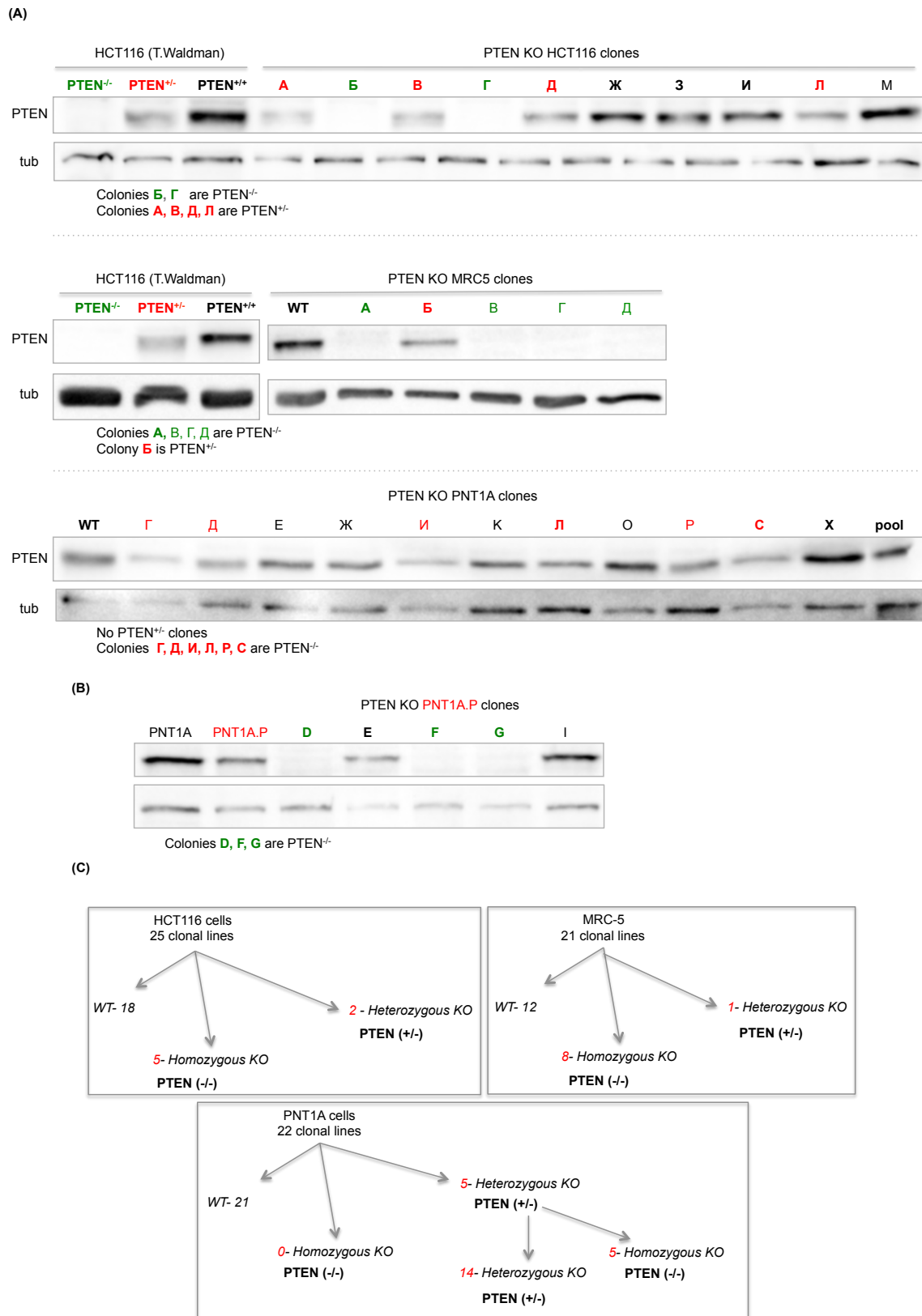


Figure 3.2. Western Blot analysis of individual clones isolated from transfected with plasmids directing PTEN KO in HCT116, MRC-5 and PNT1A cells. **(A)** Representative blots are shown for each cell line. The clones with apparent partial loss in PTEN expression (heterozygous KO lines) are highlighted in red and putative homozygous PTEN KO lines are shown in green. Letters above the gels indicate names of the clones. PTEN^{+/+}, PTEN^{+/-} and PTEN^{-/-} HCT116

Table 3.2. PCR conditions and two primer sets (TS1^{PTEN} and TS2^{PTEN}) for PCR screening of KO cell lines.

TS1 ^{PTEN}	Specific primers	T melting, °C	PCR product
	P1: GGGAGGAGAGTTATTCTGATATCCTTTG P2: TGGAAGGATGAGAATTTCAAGCAC	66.5 67.1	377 bp
TS2 ^{PTEN}	Specific primers	T melting, °C	PCR product
	P3': TTTGCTTGAGATCAAGATTGCAGA P4: TCCTTATTTGGATATTTCTCCCAATG	66.8 66.2	350 bp

Polymerase	GoTaq G2		
	T, °C	Duration	No of cycles
Initial denaturation	95	2 min	1
Denaturation	95	30 s	25
Annealing	62(TS2 ^{PTEN}) 64(TS1 ^{PTEN})	1 min	
Extension	72	1 min	
Final extension	72	5 min	1
Hold	4	Indefinite	-

PCR composition (50 µl)	
5 µl gDNA	(from ~12500 cells)
1x Reaction Buffer	
200 ng each primer	
500 µM each dNTP	
1 M betaine	
0.25 µl polymerase	

Initially the annealing temperature was optimised for both primer sets by performing individual PCR reactions at varying annealing temperatures (Figure 3.4 and data not shown) and afterwards all PCR products were purified, concentrated and submitted for sequencing.

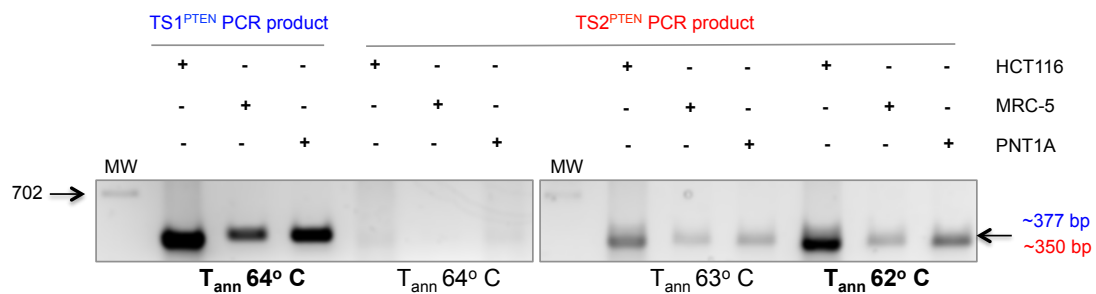


Figure 3.4. Agarose gels with PCR products amplified with TS1^{PTEN} or TS2^{PTEN} primer sets on the basis of WT HCT116, MRC-5 or PNT1A gDNA. Bands representing TS1^{PTEN} (377 bp) or TS2^{PTEN} (350 bp) amplicons are indicated with an arrow. Optimal annealing temperature (i.e. the temperature at which the single expected amplicon was maximised) was determined to be 64° C for TS1^{PTEN} primers and 62° C for TS2^{PTEN} primers. MW – DNA ladder.

Then, gDNA was purified from ten HCT116 clones, ten MRC-5 clones and eight PNT1A clones, following by PCR amplification with both primer sets (TS1^{PTEN} and TS2^{PTEN}) on each of these isolated gDNA samples. Resulting sequencing data were compared to sequencing data from WT parental lines. As expected in the case of disrupted target site mixed sequencing data were observed representing a combination of two alleles that were repaired non-identically after the Cas9-mediated DSB (Figure 3.5).

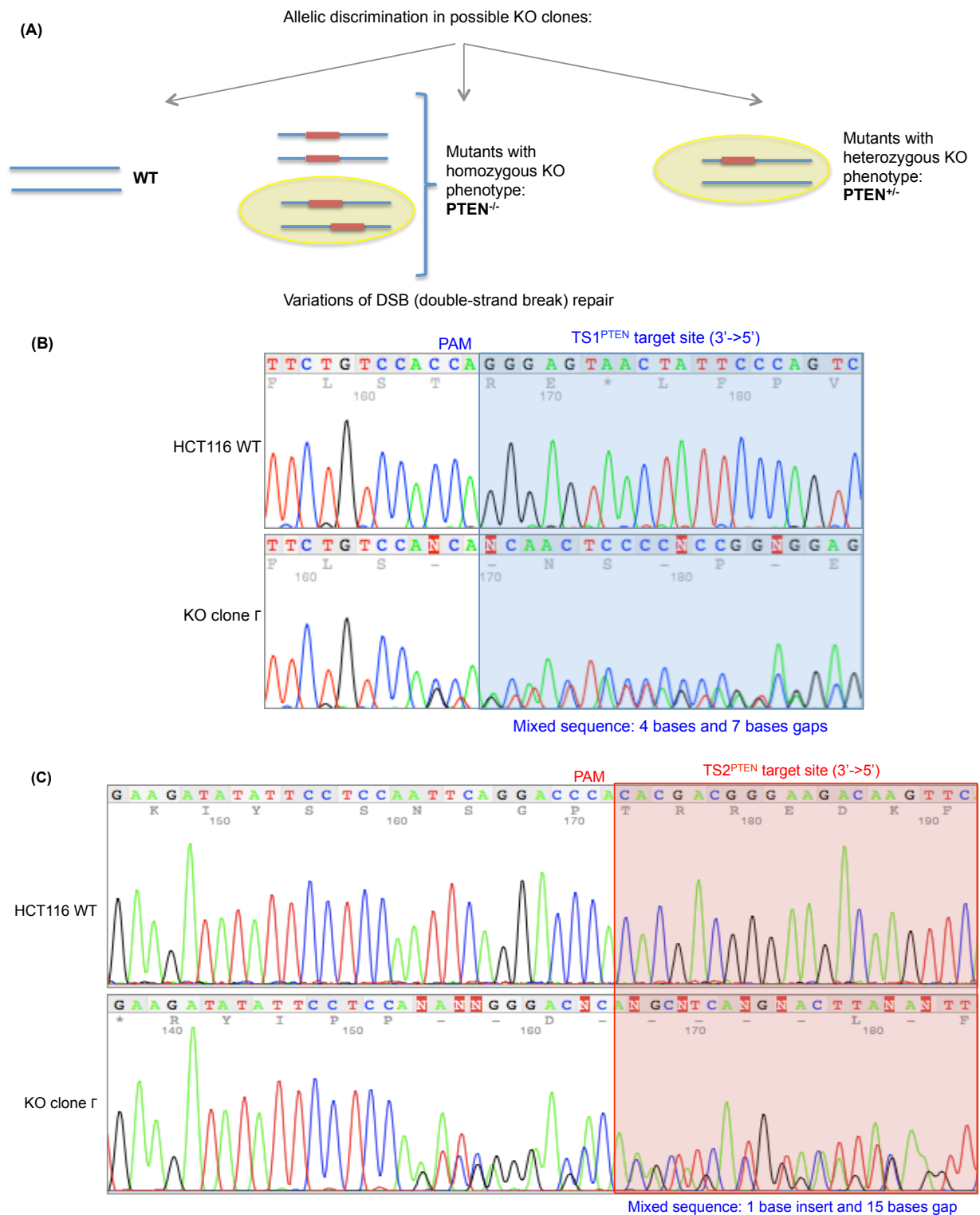
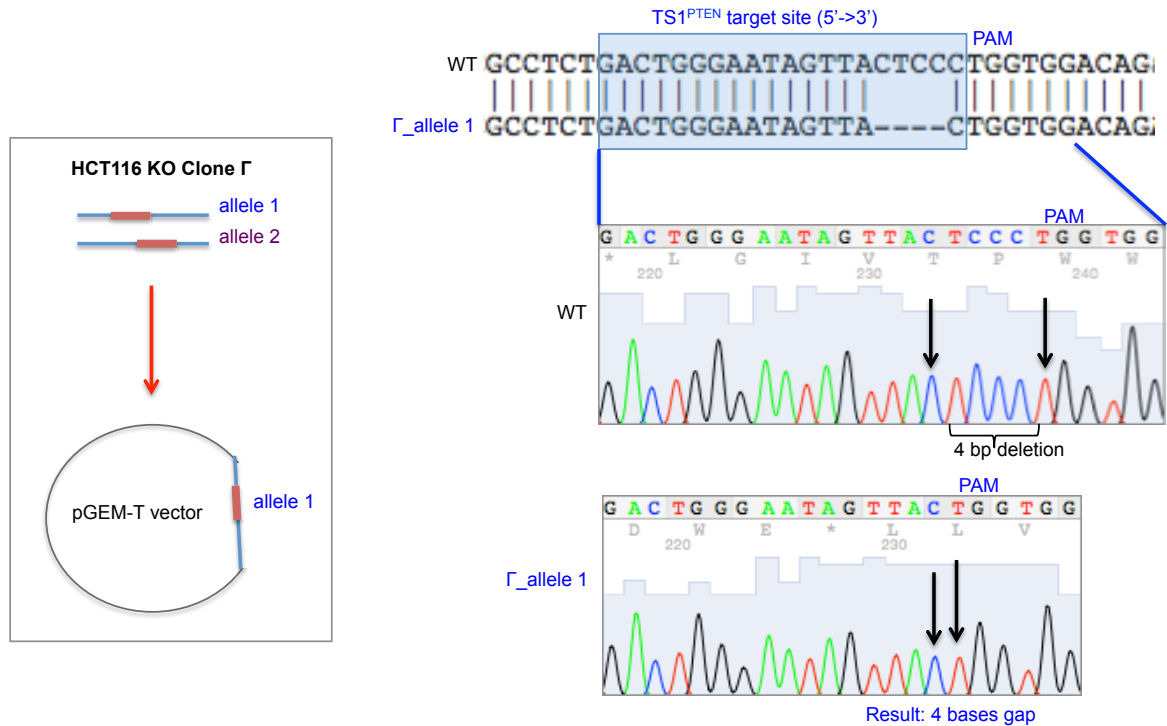


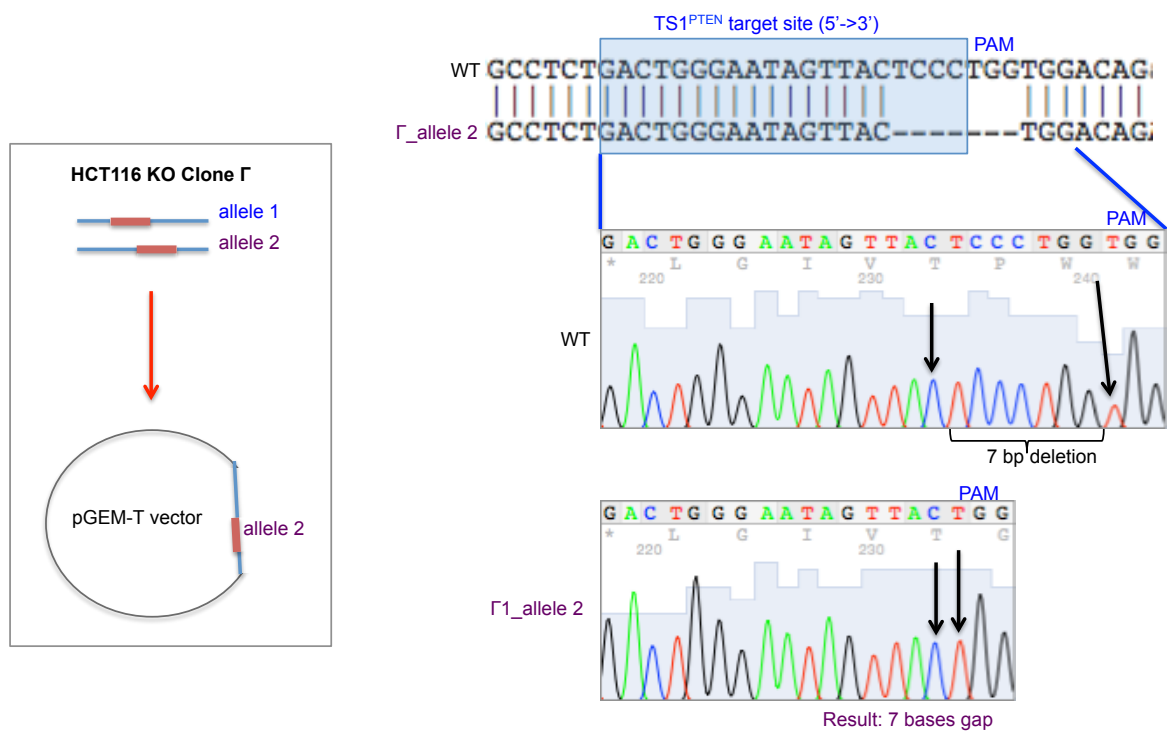
Figure 3.5. **(A)** Schematic view of DNA repair after Cas9-mediated DSB. Deletions or insertions (indels) can occur either identically in both DNA alleles or differently (highlighted with ovals). **(B)**, **(C)** In case of non-identical repair, mixed sequence is observed on sequencing chromatograms. Non-mixed DNA sequence from identical alleles in WT cells and mixed sequence from differently repaired alleles at the CRISPR target site are shown for **(B)** TS1^{PTEN} target site and **(C)** TS2^{PTEN} target site in HCT116 WT compared to HCT116 KO Clone Γ .

To discriminate between non-identical alleles, all PCR amplicons were cloned into empty pGEM-T vector backbone and transformed into E.coli DH5 α . This method would allow to distinguish between different alleles as only single allele can be integrated into the vector backbone and subsequently transformed into a bacteria cell. Plasmid integrity was confirmed by restriction analysis (with SphI/SacI) and the DNA was sequenced. About 10 mini-preps for each KO clone were analysed and insert KO DNA sequence was compared to the WT PCR sequence. A representative scheme of KO Clone Γ alleles is shown on Figure 3.6.

(A)



(B)



(C)

	TS1 ^{PTEN} target site (5'→3')	PAM
HCT116_WT	GCCTCTGACTGGGAATAGTTACTCCCTGGTGGAC	
HCT116_Γ.1	GCCTCTGACTGGGAATAGTTA-----CTGGTGGAC	
HCT116_Γ.6	GCCTCTGACTGGGAATAGTTA-----CTGGTGGAC	
HCT116_Γ.3	GCCTCTGACTGGGAATAGTTAC-----TGGAC	
HCT116_Γ.4	GCCTCTGACTGGGAATAGTTAC-----TGGAC	
HCT116_Γ.5	GCCTCTGACTGGGAATAGTTAC-----TGGAC	

Figure 3.6. Representative scheme of sequencing results for two non-identical alleles based on HCT116 Clone Γ . After discrimination via pGEM-T cloning, one allele demonstrated a 4 bp

deletion **(A)**, and the second allele housed a 7 bp deletion **(B)** in the TS1^{PTEN} PTEN target locus. **(C)** 5 different pGEM-T preps from the HCT116 Clone Γ confirmed the presence of only these two indels: 4 or 7 bp gaps in the Clone Γ alleles. (Under a tolerance of 5%, a positive result obtained in all 5 samples implies a probability of error equal to $1 - 0.95^{1/5} = 1\%$).

Allele discrimination confirmed that the KO clones could be divided in two groups according to the type of DSB repair: (i) non-identical repair in both alleles (Figure 3.6); and (ii) identical repair in both alleles (Figure 3.7).

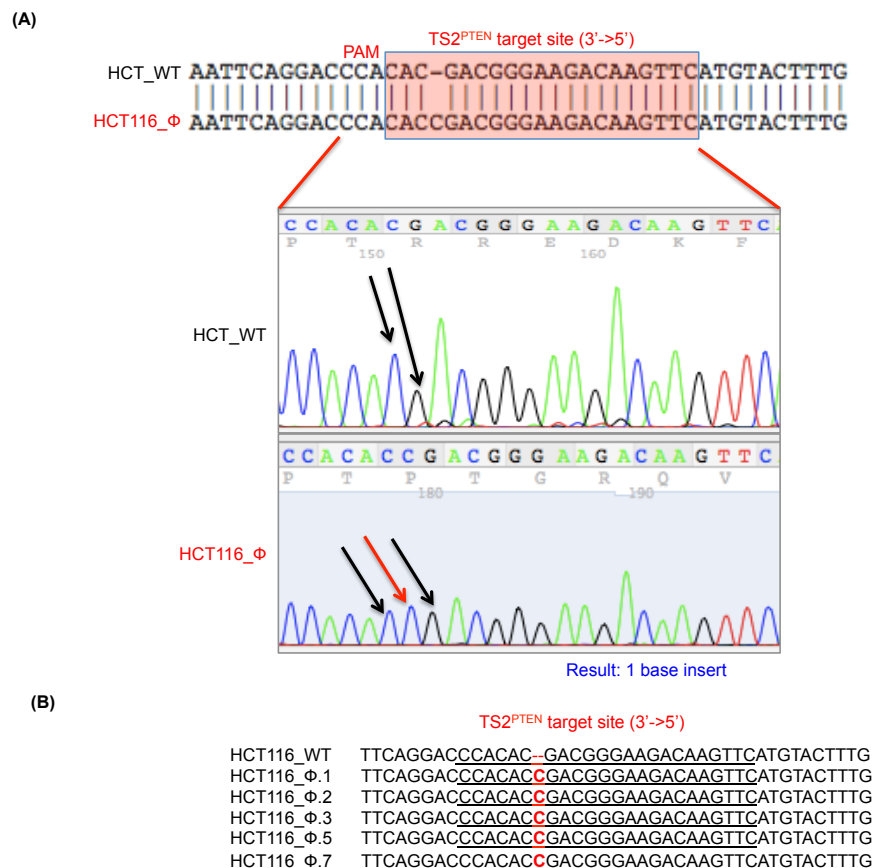


Figure 3.7. TS2^{PTEN} Target PCR product on the basis of Clone Φ HCT116. **(A)** Comparison of the TS2^{PTEN} Target PCR product sequence taken from Clone Φ HCT116 vs WT HCT116. **(B)** All individual alleles incorporated into pGEM-T vector were carrying a 1 bp insert.

Interestingly, both TS1^{PTEN} and TS2^{PTEN} guide sequences worked well in HCT116 and MRC-5 cells. Due to this there were lots of KO clones with the disruption in both alleles via these two gDNA, making it hard to find a clone that had one unaffected allele to get a PTEN^{+/-} genotype. Luckily, Clone Δ for HCT116 and Clone E for MRC-5 cells had one allele unaffected, which was confirmed by both sequencing and Western Blot analysis.

However, all PNT1A clones from the first KO round were determined to have only wild type PTEN when PCR sequencing analysis was applied. That was surprising considering that Western Blot analysis showed an obvious decrease of PTEN expression levels in several clones including Clone P that was used as a template cell line for the second KO round (Figure

3.2(A,B)). One possible explanation for this discrepancy was that the deletion caused by genome editing extended into one of the PCR primers so that the mutation would not be amplified upon PCR. To check this, new sets of TS1^{PTEN} and TS2^{PTEN} PCR primers were designed to yield longer products (Figure 3.8, Table 3.3).

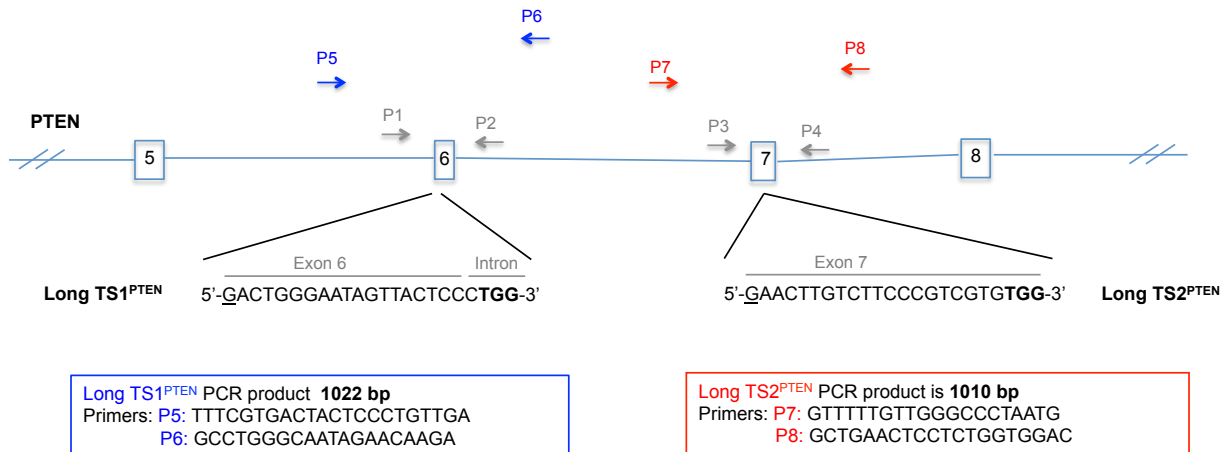


Figure 3.8. The location of two primer sets (Long TS1^{PTEN} and Long TS2^{PTEN}) to amplify longer PCR products for screening of PTEN KO lines. Two primer sets used before (TS1^{PTEN} and TS2^{PTEN}) shown in grey font.

Table 3.3. PCR conditions and two primer sets (TS1^{PTEN} Long and TS2^{PTEN} Long) for PCR screening of PNT1A clones. Annealing temperature was optimised as 64° C for both primer sets.

Long TS1 ^{PTEN}	Specific primers		T melting, °C	PCR product
	P5: TTTCGTGACTACTCCCTGTTGA	P6: GCCTGGGCAATAGAACAAGA	63.5 64.0	1022 bp
Long TS2 ^{PTEN}	P7: GTTTTTGTGGGCCCTAATG	P8: GCTGAACTCCTCTGGTGGAC	62.9 63.9	1010 bp

Polymerase	GoTaq G2		
	T, °C	Duration	No of cycles
Initial denaturation	95	2 min	1
Denaturation	95	30 s	25
Annealing	64	1 min	
Extension	72	70 s	1
Final extension	72	5 min	
Hold	4	Indefinite	-

PCR composition (50 µl)
5 µl gDNA (from ~12500 cells) 1x Reaction Buffer 200 ng each primer 500 µM each dNTP 1 M betaine 0.25 µl polymerase

Unfortunately, neither the agarose gel (Figure 3.9) nor sequencing could distinguish the difference between KO clones and WT PNT1A PCR products using the longer primer sets.

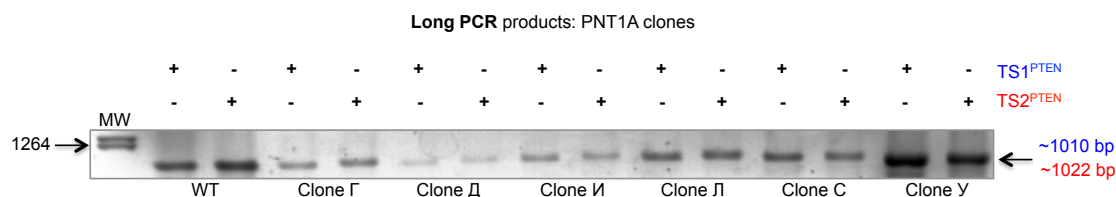


Figure 3.9. Representative agarose gel with PCR products from several PNT1A clones from the first KO round amplified with Long TS1^{PTEN} or TS2^{PTEN} PCR primers. Only a single band corresponding to the full-length WT products (1010 for TS1^{PTEN} product or 1022 for TS2^{PTEN} product) was observed in each case indicating that large deletions or insertions were unlikely to have occurred within these target sequences.

Thus, the original hypothesis was highly unlikely suggesting that another mechanism was responsible for the consistent reduction in the level of PTEN protein expression observed. There is a possibility that because individual clones were selected then it is likely that some of these cells express less PTEN normally (i.e. the starting population is heterogeneous). However, this PNT1A clone P with apparent heterozygous levels of PTEN showed stable decreased expression levels of PTEN compared to WT over time and so was potentially useful as a heterozygous mimic for further experimentation (even if we could not prove the genomic heterozygous status). Interestingly, even though the second KO round gave far greater efficiency for the homozygous KO than the first round, no heterozygous clones were found. The summary of all KO clones analysed by PCR sequencing is given in Table 3.4, representing the sequence analysis of 24 individual clones from HCT116, MRC-5 and PNT1A cells, each of which subject to allele discrimination via sequencing of around 10 individual pcr products cloned into pGEM-T vector backbones for both TS1^{PTEN} and TS2^{PTEN} primer sets per clone. The majority of indels were short insertions or deletions, although there were cases when long insertions either from pGEM-T vector backbone (as in Clone PNT1A-PG) or from *E.coli* gDNA (as in Clone MRC-5-Π) were detected.

Table 3.4. Summarised results from the sequencing analysis of KO clones established in HCT116, MRC-5 and PNT1A cells. The TS1^{PTEN} and TS2^{PTEN} PCR products were cloned into the pGEM-T vector and DNA alleles were sequenced individually. The clones that were selected for further experiments are highlighted in green (PTEN^{-/-} clones) and orange (PTEN^{+/-} clones). Each allele was confirmed out of at least five PCR products. Under a tolerance of 5%, a positive result obtained in ≥ 5 samples implies a probability of error equal to $\leq 1 - 0.95^{1/5} = 1\%$.

HCT116	TS1 ^{PTEN} target site		TS2 ^{PTEN} target site		Genotype
	Allele 1	Allele 2	Allele 1	Allele 2	
Clone Г	4 bp gap	7 bp gap	1 bp insert	15 bp gap	PTEN ^{-/-}
Clone Ф*	1 bp insert	1 bp insert	1 bp gap	1 bp gap	PTEN ^{-/-}
Clone Ц*	1 bp gap	1 bp gap	1 bp insert	1 bp insert	PTEN ^{-/-}
Clone Д	WT	17 bp gap	WT	3 bp gap	PTEN ^{+/-}
Clone Л	Appeared to be a mixture of 2 or more clones				-
Clone A	WT	WT	WT	WT	PTEN ^{+/+}
Clone У	WT	WT	WT	WT	PTEN ^{+/+}
Clone X	WT	WT	WT	WT	PTEN ^{+/+}
Clone Ш	WT	WT	WT	WT	PTEN ^{+/+}
Clone B	WT	WT	WT	WT	PTEN ^{+/+}
MRC-5	TS1 ^{PTEN} target site		TS2 ^{PTEN} target site		Genotype
	Allele 1	Allele 2	Allele 1	Allele 2	
Clone B	1 bp gap	3 bp gap	1 bp gap	8 bp gap	PTEN ^{-/-}
Clone Д*	1 bp gap	1 bp gap	1 bp insert	1 bp insert	PTEN ^{-/-}
Clone П*	1 bp gap	1 bp gap	320 bp insert	320 bp insert	PTEN ^{-/-}
Clone Г*	2 bp gap	2 bp gap	1 bp insert	1 bp insert	PTEN ^{-/-}
Clone H*	1 bp gap	1 bp gap	1 bp insert	1 bp insert	PTEN ^{-/-}
Clone И*	2 bp gap	2 bp gap	3 bp gap	3 bp gap	PTEN ^{-/-}
Clone Ё	WT	WT	WT	33 bp gap	PTEN ^{+/-}
PNT1A	TS1 ^{PTEN} target site		TS2 ^{PTEN} target site		Genotype
	Allele 1	Allele 2	Allele 1	Allele 2	
1st KO round					
All clones**	WT	WT	WT	WT	PTEN ^{+/+}
2nd KO round					
Clone PG	1 bp insert	1 bp gap	1 bp insert	593 bp insert	PTEN ^{-/-}
Clone PA	1 bp insert	1 bp gap	1 bp insert	17 bp gap	PTEN ^{-/-}
Clone PB*	1 bp insert	1 bp insert	WT	WT	PTEN ^{-/-}

* Identical alleles in both alleles

** But some of these clones were clear PTEN^{+/-} by Western Blotting

We originally employed two gRNAs as we had found that only about 50% of designed guides were functional and so employing two gRNAs in one experiment would substantially reduce the cell culture involved. However, in hindsight using the 2 gRNAs simultaneously was not ideal as it complicated the subsequent analysis of mutants. One illustration of this comes through analysis of Clone Д. This clone had one wild type allele for each PCR reaction and one allele housing an indel. Thus there are two possible functional combinations of these indels, as shown in Figure 3.10: both may be in the same allele or they may be in different alleles, making genetic assignment of the PTEN status difficult.

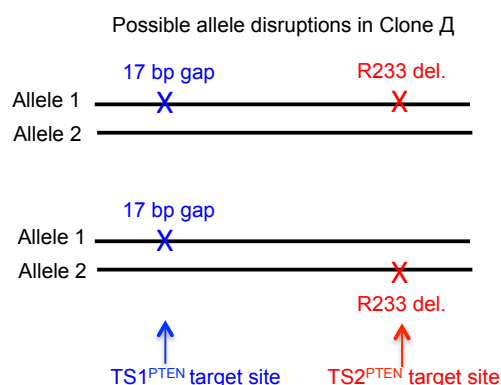


Figure 3.10. Possible allele compositions in HCT116 Clone D.

This clone has the further compounding difficulty in that one of the indels was a 3 bp gap. This gap occurred in-frame causing Arg233 deletion in PTEN protein. Arg233 is located within the lipid-binding C2 domain and its nonsense mutation (i.e. inactivating mutation onto chain-terminating codon (R233*)) is frequently found in various human cancers arising in the endometrium, brain, cervix, colorectal, breast and prostate (Duerr et al. 1998, Song, Salmena & Pandolfi 2012, M Dillon, W Miller 2014, Loures et al. 2014, Ngeow et al. 2014). Moreover, this mutation is commonly correlated with Cowden Syndrome (also known as multiple hamartoma syndrome) (Neychev et al. 2015). Neychev *et al.* speculated that the R233* mutation in one of the PTEN alleles could be sufficient cause of an abnormal cytoplasmic subcellular compartmentalisation or exercise a dominant-negative effect over wild-type protein, triggering the development of the pancreatic neuroendocrine tumour in patients with Cowden Syndrome. Thus, R233* mutation was relatively well characterised in literature, but there are no data of the functional effects of deletion of this amino acid to our knowledge. It is difficult to conclude effects of this deletion as R233 is in a short stretch of beta sheet amid loops that may or may not tolerate its deletion (Figure 3.11).

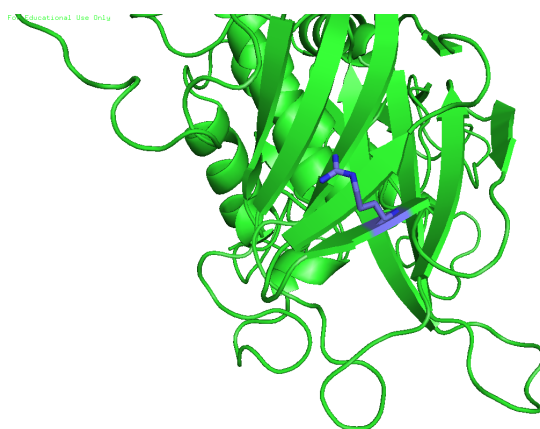


Figure 3.11. Position of the R233 amino acid on the PTEN structure diagram. R233 is highlighted in purple. The diagram is modelled on Pymol (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC).

Thus there are several possibilities of allele disruption and their functional impact on HCT116 Clone Д. The combination of these two indels may disrupt protein stability, affect protein expression or completely stop PTEN function (in case if 14 bp indel and R233* mutation occurred on separate alleles and the R233* mutation disrupted correct folding of the protein or protein activity). Alternatively, Clone Д could have a *PTEN*^{+/-} genotype (if two indels happened on the same allele or if R233* would not disrupt PTEN activity when on separate alleles).

On the basis of the immunoblotting data and PTEN activity assay (discussed later in this work, see Chapter 5.5.2, Figure 5.26(A)), it appears likely that the later possibility has occurred and genetic lesions were both within a single allele or if on different alleles, than R233* mutation did not affect PTEN activity.

For further studies following clones were chosen:

- 1) HCT116_WT (*PTEN*^{+/+}); HCT116_Д (*PTEN*^{+/-}); HCT116_Г and HCT116_Ф (*PTEN*^{-/-})
- 2) MRC-5_WT (*PTEN*^{+/+}); HCT116_Б (*PTEN*^{+/-}); HCT116_В (*PTEN*^{-/-})
- 3) PNT1A_WT (*PTEN*^{+/+}); PNT1A_П (*PTEN*^{+/-}); PNT1A_РГ (*PTEN*^{-/-})

Summarised analysis of these chosen clones by PCR and Western Blot assay is shown on Figure 3.12.

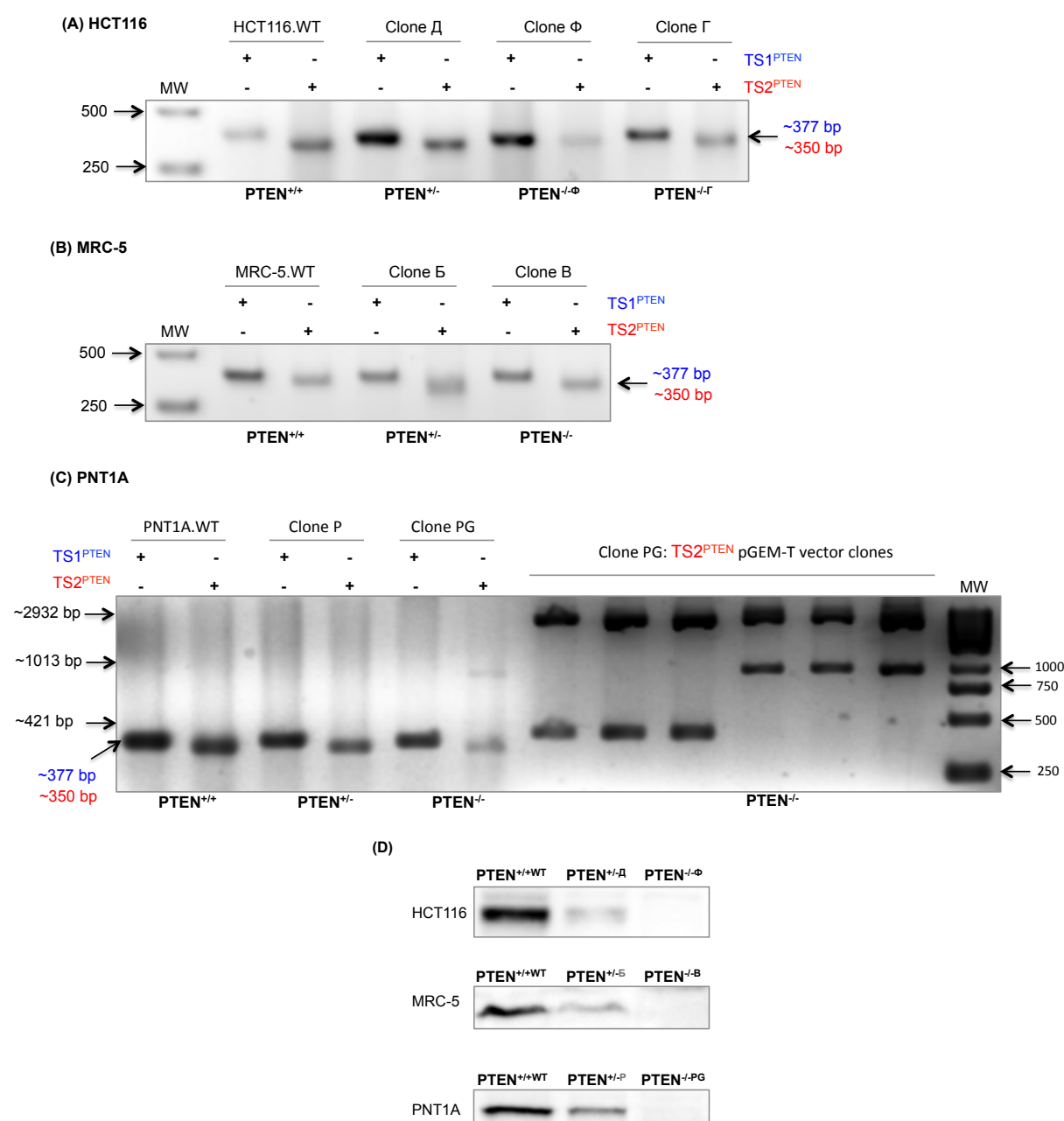


Figure 3.12. PCR and Western Blot assay summary for the KO clones selected for further study. **(A) – (C)** Agarose gels with TS1^{PTEN} or TS2^{PTEN} PCR products for each clone based on HCT116 **(A)**, MRC-5 **(B)** and PNT1A **(C)** cells. The PNT1A-PG clone harboured a 593 bp insert within one allele and 1 bp insert - in another allele (within TS2^{PTEN} target PCR site), thus there were two well-distinguished TS2^{PTEN} PCR products visible on the gel. These products were separated via cloning into the pGEM-T vector and then digested with SphI/SacI enzymes. The resulting products were 2932 bp (empty vector backbone), 1013 bp (one TS2^{PTEN} PCR allele with 593 bp) and 421 bp (another TS2^{PTEN} PCR allele with 1 bp insert). MW – DNA ladder (GeneRuler 1 kb, ThermoFisher Scientific). **(D)** Western Blot results for selected PTEN KO clones.

3.3. Conclusions

Using the CRISPR-Cas9 system, genome editing was applied to the PTEN gene in three human diploid cell lines (HCT116, MRC-5 and PNT1A) and heterozygous ($PTEN^{+/-}$) and homozygous ($PTEN^{-/-}$) KO lines were generated. Several homozygous KO were created in each cell line but it proved much more difficult to isolate cells in the heterozygous state. This result was likely to have arisen through the use of two distinct gRNAs. The mutations generated were characterised at the protein level and the genomic level for each KO event. The strategy of using two gRNAs appeared to maximise the likelihood of generating complete KO of the target gene and reducing the number of transfectants to screen. However these advantages must be offset by the difficulty in assigning allele specific changes and the potential for increased off target effects when using multiple guides.

4. Results and Discussion: PTEN Mutant Construction

4.1. Introduction

PTEN has multiple functions and intracellular properties and other reports have demonstrated that several of these can be specifically inactivated by introducing individual point mutations into the PTEN coding sequence. These mutations are summarised in Table 4.1 and schematically shown in Figure 4.1. These point mutations have been generated in order to introduce them into PTEN^{-/-} cell lines and study PTEN structural requirements in cellular senescence and lethal sensitivity experiments.

Table 4.1. PTEN mutants for integration into PTEN^{-/-} cell lines

Mutant	References	Characteristics
C124S	Li <i>et al.</i> 1998; Lee <i>et al.</i> 1999	Catalytically dead (lacking both lipid and protein phosphatase activity)
G129E	Vivanco <i>et al.</i> 2002	Lacking lipid phosphatase activity only
H93A	Lee <i>et al.</i> 1999	Reducing phosphatase activity towards PtdIns(3,4,5)P ₃ but not to PtdIns(3,4)P ₂
V403A	Valiente <i>et al.</i> 2005	Mutation in the functional PDZ domain
K13E/K289E	Song <i>et al.</i> 2008	Cytoplasmically constrained mutant (no nuclear localisation)

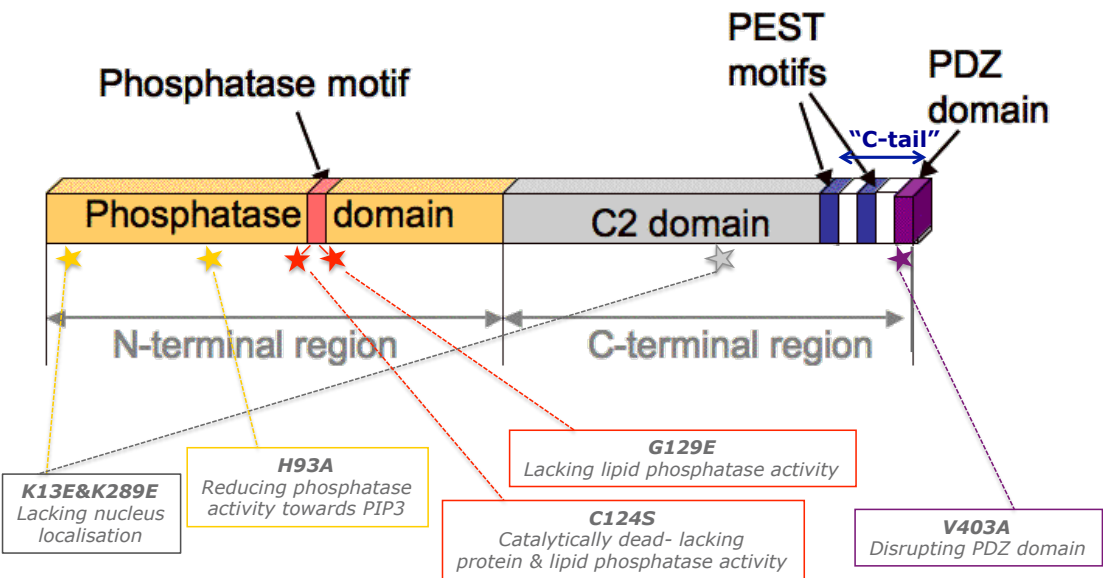


Figure 4.1. Schematic localisation of PTEN point mutations. PIP3 – PtdIns(3,4,5)P₃.

The K13E, H93A, C124S, G129E and K289E PTEN mutants have been identified in human cancers. The C124S and G129E mutations located in the phosphatase domain have been found in patients with Cowden Syndrome (also known as multiple hamartoma syndrome). Indeed, the C124S mutant, that lacks both lipid and protein phosphatase activity, and G129E mutant, that lacks the lipid phosphatase activity only, do not inhibit cell proliferation and abolish

PTEN tumour-suppressor function (Li, Sun 1998, Lee et al. 1999, Vivanco, Sawyers 2002, Eng 2003). These two mutations allowed distinguishing between two phosphatase functions of PTEN. Thus, the G129E mutation does not affect PTEN's ability to dephosphorylate protein targets but still is found in patients with Cowden Syndrome, suggesting that loss of lipid-phosphatase activity is sufficient to lead to the clinical cancer phenotype (Myers et al. 1998). The G129E mutant also fails to initiate G1 arrest, implying that the protein-phosphatase activity is not sufficient for inhibition of cell cycle progression. On the other hand, the protein-phosphatase activity of PTEN is sufficient for inhibiting cell migration, because the G129E PTEN mutation does not disrupt the inhibition of cell spreading, while the C124S mutant failed to inhibit cell spreading (Tamura et al. 1998).

A highly frequent cancer-derived H93A mutation, that is located in the WDP-loop, disrupts substrate specificity towards $\text{PtdIns}(3,4,5)_3$ over $\text{PtdIns}(3,4)\text{P}_2$ (Lee et al. 1999). This mutation leads to $\text{PtdIns}(3,4,5)_3$ build-up and failure to suppress cell proliferation in LNCaP cells expressing the H93A mutant (Georgescu et al. 2000); while U87-MG glioblastoma cells transfected with the same PTEN mutant proliferated in clusters lacking cell-cell contact inhibition and formed big colonies in soft agar compared to cells transfected with PTEN_WT (Lee et al. 1999). These findings suggested that the dephosphorylation of $\text{PtdIns}(3,4,5)_3$ was more important than the dephosphorylation of $\text{PtdIns}(3,4)\text{P}_2$ for the tumour suppression of PTEN.

The V403A mutation is responsible for PTEN PDZ domain disruption. This domain ensures PTEN stabilisation at particular subcellular localisations and PTEN protein-protein interactions. Mutation of the V403 residue to alanine disrupts PTEN stability and its ability to bind to consensus motifs of partner proteins that facilitate PTEN-mediated $\text{PtdIns}(3,4,5)_3$ hydrolysis such as MAGI-1,2,3 (members of the membrane-associated guanylate kinase family) and MAST205 (Ser/Thr kinase) (Simpson, Parsons 2001, Valiente et al. 2005).

K13E PTEN mutant has been isolated from cases of sporadic glioblastoma (Walker et al. 2004). This mutation blocks PTEN-lipid binding and thus its recruitment to the membrane. Another lysine mutant of PTEN, K289E associated with Cowden Syndrome, also fails to accumulate in nuclei due to an import effect (Eng 2003, Trotman et al. 2007). The combination of K13E and K289E impairs PTEN nuclear localisation and nuclear function (Song et al. 2008). This is possible due to the fact that K13 and K289 residues are the main PTEN monoubiquitylation sites. In wild type PTEN K13 and K289 residues are monoubiquitinated by an E3 ligase for further trafficking to the nucleus where it is deubiquitinated by HAUSP (herpesvirus-associated ubiquitin-specific protease). Mutations of these residues to Glu constrain PTEN to cytoplasmic localisations only.

These PTEN mutants were chosen because they each disrupt a single PTEN function/property that can be studied individually and then associated to a particular cancer phenotype.

Introducing these mutants in the generated PTEN KO cell systems would allow analysis of various PTEN functions in isolation and provide an understanding of the PTEN properties that are involved in PICS and lethal sensitivity mechanisms.

Two techniques based on the polymerase chain reaction (PCR) were tested for the generation of PTEN mutants: a) QuikChange site-directed mutagenesis and b) Two-step site-directed mutagenesis. Two-step PCR method was more reliable in generating the desired mutations.

4.2. Mutagenesis strategies

Two mutagenesis methods were carried out to generate six PTEN mutants: C124S, G129E, H93A, V403A and K289E with this latter mutation being used as the template for creating the double K289E/K13E mutant. The methods are schematically described on a Figure 4.2.

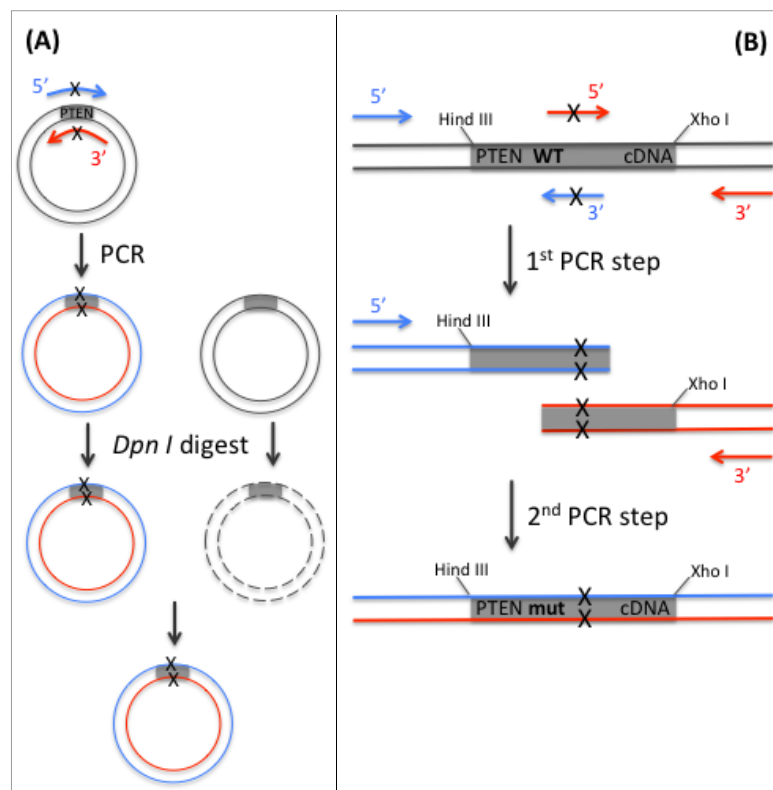


Figure 4.2. PTEN mutagenesis strategies. **(A)** QuikChange mutagenesis. PCR elongation of template DNA with mutagenic primers 5' and 3' is followed by DpnI digestion, a restriction enzyme targeting methylated (parental) DNA only **(B)** Two-step site-directed mutagenesis. On the first PCR round, two mutant products are amplified using two sets of oligonucleotide primers: 1) 5' (vector-specific) and 3' (PTEN-specific carrying mutation) – shown in blue; 2) 5' (PTEN-specific carrying mutation) and 3' (vector-specific) – shown in red. These two amplification products can hybridise through the complementary region and serve as a template in the second PCR round with external vector-specific 5' and 3' primers. Final amplification product carries the desired PTEN mutation (indicated by crosses). HindIII and XhoI restriction sites flank PTEN cDNA.

In the first instance, the QuikChange site-directed mutagenesis was chosen to introduce all mutations as it was previously used for constructing PTEN mutations in the literature: G129E and C124S in (Myers et al. 1997) ; C124S in (Shen et al. 2007). QuikChange is a fast and relatively straightforward one-day mutagenesis method (Figure 4.2(A), (Papworth, Greener & Braman 1994, Carrigan, Ballar & Tuzmen 2011)). Briefly, mutant-strand PCR amplification is followed by DpnI digestion of the parental methylated DNA template. This technique was carried out with PfuUltra II fusion HS DNA polymerase (Agilent, USA) following the reaction conditions listed in Materials and Methods (Table 2.3(A)) according to the recommendations in the Agilent manual (QuikChange Site-Directed Mutagenesis Kit, Agilent, USA). All mutagenic oligonucleotide primers were designed to carry a specific PTEN mutation and generate silent mutations to introduce a diagnostic restriction site for a quick screening of resulting constructs. The complete list of specific primers is shown in Table 4.2. PTEN cDNA flanked with HindIII and XhoI restriction sites within the pcDNA3 vector was chosen as a template construct (pcDNA3-PTEN was previously synthesised by Ms Harriet Rungay, Figure 4.3(A)).

Table 4.2. Primers designed for site-directed PTEN mutagenesis. Desired mutations are shown in red and silent mutations introducing diagnostic restriction sites are shown in blue. 5' primer initiates PCR amplification on the sense DNA strain in 5' -> 3' direction, while 3' primer was designed complimentary to the anti-sense DNA allele. pcDNA3 vector-specific primers are the same for all mutants except K13E and V403A due to their close proximity to the start and end of the PTEN cDNA. Thus these mutations have been incorporated in the external primers (5' for K13E and 3' for V403A). The primers read from 5' to 3'. Specific restriction enzymes are provided for each mutant.

Mutant		Specific primers	T melting, °C	Restriction enzyme
C124S	Short	5': CAATTCAC TCT AAAG CCGGC CAAGGGACG	76.5	NaeI
		3': GTCCTT GCCGGC TTT AG AGTGAATTGC	75.9	
	Overhang	5': CAATTCAC TCT AAAG CCGGC CAAGGGACGAACTGGTG	82.0	
		3': GTCCTT GCCGGC TTT AG AGTGAATTGCTGCAAC	80.8	
G129E	QuikChange recommended	5': ATGTTGCAGCAATTCA TCT AAAG CCGGC CAAGGGACGAACTGGTGT	86.7	NaeI
		3': ACACCA GTTCG TCCCTT GCCGGC TTT AG AGTGAATTGCTGCAACAT	86.7	
	Short	5': CACTGTAAAG CCGGC CAAG GAA CGAACTGGTG	79.5	
		3': CACCA GTTCG T TCTT GCCGGC TTTACAGTG	79.5	
H93A	Overhang	5': CACTGTAAAG CCGGC CAAG GAA CGAACTGGTGTAATGATATG	80.1	—*
		3': CAGTT CGT TCTT GCCGGC TTTACAGTGAATTGCTG	81.4	
	Short	5': CCTTTTGAAG GAT GCT AATC CACCACAGC	76.5	
		3': GCTGTGGTG GATT AGCATC TTCAAAGG	75.9	
K289E	Overhang	5': CTTTGAAG GAT GCT AATC CACCACAGCTAGAAC	72.1	AatII
		3': CTGTGGTG GATT AGCATC TTCAAAGGATATTG	72.4	
	Short	5': GACCAGAGGAG ACGTC AGAA GAA GTAGAAAATG	72.0	
		3': CATTTTCTAC TTCTCT GACGTC CCTCTGGTC	72.0	
V403A	Overhang	5': GAGGAG ACGTC AGAA GAA GTAGAAAATGG	68.5	StuI
		3': CTAC TTCTCT GACGTC CCTCTGGTCCTG	72.1	
K13E		3': GCTCGAGTCA GGCCT TTGTAATTTGTGTATG	73.0	XhoI
Vector-specific primers		5': CAAGCTTATGACAGCCATCATCAAAGAGATCGT CTCGAG AAAC GAA AGG	83.9	
		5': CGGTGGGAGGTCTATATAAGCAGAGCTCTC	72.1	
		3': CTGGCAACTAGAAAGCAGTCGAGGC	75.5	

* There was no silent mutation generated for H93A PTEN mutant due to lack of possible restriction sites with the common enzymes that could be used for the diagnostic digestion of this mutant.

It was decided to start with only one mutation: PTEN_C124S. In order to introduce this mutation into pcDNA3-PTEN construct via QuikChange method, three sets of specific primers

were tested: a) short primers; b) primers with longer overhang to promote annealing to the template (Liu, Naismith 2008) ; and c) significantly longer primers designed according to Agilent's recommendations (Table 4.2). PCR mixture after template DNA digestion with DpnI (enzyme which is specific for methylated DNA) was transformed into *E.coli* DH5 α competent cells. Very few colonies were observed in experiments with all primers and when the plasmids isolated from these colonies were analysed by restriction digest, they did not correspond to the expected product. An attempt to use higher concentration of template plasmid (15-75 ng) lead to the increased number of colonies formed after transformation. However, they appeared to carry a wild type PTEN template. Thus, neither of the tested QuikChange conditions resulted in a successful mutated construct.

Having attempted QuikChange mutagenesis with little success, a two-step PCR mutagenesis method (Figure 4.2(B), (Ho et al. 1989, Carrigan, Ballar & Tuzmen 2011)) was carried out with several primers sets, polymerases and thermocycle conditions.

Initially, the annealing temperature was optimised for each mutant on the first round of PCR amplification with PfuUltra II Fusion HS polymerase. The sharpest and brightest bands of predicted size were observed for both 5' and 3' fragments of each mutant at the optimal annealing temperatures with overhang primers (Figure 4.3(B-E), (Materials and Methods (Table 2.3(B)).

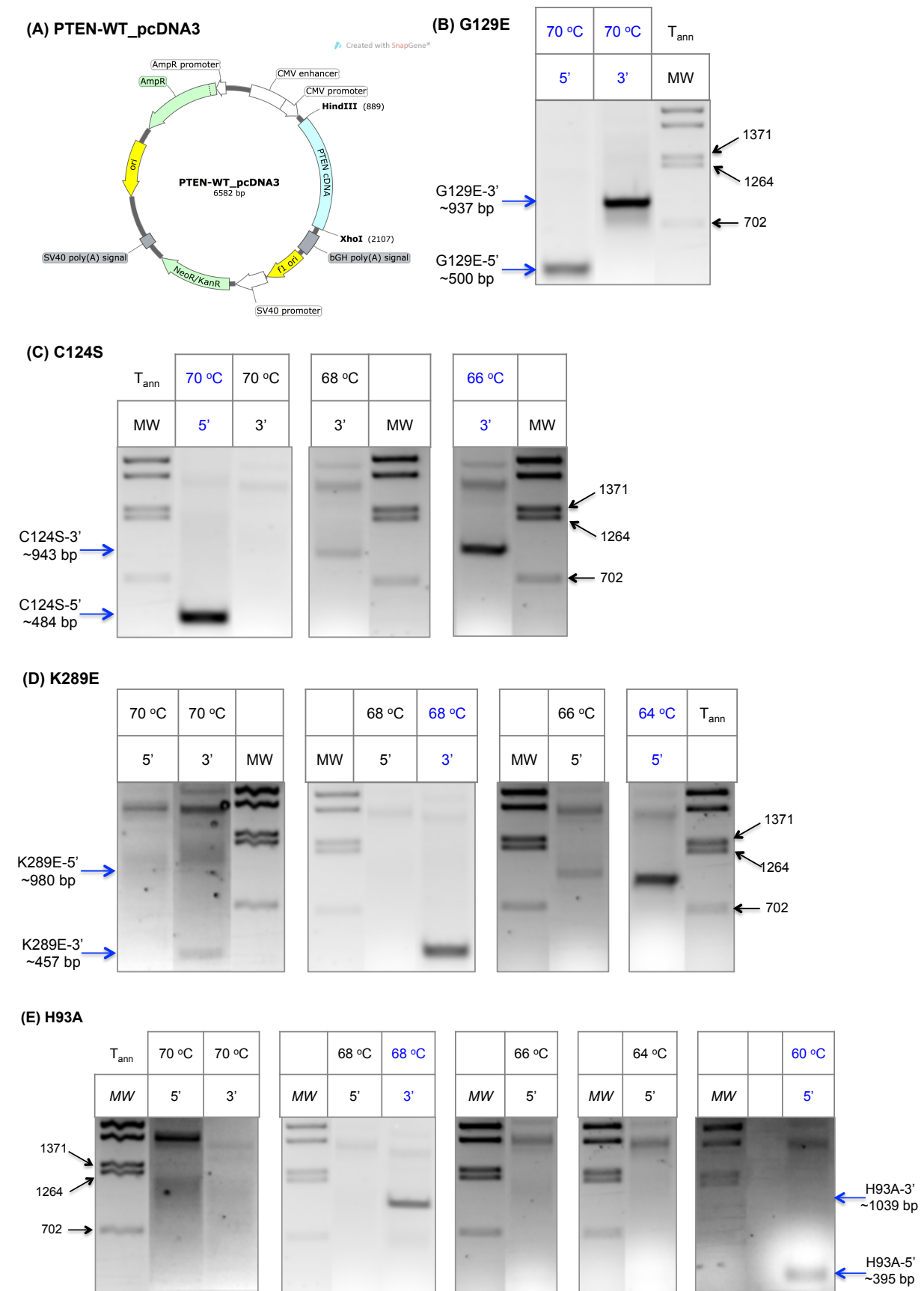


Figure 4.3. **(A)** Plasmid map of the pcDNA3-PTEN-WT used as template for mutagenesis. PTEN cDNA (blue) flanked by HindIII and XhoI restriction sites is located within the MCS of the

pcDNA3 vector. Other vector features (such as origin, drug resistance, promoters and poly(A) site) are also shown on the map (created using SnapGene, USA). **(B)-(E)** First round of two-step PCR mutagenesis with PfuUltra II Fusion HS polymerase and overhang primers. Optimal annealing temperature (T_{ann}) is highlighted with blue font for 5' and 3' amplicons. Desired bands on an agarose gel are indicated by blue arrows. Expected bands sizes: **(B)** 500 bp (5') and 937 bp (3') for G129E; **(C)** 484 bp (5') and 943 bp (3') for C124S; **(D)** 980 bp (5') and 457 bp (3') for K289E; **(E)** 395 bp (5') and 1039 bp (3') for H93A. V403A and K13E mutants were not considered at this stage. Some samples were run on separate gels alongside DNA ladder (MW) for comparison.

Subsequently, purified 5' and 3' fragments were utilised as a template for the second round of PCR giving strong bands corresponding to a full size mutated PTEN cDNA on an agarose gel (Figure 4.4).

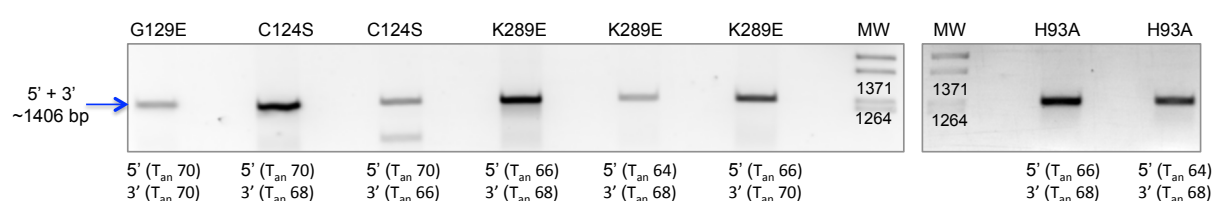


Figure 4.4. Second round of two-step PCR mutagenesis with PfuUltra II Fusion HS polymerase. Bands corresponding to the full size constructs (the expected size is 1406 bp) are indicated with a blue arrow. 5' and 3' fragments synthesised using different annealing temperature values demonstrated equal template potency for the second amplification step.

Then, these PCR products were digested with HindIII and XhoI and ligated back into the pcDNA3 vector, following by transformation of *E. coli* DH5 α cells. Plasmid DNA from resulting colonies was purified (mini-preparation), digested and screened for the presence of the insert of the correct size (Figure 4.5). At this stage a quick screening with HindIII and XhoI digestion was used instead of characteristic enzymes. The digestion result looked good for several samples that were then submitted for sequencing. Unfortunately, only few samples contained the correct insert and the sequencing results of positive plasmids suggested that no mutation was harboured and all constructs contained wild type PTEN.

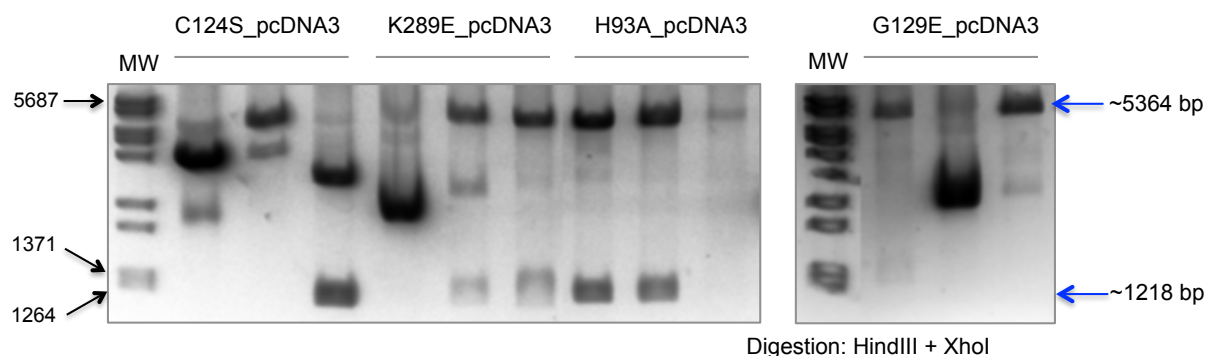


Figure 4.5. Representative agarose gel of purified pcDNA3-PTEN-mut (6582 bp) samples digested with HindIII and XhoI. Expected bands of a correct size (1218 bp and 5364 bp) were visible in some samples (indicated by blue arrows).

The method was further carried out with GoTaq G2 and GoTaq G2 Flexi polymerases. A similar approach of optimising the annealing temperature for each set of primers was carried out (Materials and Methods (Table 2.3(B))). This time PCR enhancing agents such as Betaine and DMSO were used because of their previously reported property to minimise possible secondary structures formation in primers and template plasmid (Frackman et al. 1998, Musso et al. 2006). However all conditions also gave no desired mutants with these polymerases (data not shown).

Finally, four out of six mutants were successfully generated with overhang set of mutagenic primers and Phusion High-Fidelity polymerase. An agarose gel containing PCR products from both rounds of mutagenesis is shown on Figure 4.6(A). Amplicons from the second PCR round were A-tailed and ligated into the pGEM-T vector (according to manufacturer's recommendations, Promega) instead of pcDNA3 vector in order to improve ligation efficiency (Marchuk et al. 1991). Ligated DNA constructs were transformed into competent cells. pGEM-T vector contains part of the LacZ coding sequence that is deleted in the bacterial host strain such that the non-recombinant plasmid expresses the missing part of LacZ which complements the loss in the host cells and subsequently generates β -galactosidase activity. This allows distinguishing between colonies with successfully ligated constructs (white) from the colonies with an empty self-ligated vector (blue) when grown on a chromogenic substrate. Plasmids from white colonies were purified and analysed by restriction digestion (examples of agarose gels with characteristic digestions of G129E, C124S, V403A and H93A pGEM-T constructs are presented on Figure 4.6(B-E)).

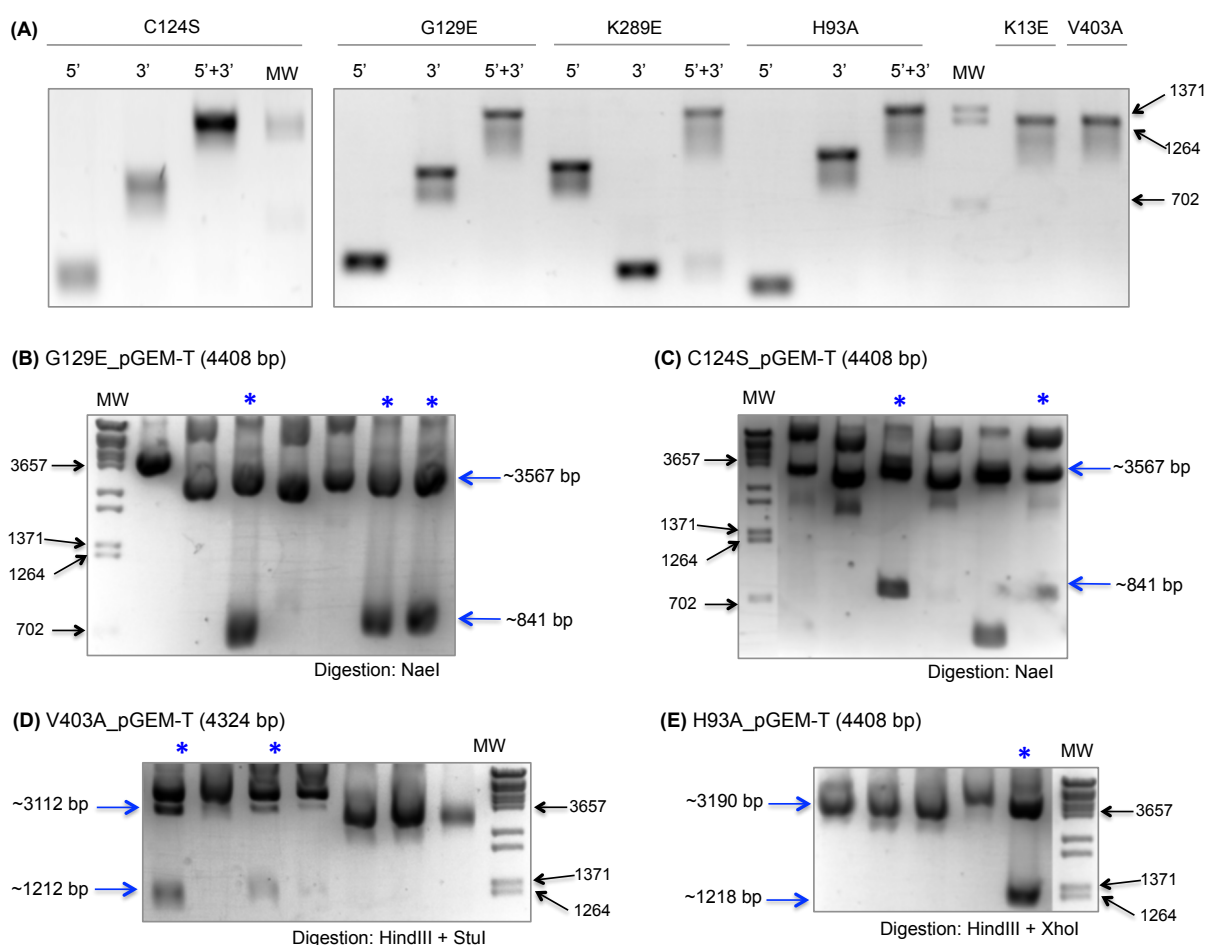


Figure 4.6. **(A)** First and second rounds of two-step PCR mutagenesis with Phusion High-Fidelity polymerase and overhang primers. **(B)-(D)** Representative gels restriction enzyme digestions of potential mutant plasmids are shown. **(B)** G129E and **(C)** C124S constructs were digested with the mutation-specific NaeI enzyme (NaeI also cuts PTEN cDNA resulting in two fragments only if the mutation is harboured); **(D)** V403A plasmid was digested with the mutation-specific StuI (not present in pGEM-T-PTEN-WT) and vector specific HindIII; **(E)** HindIII & XhoI digestion was used to characterise the presence of the insert for H93A plasmid. Characteristic bands are indicated with blue arrows; plasmid samples that gave the bands of correct size upon digestion are highlighted with blue stars.

Final constructs successfully harboured four desired mutations (C124S, G129E, H93A and V403A PTEN). The inserts in all constructs and the junctions with the vector backbone were verified by sequencing (data shown in Appendix 12.2). K13E and K289E mutations were attempted individually but none of the conditions were successful for these mutations.

Q5 High-Fidelity polymerase was used at the next stage, however neither K13E nor K289E were obtained by this method: K289E mutant ligated products appeared to be wild type PTEN; K13E mutant was generated but a 8 base pair deletion in the HindIII restriction site motif was detected after sequencing.

Given that none of mutagenesis attempts to generate PTEN K289E/K13E mutant gave a desired product, it was decided to order these mutations as two long oligonucleotide gBlocks

(<http://www.idtdna.com/pages/products/genes/gblocks-gene-fragments>, IDT) and ligate them together into pGEM-T-vector. Two sequential gBlocks were designed as following: 1) 373 bp pGEM-T-PTEN fragment containing K13E mutation flanked by ApaI and BglIII restriction sites; and 2) 586 bp PTEN fragment harbouring K289E mutation flanked by BglIII and ClaI restriction sites. Initially, these fragments were individually ligated into pGEM-T, followed by transformation, identification and purification. Then, these gBlocks were digested out and ligated together into a pGEM-T-PTEN-WT backbone flanked by ApaI and ClaI restriction sites. This is schematically illustrated on Figure 4.7(A). The final product was characterised by restriction digestion (Figure 4.7(B)) and sequencing of the insert (Appendix 12.2).

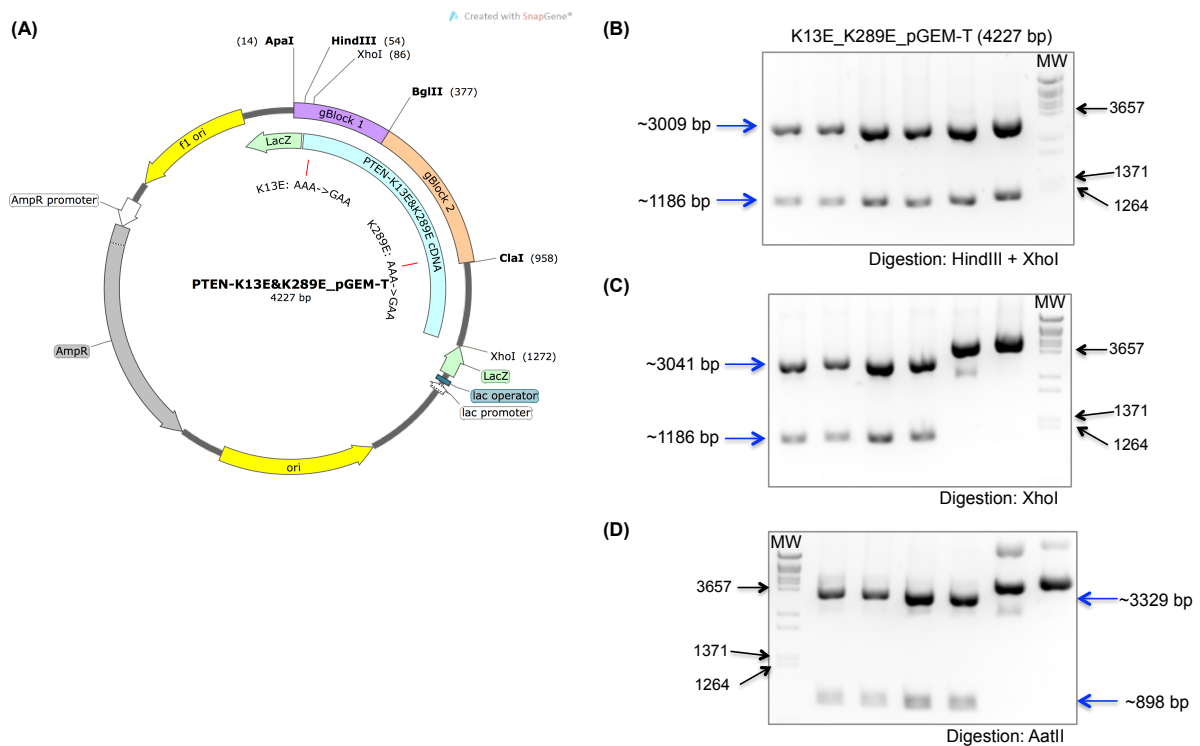


Figure 4.7. **(A)** Plasmid map of pGEM-T-PTEN-K13E&K289E construct built from two gBlocks flanked with ApaI/BglIII (gBlock 1 containing K13E mutation) and BglIII/ClaI (gBlock 2 containing K289E mutation). These gBlocks were ligated to each other and to the linearised PTEN_pGEM-T construct digested with ApaI/ClaI. HindIII and XhoI (1272) sites mark PTEN sequence. XhoI (86) and AatII (918) sites are specific for K13E and K289E mutations respectively. **(B)-(D)** Representative agarose gels of pGEM-T-PTEN-K13E&K289E plasmid digested with **(B)** HindIII & XhoI (confirming successful ligation); **(C)** XhoI (specific for K13E mutation); and **(D)** AatII (specific for K289E mutation). Blue arrows highlight the bands of the correct size for each digestion experiment.

4.3. Conclusions

Four out of six PTEN mutants (H93A, C124S, G129E and V403A) were successfully synthesised using a two-step PCR mutagenesis technique. The K13E & K289E mutant was

generated by ligation of two gBlocks. All constructs were fully characterised and sequenced at the nucleic acid level. Schematic maps of all PTEN mutants are shown on Figure 4.8.

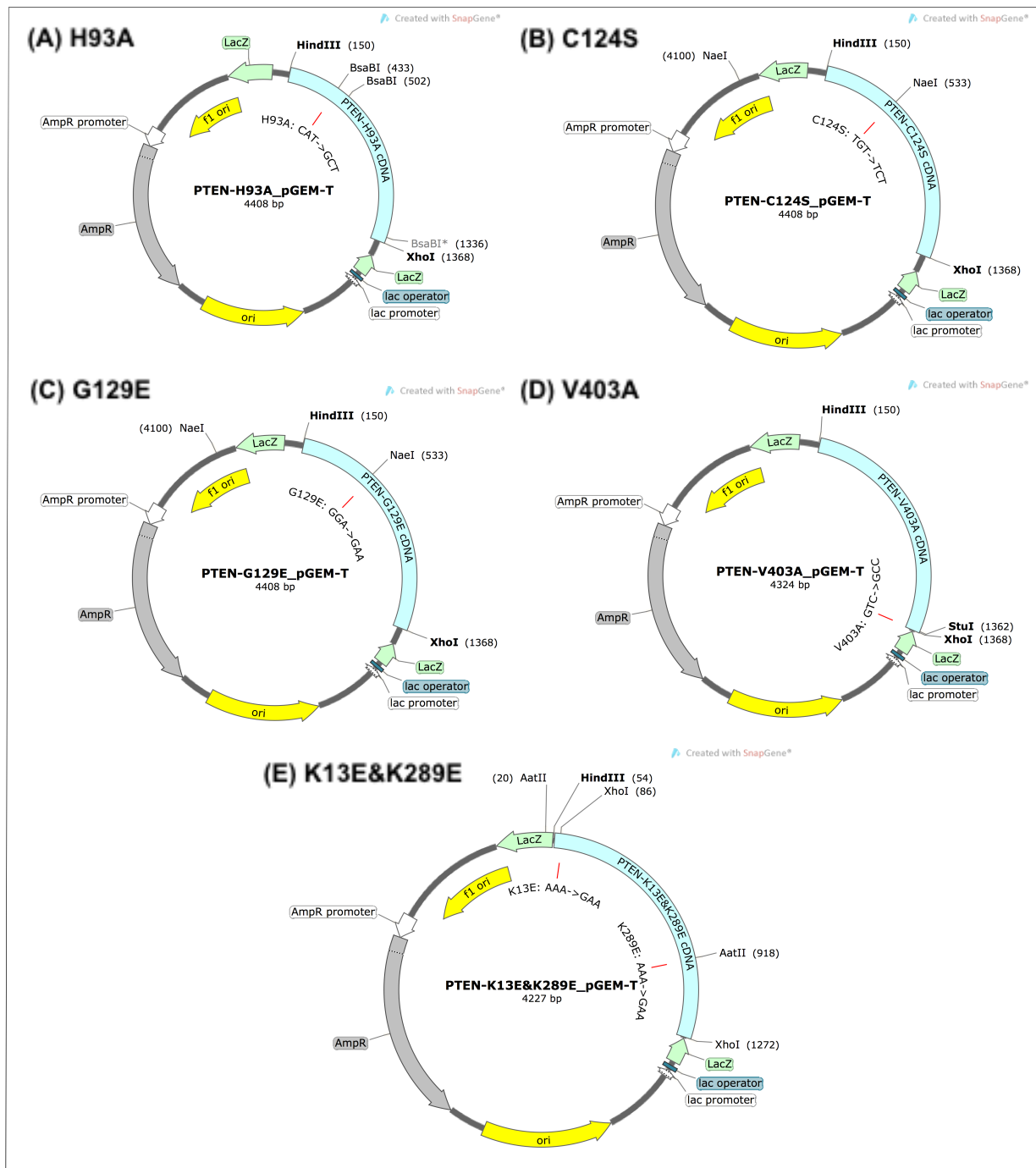


Figure 4.8. Plasmid maps for all pGEM-T-PTEN-mut constructs. The PTEN sequence is flanked with HindIII and XhoI sites in each case. Restriction enzymes specific for each mutation are also shown. Red lines inside maps indicate the position of mutations and change in base pairs.

At the next stage all successful PTEN mutants were cloned into the target vectors (via the flanking HindIII and XhoI sites) for cellular experiments.

5. Results and Discussion: PTEN Cell Line Generation (PTEN Knock-In)

5.1. Introduction

In order to identify the PTEN requirements for the synthetic lethality and PICS phenotypes, it was planned to introduce a single copy of PTEN mutants and wild type PTEN as a control in PTEN^{-/-} cells thereby recreating the heterozygous state. In this chapter several methods of introducing PTEN into the genome of PTEN null cell lines were investigated.

There are several common techniques for cloning the gene of interest (in this case, PTEN) into human cells (Adamson, Jackson & Davis 2011).

- (i) The simplest method is based on cell transfection with a DNA plasmid, containing PTEN. This method is the quickest and highly efficient, however the transfection copy numbers and genomic insertion site can not be pre-determined. Thus, this method is not suitable for this project. Indeed, an effect of PTEN mutations can be assessed accurately and compared to the wild type PTEN only if a single copy of each PTEN variant would be introduced in the same genome position.
- (ii) Sleeping Beauty transposon system is very similar to the plasmid transfection method but it utilises transposable elements to integrate a gene of interest into the genome (Mátés et al. 2009, Kowarz, Löschner & Marschalek 2015). This system has a specific site of integration (TA-dinucleotides) and allows robust long-term gene expression, often with a higher efficiency than plasmid transfection. However, this method does not allow regulating the copy numbers or location of integrated transgene.
- (iii) A gene knock-in method based on Cre-Lox homology recombination allows inserting gene of interest at the exact position on the chromosome (so PTEN can be cloned in the endogenous sequence giving no integration effects or effects on protein expression) (Topaloglu et al. 2005, Rago, Vogelstein & Bunz 2007). This method was used in several reports for generating PTEN KOs (Liliental et al. 2000, Kwon et al. 2001, Lee, Kim & Waldman 2004, Chen et al. 2013). However, the low efficiency of transfection and a very tedious construct generating for this method make it unfavourable for analysing multiple mutants.
- (iv) Site-specific endonuclease technologies utilising ZFNs (Zinc-finger nucleases) (Kim, Cha & Chandrasegaran 1996, Smith, Berg & Chandrasegaran 1999, Bibikova et al. 2001), TALENs (transcription activator-like effectors) (Boch et al. 2009, Moscou, Bogdanov 2009) or newly developed CRISPR/Cas9 (Charpentier, Doudna 2013, van

der Oost 2013) systems are very powerful techniques for targeted gene knock-in. However, ZFNs and TALENs are quite laborious and would require individual knock-in experiments for each PTEN mutation, which would be very time-consuming. Whereas CRISPR/Cas9 system was not so well-developed for knock-in applications in the beginning of this study.

- (v) Finally, a targeting integration method, known as recombinase-mediated cassette exchange (RMCE) (Schlake, Bode 1994, Thomas et al. 2004), was chosen for this project (initially) as it has benefits from other methods listed above (Adamson, Jackson & Davis 2011) : a single PTEN copy can be integrated into a pre-determined single genomic position with a high transfection efficiency. Moreover, once the cell clones containing a single Flp recombination target (FRT) site are produced, various constructs carrying a complimentary recombinase site and non-mutated or mutated PTEN can be introduced quite rapidly. Thus, stable cell lines containing various single copy PTEN variants can be efficiently generated and compared to each other. In order to insert a single PTEN copy (WT or mutants) into PTEN null cells Flp recombinase (FRT/Flp) system (Invitrogen) was used.

FRT-Flp recombination was attempted originally but this method did not result in successful knock-in cells. At the next stage, the CRISPR/Cas9 technique was applied giving several clones with PTEN integration but unfortunately not all mutants were created with this method. Thus, a third attempt using the Sleeping Beauty transposon-based recombination was adopted as a quick and simple alternative. This method was successful for incorporating all PTEN mutants into HCT116 and MRC-5 PTEN^{-/-} cells.

5.2. PTEN knock-in via FRT-Flp recombination

FRT recombination methodology allows insertion of a single copy of wild type or mutated gene of interest into a cell line containing a single FRT site, called Flp-In host cell line (Schlake, Bode 1994, Thomas et al. 2004). Therefore, the experiments were divided into two stages and initially investigated only for HCT116 PTEN^{-/-} cell line. (i) The first stage involved generation of a Flp-In stable cell line by transfecting the PTEN null HCT116 cells with the FRT-site containing plasmid (pFRT//acZeo, Invitrogen) and choosing single integrants. (ii) In the second stage the plasmid containing both a complimentary FRT-site and the PTEN mini-gene (wild type or mutated) would be introduced (together with a plasmid encoding Flp recombinase) into the newly generated Flp-In host cell line (Figure 5.1).

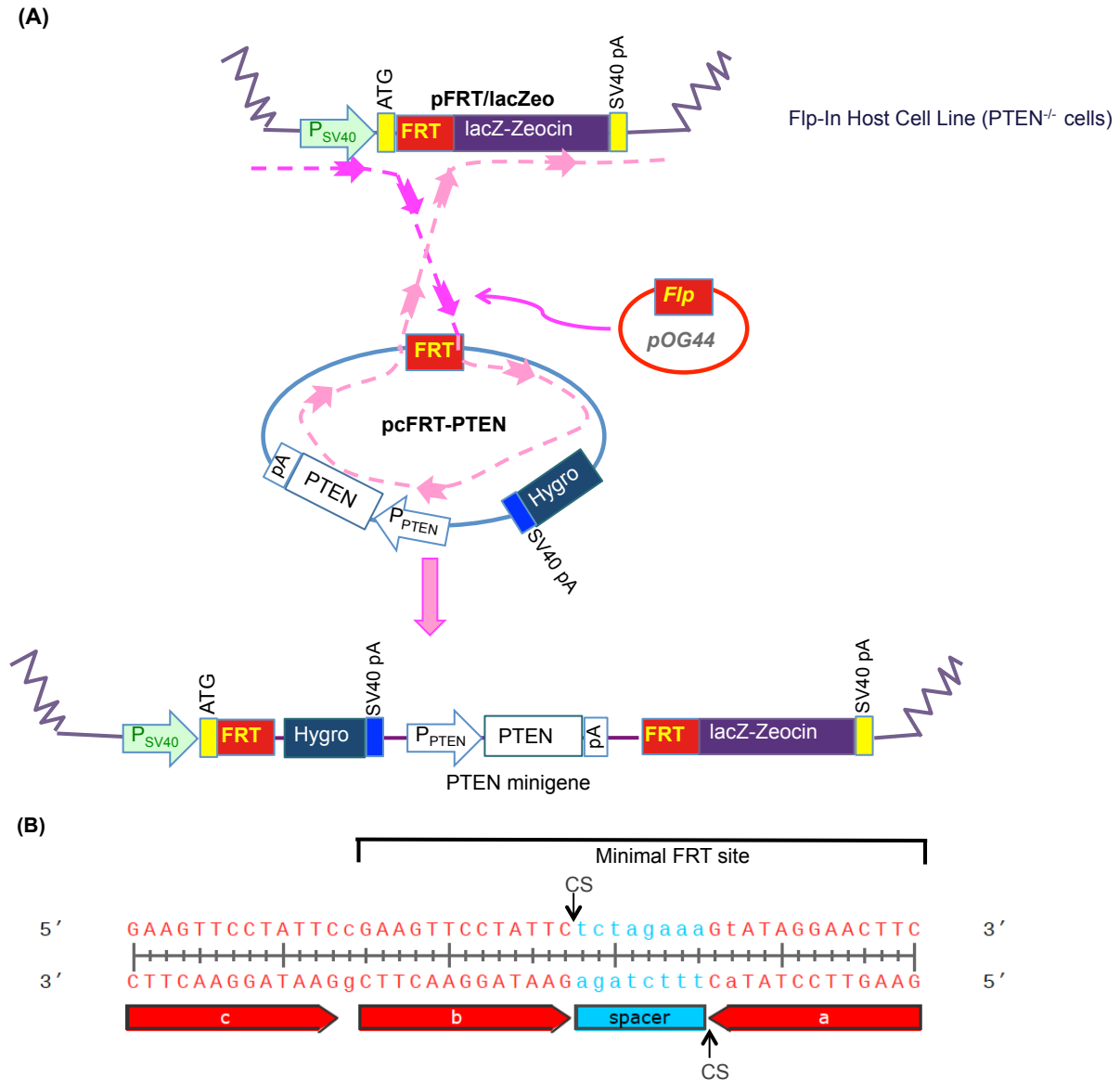


Figure 5.1. **(A)** Diagram of the FRT/Flp recombinase system. The Flp-In host cell line (constructed in HCT116 PTEN^{-/-} cells) containing SV40 promoter and ATG-FRT site from the pFRT/lacZeo plasmid is co-transfected with the pOG44 plasmid expressing Flp recombinase and an pFRT-PTEN plasmid containing a PTEN minigene (i.e. PTEN promoter, cDNA and PolyA sites). Flp recombinase promotes homologous recombination between the FRT sites in the Flp-In host cell line and pFRT-PTEN expression vector, thus integrating the PTEN minigene into a pre-determined genomic location. The hygromycin resistance gene (Hygro) in pFRT-PTEN is lacking a promoter and an ATG initiation codon and is only generated in an active form by successful recombination allowing these cells to be resistant to hygromycin. **(B)** The FRT site usually consists of a 47 bp sequence containing three 13 bp repeats (a-c) separated by an 8 bp spacer. Flp recombinase binds to the FRT site and catalyses a double strand cleavage at the boundaries of the 8 bp spacer. Only two 13 bp FRT repeats are essential (**a** and **b**), while an additional 13 bp repeat **c** is not required for cleavage. CS, Cleavage Site.

Within this project The PTEN FRT/Flp knock-in strategy involved (i) generation and characterisation of the Flp-In host cell line (initially in HCT116 PTEN^{-/-} cells) integrating a single

copy of a pFRT//lacZeo plasmid; (ii) making a construct containing PTEN gene (initially, wild type) and a complementary FRT-site, which required engineering of PTEN minigene (see below) and its subsequent cloning into the modified pcDNA5/TO/FRT vector; and (iii) co-transfection of the PTEN-FRT construct and Flp recombinase into the Flp-In HCT116 cells and selection of final integrants containing one copy of PTEN.

5.2.1. Making stable Flp-In host cell line: integrating FRT site into PTEN^{-/-} HCT116 cells

Flp-In host cell line containing a single FRT site from pFRT//lacZeo plasmid was designed on the basis of PTEN^{-/-} HCT116 cells.

5.2.1.1. PTEN^{-/-} HCT116 cells transfection with pFRT/LacZeo plasmid

The pFRT//lacZeo plasmid (Invitrogen) introduces Zeocin resistance upon transfection, which can be used as a selection criterion. Initially, the pFRT//lacZeo (circular or linearised with ApaI, 8 µg per 60 mm dish) was transfected into PTEN^{-/-} HCT116 cells using Lipofectamine 2000 according to the manufacturer's protocol. This protocol was chosen over several transfection protocols including electroporation and other lipid-based reagents tested (Lipofectamine, Lipofectamine LTX): an optimised DNA:Lipofectamine 2000 ratio of 1:5 (0.8 µg DNA per well of a 24-well plates) provided with the highest transfection efficiency as judged by preliminary experiments with pFRT-LacZeo transfection into PTEN^{-/-} HCT116 cells following by calculating the percentage of blue cells upon reaction with X-Gal substrate (data not shown). After 24 hours cells were trypsinised and plated into the fresh medium. 24 hours later 10 µg/ml zeocin was added to select for clones carrying stably integrated plasmid. The concentration of zeocin used was based on a previously determined dose response and caused complete cell death in 12-14 days (detected with 2% methylene blue, data not shown). Media and antibiotic were replaced every three days. However, after several weeks of zeocin selection, it was impossible to separate colonies, as non-transfected cells would not detach from the tissue culture dish. It is known that the cells sensitive to zeocin do not round up and detach from the dish (Zeocin Selection Reagent manual, Invitrogen). Zeocin causes cell death by intercalating into and cleaving DNA. Initially, affected cells stop dividing, they can be bigger in size and abnormal in shape. Eventually, these cells will be dead but remain as cellular debris. At the same time, zeocin-resistant cells should not undergo any morphological or division changes and should overgrow non-resistant cells. However, some cells can be more resistant to zeocin. In this case it is suggested to keep cells at +4° C for 2 hours in media buffered with HEPES (Zeocin Selection Reagent manual, Invitrogen). This step should overcome resistance by stopping the cell division process temporarily, allowing zeocin to act more effectively. This step was tried with HCT116 cells as well but did not improve the selection process: there was still too much

cell debris on the dish covering all potential resistant colonies. Replating cells to the fresh dishes also did not improve the selection.

5.2.1.2. PTEN^{-/-} HCT116 cells co-transfection with pFRT/LacZeo and pBabePuro

In order to overcome this problem, it was decided to use another drug selection for separating clones carrying integrated plasmid. The same protocol was used and the mixture of one part pBabePuro plasmid and 9 parts of circular or linearised pFRT/LacZeo plasmid were co-transfected into cells. pBabePuro is a commonly used, mammalian expression plasmid containing puromycin resistance. Cells colonies were selected on the basis of puromycin resistance. The drug concentration (0.2 µg/ml) was determined on the basis of a dose response assay. Around 120 puro-resistant colonies were screened for the presence of pFRTLacZeo using staining for β-Galactosidase activity due to LacZ expression from the plasmid. Unfortunately, even though the co-transfection was made in a ratio 9:1, none of the pBabePuro positive colonies (Puro resistant) contained pFRT/LacZeo plasmid (negative data not shown).

5.2.1.3. Construction of pEGFP-puro-FRT plasmid and its use in PTEN^{-/-} HCT116 cells

pEGFP-puro-FRT construction

One simple method to enforce maintenance of the pFRT/LacZeo plasmid upon antibiotic selection was to introduce the puromycin resistance gene into an FRT-containing plasmid. For this, the new pEGFP-puro-FRT plasmid was designed and cloned. For this, an FRT site was designed according to the Invitrogen manual with the addition of ATG site and flanked within NheI/AgeI restriction sites, following by its cloning into pEGFP-puro plasmid.*5' and 3' primers consisting of a [NheI restriction site](#), an initiating methionine codon, the FRT site (underlined) and a [AgeI restriction site](#) were designed:

5' primer:

5'-[CTAGC](#)ATGGAAGTTCCTATTCCGAAGTTCCTATTCTctagaaaGtATAGGAACTTC[A](#)-3';

3'primer:

5'-[CCGGT](#)GAAGTTCCTATaCtttctagaGAATAGGAACTTCgGAATAGGAACTTCCAT[G](#)-3')

and annealed together to yield a double stranded adaptor which was then phosphorylated with polynucleotide kinase. pEGFP-puro plasmid containing the puromycin-resistance gene and the enhanced GFP gene was obtained from AddGene (Abbate et al. 2001) ; this plasmid expresses both marker genes from a single promoter with the coding sequences separated by a P2A site (P2A is 2A peptide from porcine teschovirus-1 polyprotein that is utilised for

separating genes as eukaryotic ribosomes fail to insert peptide bond between the Gly and Pro residues) and has NheI and AgeI restriction sites that can be used to fuse additional sequences to the 5' end of the EGFP coding sequence. pEGFP-puro was digested with NheI and AgeI and de-phosphorylated and ligated with the double stranded NheI-FRT-AgeI adaptor. *E. coli DH5α* were transformed with the ligated vector, 15 colonies were inoculated and screened for FRT insertion by determining the presence of an additional XbaI restriction site (TCTAGA) introduced with the double stranded adaptor. Two successful colonies were identified containing the appropriate XbaI fragment (see schematic Figure 5.2) and the FRT insertion was subsequently confirmed qby DNA sequencing (Appendix 12.2).

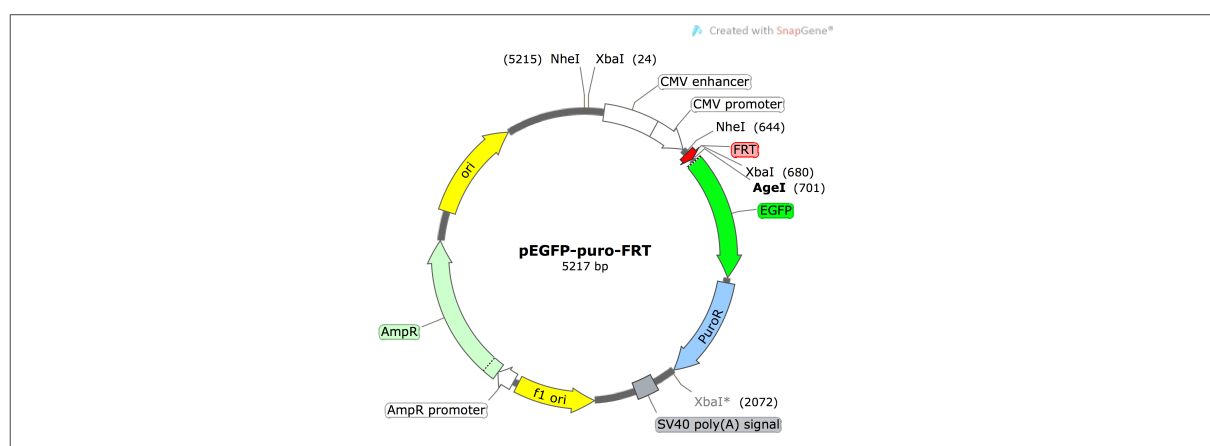


Figure 5.2. Schematic map of pEGFP-puro-FRT plasmid. FRT, EGFP and puromycin sequences are highlighted in red, green and blue respectively.

PTEN^{-/-} HCT116 cells transfection with pEGFP-puro-FRT

Cells were transfected with the new pEGFP-puro-FRT. Optimal transfection method was determined comparing electroporation and Lipofectamine 2000-based transfection. The second method gave better transfection efficiency as judged by preliminary data from transient pEGFP-Puro transfection into cells (not shown). The ratio of DNA:Lipofectamine 2000 which provided the maximum number of transfectants per ug DNA was determined as 1:2. Cells were then transfected with pEGFP-puro-FRT under these optimised conditions and clones harbouring plasmid were selected after selection with puromycin (0.2 µg/ml). Resistant clones were then screened for GFP expression as described below.

5.2.1.4. Colony screening for pEGFP-puro-FRT integration

In order to determine clones with a single integrated copy of pEGFP-puro-FRT, around 200 individual clones carrying integrated pEGFP-puro-FRT plasmid were screened on a number of criteria: i) puromycin resistance (0.2 µg/ml) ii) under the microscope, iii) Western blotting and iv) flow cytometry using GFP signal as a read-out.

Fluorescence microscopy

All puromycin resistant cell colonies were investigated using a fluorescent microscope but the GFP signal was too dim to visualise.

Western Blotting

To attempt to detect GFP protein, GFP levels were assessed by Western blotting. Non-transfected HCT116 PTEN^{-/-} cells and GFP-transfected U2OS^{D1-GFP} cells were used as negative and positive controls respectively. Individual colonies were lysed and resolved on SDS-PAGE, immunoblotted and probed with an anti-GFP antibody. The specific GFP signal of colony lysates was weak compared to the positive control signal and not substantially above the background evident in the negative control lane (Figure 5.3). Colonies that showed any hint of GFP signal over background were chosen for further screening by flow cytometry.

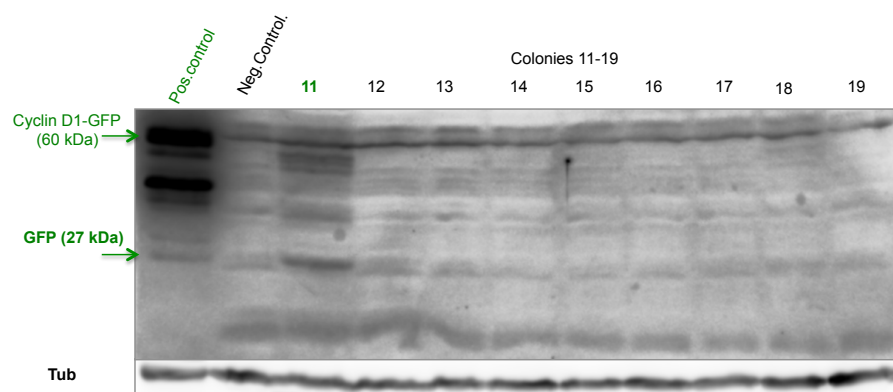


Figure 5.3. Assessing GFP expression in puromycin resistant cell colonies transfected with pEGFP-puro-FRT by Western Blotting. One representative blot with nine colonies lysates is shown. U2OS^{D1-GFP} cells stably expressing GFP (transfected with Cyclin D1-GFP plasmid (N. Suwaki, unpublished)) was used as a positive control. Non-transfected HCT116 PTEN^{-/-} cells were used as a negative control. Colony 11 demonstrated a detectable GFP signal in comparison with the negative control. Tubulin levels were determined for all samples as the loading control.

Flow cytometry

165 colonies were grown in the 6-well plates until near confluency then trypsinised, washed with PBS and filtered to remove aggregates. The samples were analysed by flow cytometry using the laser excitation at 488 nm, emission collected at 530 nm and 10,000 events per sample were acquired. Non-transfected HCT116 PTEN^{-/-} cells were used as a negative control to determine autofluorescence. After removal of doublets, data were plotted as GFP-fluorescence against number of events. Each colony was plotted on a fluorescence histogram and those colonies exhibiting GFP fluorescence greater than the autofluorescence of the negative control were chosen for further work (two colonies are shown as an example on Figure 5.4).

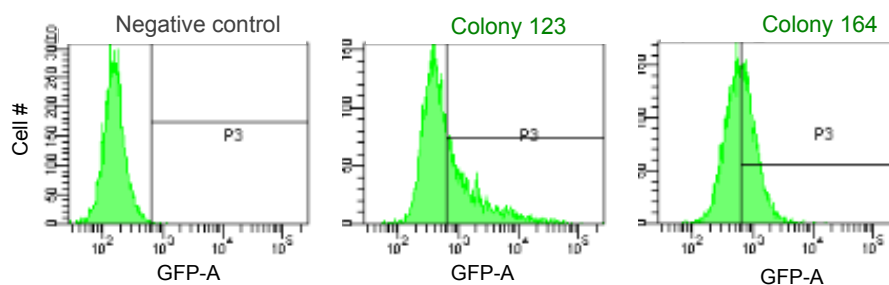


Figure 5.4. Assessing GFP expression in puromycin resistant cell colonies transfected with pEGFP-puro-FRT by Flow Cytometry. Two colonies are shown as representative examples of positive colonies. GFP signal throughout cells colonies populations was plotted on a histogram after excluding all aggregates and non-single cells. Non-transfected host cells were used as a negative control for measuring determining the threshold of autofluorescence.

Approximately 50 individual GFP-positive colonies were selected for further analysis to determine the number of plasmids integrated into the host genome using Southern blotting.

5.2.2. Making stable Flp-In host cell line: characterising single integrants by Southern Blotting

The Southern blotting method is based on transferring fragments of genomic DNA separated on an agarose gel to a nylon membrane, followed by hybridisation to a labelled DNA probe (Figure 5.5(A)). Subsequent detection allows locating the positions of bands complementary to the target of the probe. If a single integration event has occurred, only one band will be visible in that sample whereas multiple integrations will result in multiple bands being detected. Four different restriction enzymes were used to digest the genomic DNA; this would minimise the likelihood that multiple insertions occurring at different locations would be classed as single insertion colonies.

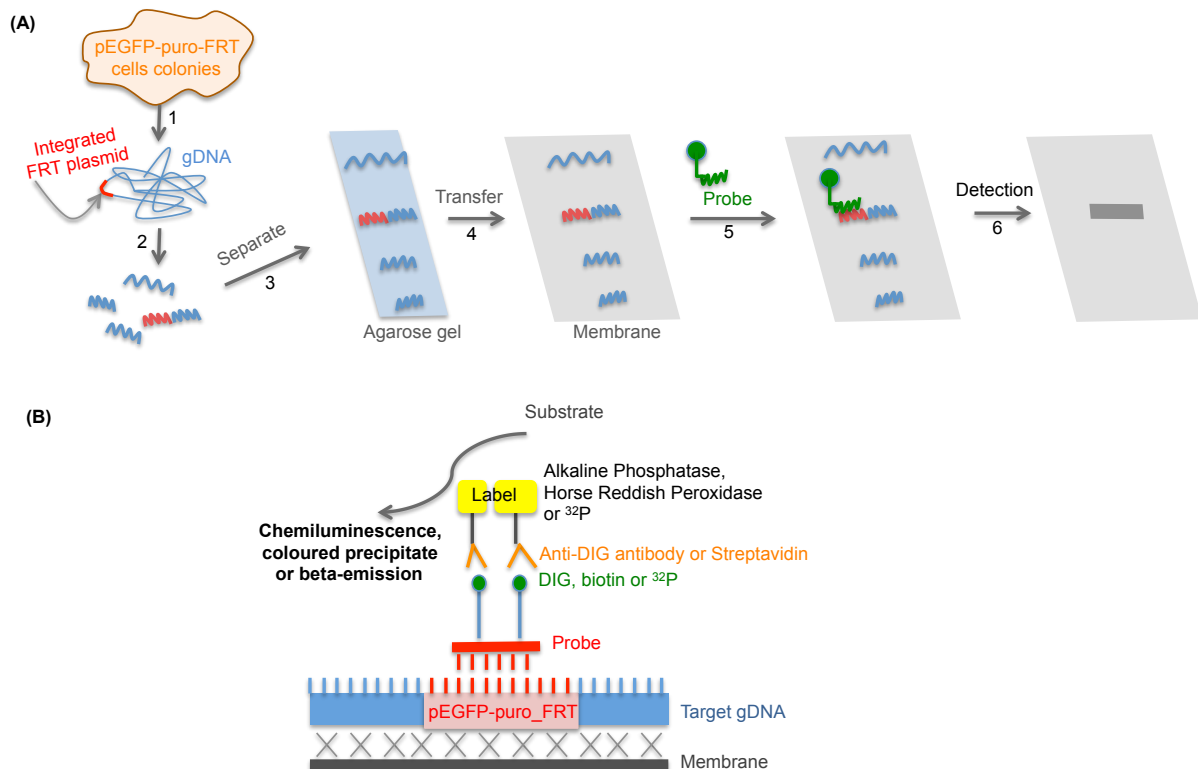


Figure 5.5. Schematics showing the Southern blotting process. **(A)** Stages: 1) Extraction of gDNA from puromycin resistant/GFP positive cell colonies; 2) Digestion of gDNA with restriction enzymes; 3) Separation of DNA fragments on an agarose gel; 4) Transfer of the DNA from the gel to nylon membrane; 5) Annealing target fragments to biotin/DIG or ^{32}P – labelled probes (complementary to integrated plasmid); 6) Detection. **(B)** Southern blotting with biotinylated, DIG or ^{32}P -labelled probes.

Several methods were optimised for probe labelling and detection. First was based on biotinylation with detection via Streptavidin conjugated with an enzyme that gives a detectable signal upon reaction with a substrate (Alkaline Phosphatase, AP; or Horse Reddish Peroxidase, HRP). The second method was based on digoxigenin (DIG) labelling and sequential detection by anti-DIG Fab-fragments coupled to AP. And the third method utilised ^{32}P -labelled dCTP. These methods are schematically shown on Figure 5.5(B). Initially Southern blotting with biotinylated probes was chosen as the cheapest and fastest.

5.2.2.1. Biotin probe labelling using Biotin DecaLabel DNA labeling kit

Probes were labelled with Biotin-11-dUTP according to the manufacture's instructions (Biotin DecaLabel DNA labeling kit, Thermo Fisher). To optimise the protocol and for use as a positive control, we targeted the *CDKN1A* gene encoding p21^{Cip1} (further addressed as p21) as a single copy gene in human genome. The probe was prepared by excising the *CDKN1A* cDNA fragment (600 bp) with EcoRI/BamHI from 1 µg of pcDNA3-p21 (Jarviluoma et al. 2006) by restriction digestion with EcoRI and BamHI and then purified by gel electrophoresis (2%

agarose gel). Probes were labelled overnight with biotin by random primed DNA synthesis with the Biotin DecaLabel DNA labeling kit.

To estimate the yield of biotin-labelled probe at the end of the reaction, serial dilutions of the reaction were prepared and spotted onto a positively charged nylon membrane. The membrane was then blocked and incubated with the Streptavidin-AP conjugate and detected using BCIP/NBT as substrate to produce a blue precipitate. Probe yield was estimated by comparing to the biotin-labelled control DNA template (component of the labelling kit) (Figure 5.6).

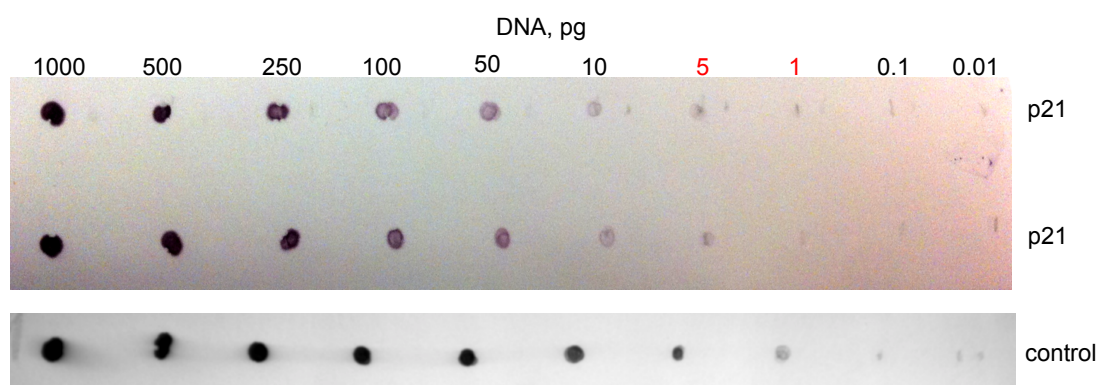


Figure 5.6. Estimation of biotinylated p21 probe labelling. Various concentrations of p21 probe and biotinylated DNA template control were spotted to the positively charged nylon membrane and developed using the Thermo Fisher kit. 1-5 pg was easily visualised with the colourimetric detection.

Thus, labelling was done in a good yield comparable with the control DNA template. This biotinylated probe was used for testing the Southern blotting protocol.

5.2.2.2. Southern blotting with biotin-labelled probe

Genomic DNA from HCT116 cells was extracted by long protocol (see Chapter 2.1.3.2) according to The Hedrick Lab protocols (The Hedrick Lab, 2016). 5 µg of purified DNA was digested with HindIII and separated on a 0.7% agarose gel (18 V, 5 hours). Linearised with EcoRI pcDNA-p21 plasmid and lambda DNA digested with Eco 91I were run on a gel as a positive control and molecular weight marker respectively. Then the samples were transferred by capillary transfer onto positively charged nylon membrane and hybridised in a solution containing the biotinylated p21 probe (600 bp, 250 ng) and digested lambda DNA probe. Subsequently the membrane was stained with Streptavidin-AP and hybridisation locations detected using the BCIP/NBT substrates. Unfortunately, no signal corresponding to p21 was detected in gDNA samples by this method (Figure 5.7 (A)). To determine hybridisation sensitivity, dilutions of linearised pcDNA3-p21 plasmid were analysed using the same Southern blotting protocol (Figure 5.7 (B)). The limit of detection was determined to be ~500 pg of the plasmid, which was hundreds times higher than concentration of the probe in gDNA sample.

This assumption is based on calculations that 5 µg of human gDNA sample would contain about 0.93 pg of a single-copy 600 bp length sequence that corresponds to p21 probe****. This calculation and the observed results indicated that the use of colorimetric detection was not sensitive enough to detect single copy targets in genomic Southern blots.

In order to increase sensitivity chemiluminescent detection was tested. The same experiment with pcDNA3-p21 dilutions was carried out but with a neutravidin-HRP conjugate being employed along with the chemiluminescent ECL substrate (Figure 5.7 (C)). Chemiluminescent detection increased the sensitivity only two times in comparison with colorimetric detection, which was not enough for detection a single-copy gene in a gDNA sample.

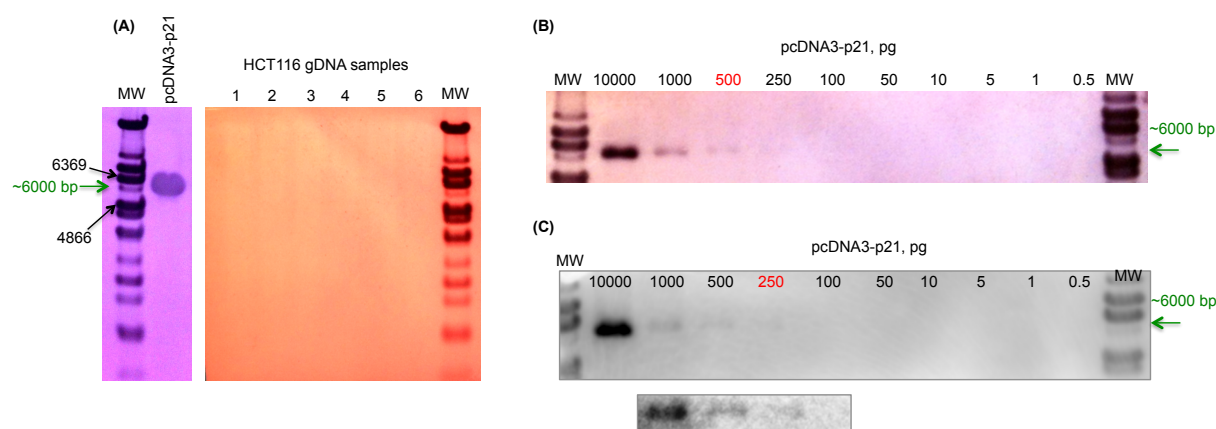


Figure 5.7. Southern blotting with biotinylated p21 probe (600 bp, 250 ng). **(A)** Six samples of gDNA extracted from ~12,500 HCT116 cells were analysed. Unfortunately, no signal was detected for any of these samples. Linearised with EcoRI/BamHI pcDNA3-p21 plasmid (0.5 µg) was used as a positive control (expected band size – 6000 bp). **(B)** Southern blotting of pcDNA3-p21 diluted samples with biotinylated p21 probe and colourimetric detection: 0.5 ng was the smallest concentration that could be detected. **(C)** Southern blotting of pcDNA-p21 diluted samples with biotinylated p21 probe and chemiluminescent detection: 0.25 ng was the smallest concentration that could be detected. The lanes 1000-100 also shown with electronically enhanced detection (bottom panel). MW is DNA ladder (biotin-labelled Lambda DNA (100 ng) digested with 5 ng of Eco91I labelled with Lambda DNA/HindIII probe).

5.2.2.3. DIG probe labelling using DIG-High Prime DNA Labelling kit

In an attempt to increase detection sensitivity further, tests were conducted using the DIG-High Prime DNA Labeling and Detection Starter Kit II from Roche. According to manufacturer, the DIG-system can detect down to 0.5 pg of gDNA in a Southern blot format via chemiluminescence detection, which should be enough to detect a single copy gene. p21 DNA probe was labeled with DIG for 2 or 22 hours according to the kit protocol based on random-primed DNA labeling (Digoxigenin-11-dUTP is reported to be incorporated once in every 20-25 nucleotides). Labeling yield was determined in a similar manner to the previously described

**** Human diploid genome is 6 billion bp, human haploid genome is 3.0-3.4 billion bp. Thus 600 bp (CDKN1A gene length) from approximate 3,200,000,000 bp haploid gDNA represent 5,333,333 dilution, i.e. 5 µg gDNA contains 0.93 pg of p21.

dot-assay (Figure 5.8). Anti-DIG antibodies conjugated with AP were used with a CSPD substrate were used for chemiluminescence detection. For the highest signal level, the membrane was incubated for 15 hours with the substrate and then exposed for 1 hour. 0.03 pg of the probe was easily detected in all cases. Probes with the longer labelling reaction time (> 20 hours) gave slightly better signal than after 2 hours.

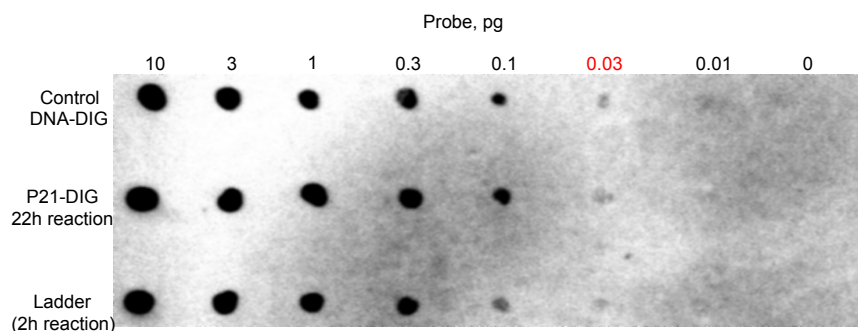


Figure 5.8. Estimation of DIG-p21 probe labelling. Various concentrations of DIG-labelled p21 probe, ladder probe (lambda DNA digested with Eco91I) and DIG-labelled DNA template control (provided within the kit) were spotted to the positively charged nylon membrane and then developed using the Roche kit.

This kit allowed detection of much lower concentrations of target sequences in samples compared to biotinylated probes.

5.2.2.4. Southern blotting with DIG-labelled probe

At the next stage various dilutions of linearised pcDNA3-p21 plasmid were separated by electrophoresis, transferred to a membrane and hybridised and detected using the DIG system according to the manufacture's manual. For the highest signal level, the membrane was incubated for 18 hours with the CSPD substrate and was exposed for 1.1 hour (Figure 5.9 (A)). The limit of detection was determined as 50 pg. Although this sensitivity was five times better than in the biotinylation-based method, it appeared unlikely to be sufficiently sensitive to detect single copy genes in human genomic DNA. In order to verify this, several gDNA samples were hybridised with the DIG-labelled p21 probe in the same Southern blotting protocol (Figure 5.9 (B)). Indeed, sensitivity was insufficient to detect the single copy CDKN1A gene in various gDNA samples prepared by three distinct methods: (i) express short protocol, (ii) longer protocol (with extra phenol:chloroform purification step) and (iii) commercial Thermo Scientific genomic DNA purification kit protocol (See Chapter 2.1.3 Materials and Methods). Using the rough calculation outlined above, the sensitivity of detection had to be improved at least 50 times in order to detect single copy gene in gDNA samples.

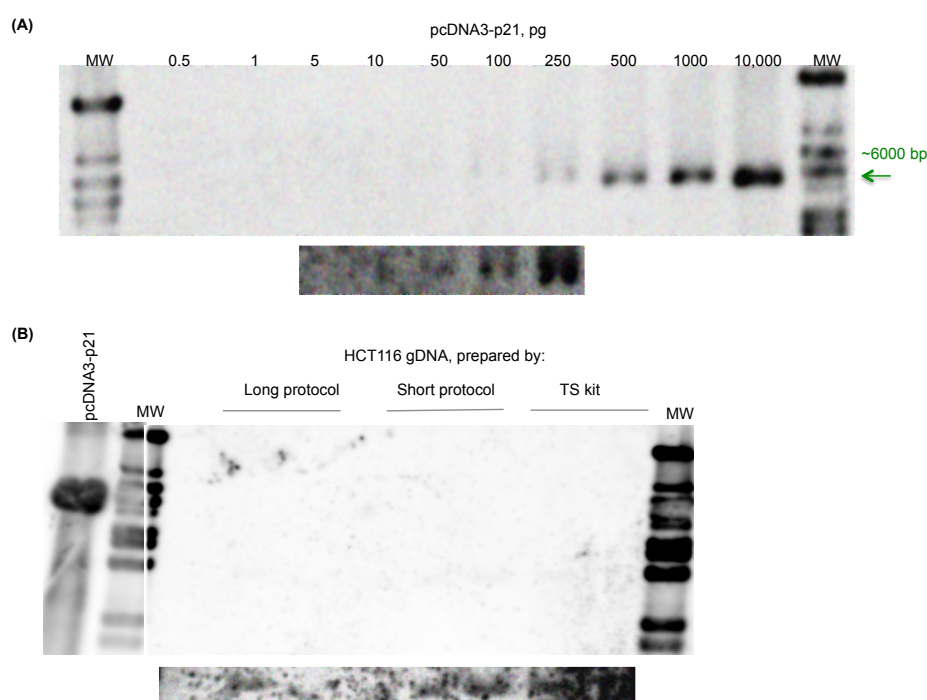


Figure 5.9. Southern blotting with DIG-labelled p21 probe (600 bp, 136 ng). **(A)** Southern blotting of pcDNA3-p21 (linearised with EcoRI) diluted samples with DIG-labelled p21 probe via chemiluminescence detection: 6000 bp bands correspond to pcDNA3-p21 plasmid. 50 pg was the smallest concentration that could be detected. The lanes 5-250 pg also shown with electronically enhanced detection (bottom panel). **(B)** Southern blotting of gDNA samples with DIG-labelled p21 probe: seven samples of gDNA extracted from HCT116 cells by different methods and digested by HindIII were hybridised to DIG-p21 probe. gDNA purification was performed by: (1) Long protocol; (2) Short protocol; (3) Thermo-Scientific Genomic DNA Purification Kit. Very weak – almost undetectable signal was observed for all samples. All lanes also shown with electronically enhanced detection (bottom panel). Linearised pcDNA3-p21 plasmid and Lambda DNA/Eco91I were run on a gel as a control and molecular weight marker (MW) respectively

In attempts to improve sensitivity, the following changes were assessed: (1) higher antibody concentration (1:1000); (2) longer DNA probe (up to 2250 bp); (3) different probe concentration (up to 300 ng); (4) reduced washing time and temperature (RT) (negative data not shown). However, none of these experiments allowed detection of any specific signal in gDNA samples. Thus, ^{32}P labelling was investigated to maximise detection sensitivity as this has been reported to allow detection down to 10 fg DNA (Osborn 2000)(Figure 5.10(A)).

5.2.2.5. Southern blotting with ^{32}P -labelled probes

The 600bp BamHI/EcoRI p21 cDNA fragment was labelled with α - ^{32}P -dCTP using the Thermo Scientific DecaLabel DNA Labeling Kit and purified from unincorporated dNTP by spinning the probe through the Illustra MicroSpin G-50 columns (GE Healthcare Life Sciences, UK). Several gDNA samples were purified from HCT116 cells using three distinct methods: (i) short protocol, (ii) long protocol and (iii) Thermo Scientific kit protocol (see Chapter 2.1.3). These samples were digested with HindIII, subject to Southern blotting and hybridised with ^{32}P -p21 at 68°C

overnight. After several washes in SSC-based buffers at 68° C for a total of 45 mins (see Chapter 2.1.4 Materials and Methods), the blot was exposed to a phosphorimager screen and subsequently visualised using a Fuji FujiFilm FLA 5000 PhosphorImager. All samples demonstrated a band of a correct size (gDNA digested with HindIII should give 1252 bp fragment that contains p21). Samples purified from near confluent cells (24-well TC plate, one well per sample, ~12,500 cells/well) by short protocol (i) or Thermo Scientific kit (iii) demonstrated better signal than long protocol samples (Figure 5.10(B)). Unexpectedly, a second band was detected around 3,500 bp in all gDNA samples but did not correspond to predicted p21 fragments upon HindIII digestion. In order to test if this was a p21 probe specific effect, a PTEN probe was labelled and tested as described below.

Herein, gDNA samples were hybridised with a 1410 bp – long probe (long PTEN promoter region from pGEM-T_PTEN^{long} plasmid digested with EcoRI and BglII). This probe should bind to a promoter fragment in the PTEN gene that is diploid gene in human gDNA. gDNA samples were prepared similar to the previous experiment and were digested with Scal and EcoRV. The ³²P-labelled PTEN^{long} probe was hybridised to the samples and generated a single ~3200 bp band as expected. The hybridisation band with the strongest specific signal was observed from 5 µg gDNA sample prepared by long protocol (ii). However, the short protocol (i) gave clear results too and because it took considerably less time, this was employed to screen colonies from genome editing experiments.

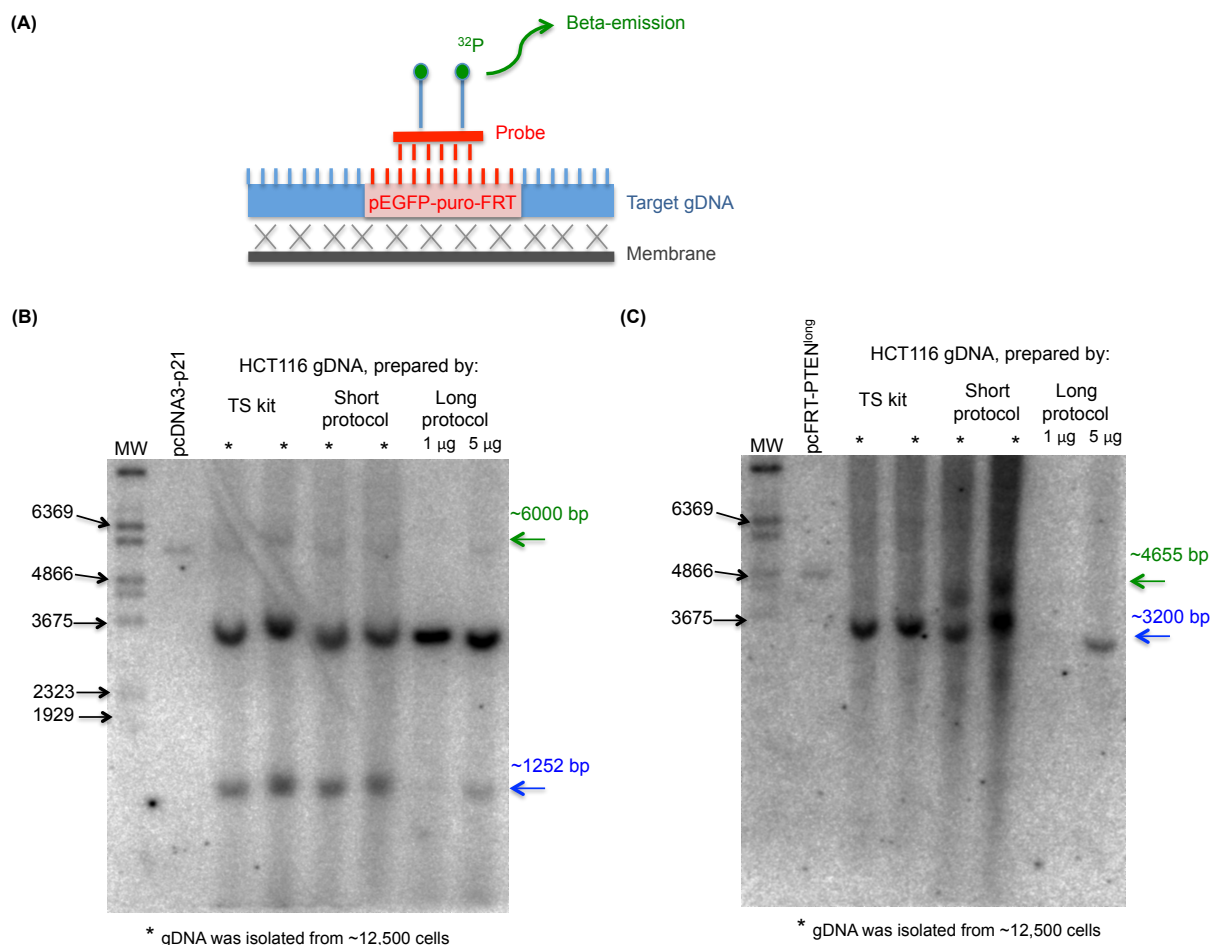


Figure 5.10. Southern blotting with ^{32}P -labelled PTEN probe. **(A)** Schematic of a Southern Blotting protocol with radioactive detection. **(B)** Six gDNA samples purified from HCT116 cells by three methods and digested with HindIII were hybridised to ^{32}P -p21 probe (600 bp, 180 ng). Linearised with EcoRI pcDNA3-p21 plasmid (0.2 μg) containing p21 fragment (identical to the probe) was loaded as a positive control giving a ~6000 bp band (green arrow). The p21 probe was successfully hybridised to a ~1252 bp fragment of all digested gDNA samples (highlighted with a blue arrow). An unknown band of ~3500 bp was also seen in all samples. **(C)** Six gDNA samples extracted from HCT116 cells by different methods and digested by Scal and EcoRV were hybridised to ^{32}P -PTEN probe (1410 bp, 300 ng). pcFRT-PTEN^{long} plasmid digested with EcoRI containing a 4655 bp PTEN fragment (0.4 μg) was loaded as a positive control. The ^{32}P -labelled PTEN probe was successfully hybridised to a ~3200 bp fragment of all digested gDNA samples (highlighted with a blue arrow). A ~4655 bp band corresponding to pcFRT-PTEN^{long} plasmid was also observed (green arrow). For all genomic samples in B and C, the purification was performed by: (1) Thermo-Scientific Genomic DNA Purification Kit (TC kit); (2) Short protocol; (3) Long protocol. For MW ladder Lambda DNA/Eco91I sample was hybridised to ^{32}P -Lambda DNA/Eco91I probe (100 ng).

Having established the methodology to determine the presence and absence of target sequences in genomic DNA, it was then possible to screen transfected cells for the number of pEGFP-puro-FRT plasmids. In order to determine the number of integrated FRT sites in each pEGFP-Puro-FRT- transfected colony, a GFP fragment (728 bp, digested from pEGFP-Puro plasmid with EcoRI and NheI) was labelled with ^{32}P -dCTP and used as a probe. Mammalian

cells do not contain an endogenous GFP gene, therefore, a GFP probe allowed facile identification of those clones that contained pEGFP-Puro-FRT plasmid. To screen all clones, purified gDNA samples were digested with PstI. This restriction enzyme is a frequent cutter of human gDNA and it cuts a single known site outside of the GFP fragment in pEGFP-Puro-FRT. Thus, PstI would allow discrimination between single integrants containing only one hybridising band corresponding to a single copy of the integrated plasmid from the samples with multiple integrants with several bands of varying sizes (Figure 5.11(A)).

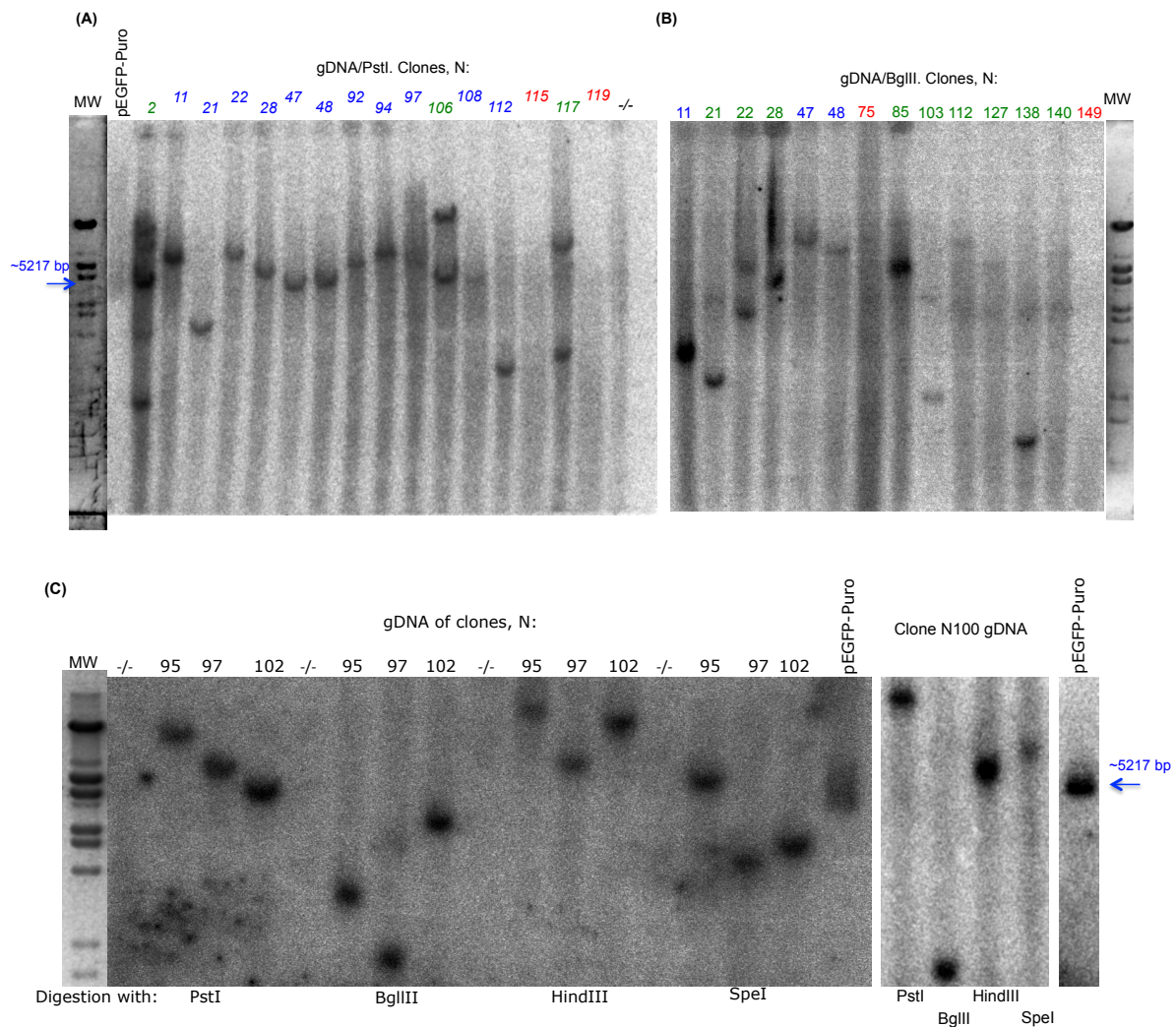


Figure 5.11. Screening for individual transfectants using Southern blotting with ^{32}P -labelled GFP probe (728 bp). **(A)** Representative examples of 16 gDNA samples extracted by short protocol from pEGFP Puro-FRT transfected clones derived from PTEN^{-/-} HCT116 cells. Samples digested by PstI were hybridised with the ^{32}P -labelled GFP probe (150 ng). Samples with multiple hybridising bands shown in green font, with no bands at all – in red font, and with a single integration – in blue font. **(B)** Examples of 14 gDNA samples (short protocol) digested with BglII and hybridised to ^{32}P -GFP probe (300 ng). **(C)** Four gDNA samples (N95, 97, 100 and 102) extracted by long protocol (10 $\mu\text{g}/\text{sample}$) digested with PstI, BglII, HindIII or SpeI demonstrating only one plasmid integration. Linearised with Scal pEGFP-Puro plasmid (3 pg) was loaded as a positive control and hybridised to 3 pg of the ^{32}P -labelled GFP probe

(expected band at ~5217 bp). gDNA extracted from non-transfected PTEN^{-/-} HCT116 cells was also digested and loaded as a negative control (labelled -/-). For MW ladder Lambda DNA/Eco91I sample was hybridised to ³²P-Lambda DNA/Eco91I probe (100 ng).

All puro-resistant clones selected after flow-cytometry screening were tested and those containing only one hybridising band were then tested again with different restriction enzymes. This step allowed elimination of false-positive clones that contained more than one integrant. BglII, HindIII and SpeI were chosen as alternative restriction enzymes based on the same criteria listed above (Figure 5.11(B,C)). Table 5.1 shows the numbers of screened clones with brief assessment of clones falling into different categories.

Table 5.1. Summary of PTEN^{-/-} HCT116 cells transfected with pEGFP-Puro-FRT. Columns indicate (i) structure of transfection plasmid (circular/linearised); (ii) GFP signal as a percentage from autofluorescence signal in parental population (determined via flow cytometry); and (iii) Southern blotting analysis with four distinct restriction enzymes. PTEN^{-/-} HCT116 cells are labelled as -/- and added as a negative control. Gaps in the table mean that such analysis was not perform for the particular clone. Stars indicate potentially positive clones with single integrants. Colonies selected for further analysis highlighted in green.

Colony N	Plasmid	GFP signal (% of parent)	Southern Blotting, Digestion with			
			Pst I	Bgl II	Hind III	Spe I
-/-	no	no	0	0	0-1	0-1
2	Linerised	27	2-3			
11	Linerised	21.5	1	1	1	
21	Linerised	+/-	1-2	1		
22	Linerised	+/-	1	2		
23	Linerised	+	1			1
28	Linerised	+	1			1
47	Circular	11	1	1	2	
48	Circular	19	1	1		
70	Linerised	9.9	1	1	1	
75	Linerised	34	1	0	1	1-2
80	Linerised	36	2		3	
85	Linerised	11.4	1	1	1-2	
92	Linerised	15	1		1-2	
94	Linerised	11	1		1	
95	Linerised	++	1	1	1	1
97	Linerised	94.4	1	1	1-2	1-2
100	Linerised	22.7	1	1	1-2-3	1
102	Linerised	89.7	1	1	1-2	1
103	Linerised	16.1	1	2	1-2	
104	Linerised	16.1	2		1	
105	Linerised	74			2	
106	Linerised	28.9	2			
107	Linerised	30			1	
108	Linerised	20		1	2	
112	Linerised	36.6	1			
113	Linerised	11.3	1		2	
115	Linerised	11.1	0			
117	Linerised	21.8	2			
119	Circular	11.3				
120	Circular	27.9	1		2	
121	Circular	19.6	1		2	
122	Circular	11				
123	Circular	36	1-4			
125	Circular	26	2			
126	Circular	21.3		1		
127	Circular	22.5	1			
138	Linerised	18.3	1	1-2		
139	Circular	14.1	1			
140	Circular	21.2	1			
142	Linerised	71.4				
143	Circular	21.7	1-2		2	
146	Circular	13.1				
149	Linerised	10.3	1			
163	Linerised	17.5	2-3			
164	Linerised	48	2		3	
165	Linerised	6.1	2-3			

Four gDNA samples (N95, 97, 100 and 102, Figure 5.11(C)) displayed only one band in all digestion experiments and thus were highly likely to contain only one FRT integration. These samples were selected as parental lines for subsequent FRT-recombination experiments with pcFRT-PTEN constructs.

5.2.3. Cloning wild type PTEN minigene into FRT-vector (making of pcFRT-PTEN plasmid)

At the next stage, PTEN was cloned into the plasmid containing FRT-homology site. The full-length *PTEN* gene is almost 109 kilobases in length, which makes it impractical to work with such a large construct as it could not be simply incorporated into a plasmid as it is. It is known, that some gene regions can be removed without affecting gene expression (Warner, Chamberlain 2002). This compact version of a gene is called minigene and usually it consists of the promoter region, cDNA and polyadenylation site. In order to make a PTEN minigene two versions of the PTEN promoter were chosen due to the fact that the full-length promoter region was characterised either as 2235 bp fragment (lying between -2233 and +1, (Stambolic et al. 2001)) or as 599 bp fragment (lying between -1344 and -745, (Teresi, Waite 2008)). Thus both of these promoters were cloned: (i) Short promoter^{†††} (517 bp, -1344/-837); (ii) Long promoter[†] (1410 bp, -2233/-837). These fragments were amplified from HCT116 cell genomic DNA by PCR using GoTaq G2 polymerase and primers derived from human genomic PTEN (GeneBank accession number AF067844) designed with 5'-BglII and 3'-EcoRI restriction sites at the ends (Table 5.2).

Table 5.2. PCR primers and conditions for short and long PTEN promoters amplification. 5'-BglII and 3'-EcoRI restriction enzyme sites are highlighted with blue and green font respectively.

	Specific primers		T melting, °C
Short promoter	5': AGATCTGAGTCAGTGACACTGCTCAACGC		73.9
	3': GAATTCGAGAGAACTGAGCGCAGTCG		73.4
Long promoter	5': AGATCTCTCCGGAGCTATCACTGGGGAG		74.1
	3': GAATTCGAGAGAACTGAGCGCAGTCG		73.4

Polymerase	GoTaq G2		
	T, °C	Duration	No of cycles
Initial denaturation	95	2 min	1
Denaturation	95	30 s	25
Annealing	65	1 min	
Extension	72	1 min	
Hold	4	Indefinite	-

^{††††} DNA fragment sizes differ from those stated earlier due to overlapping with the cDNA sequences and addition of restriction sites for cloning.

Amplified promoters were subcloned into the pGEM-T-vector after addition of a single thymine base to the fragment ends. The integrity of each promoter was confirmed by DNA sequencing, (Appendix 12.2).

Next, the structural minigene component, PTEN cDNA ending with a polyadenylation site and 836 bp from the promoter region[†] (subcloned within EcoRI and XhoI restriction sites of pOTB7 vector) was purchased commercially (pOTB7-PTEN, PTEN region: -836/+1521, Clone ID 3937787, Thermo Scientific). The XhoI site was extended with SphI site using the double strand (ds) adaptor. For this, XhoI-SphI-XhoI adaptor was annealed from two identical oligos (5'-TCGAGGCGCATGCC-3'), phosphorylated and ligated to the pOTB7 vector containing the PTEN cDNA after its digestion with XhoI restriction enzyme and dephosphorylation. The new site was confirmed by DNA sequencing (Appendix 12.2).

Finally, promoters and cDNA were cloned into the pcDNA5/TO/FRT vector consisted of complimentary FRT site and Hygromycin gene lacking promoter and ATG (Invitrogen). The vector was digested with BglI and SphI to cut out the cytomegalovirus (CMV) promoter and polyadenylation sites (Figure 5.12(A)). Schematic maps for the designed constructs are shown on Figure 5.12(B).

For this, PTEN promoters were double digested with Bgl II and Eco RI restriction enzymes, cDNA fragment was double digested with EcoRI and SphI enzymes and the fragments resolved using a 1% (w/v) agarose gel. A ~517± bp DNA band (corresponded to the short promoter), ~1410± bp DNA band (corresponded to the long promoter) and ~2357 bp DNA band (corresponded to cDNA with polyadenylation site) were purified from gel and cloned into BglII & SphI site of pcDNA5/TO/FRT vector (3864 bp) resulting in pcFRT PTEN^{short} and pcFRT-PTEN^{long} plasmids. Plasmids were characterised by restriction digestion (Figure 5.12(C)) and all plasmid junctions were sequenced successfully (Appendix 12.2).

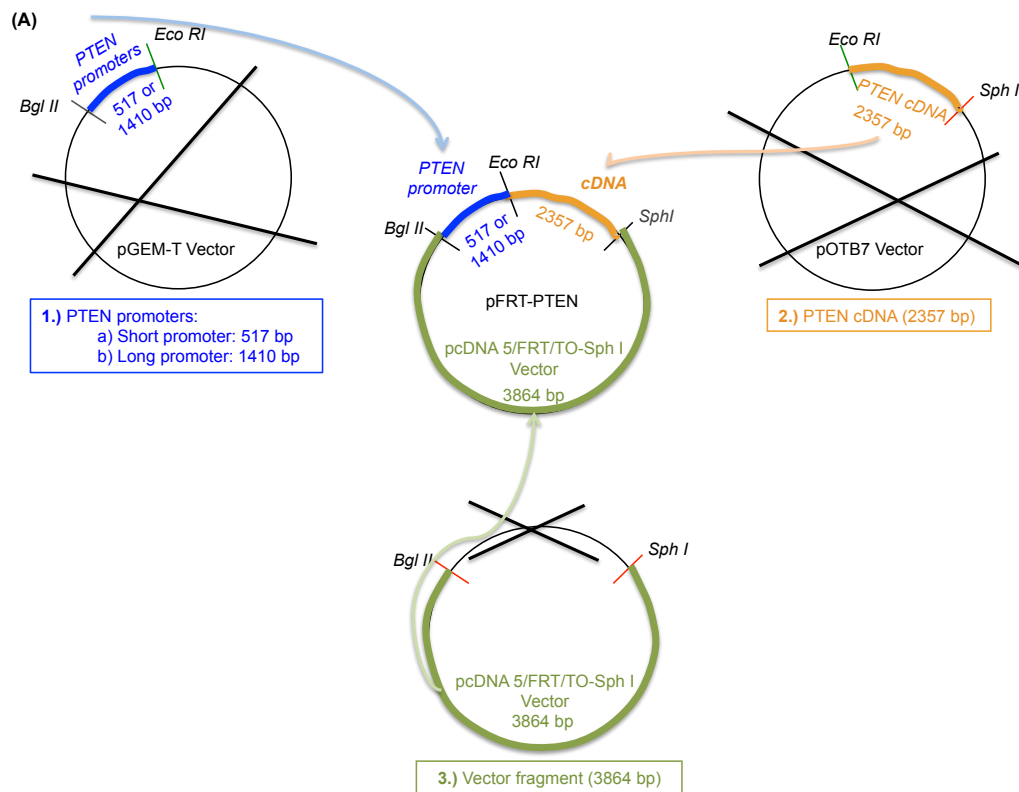
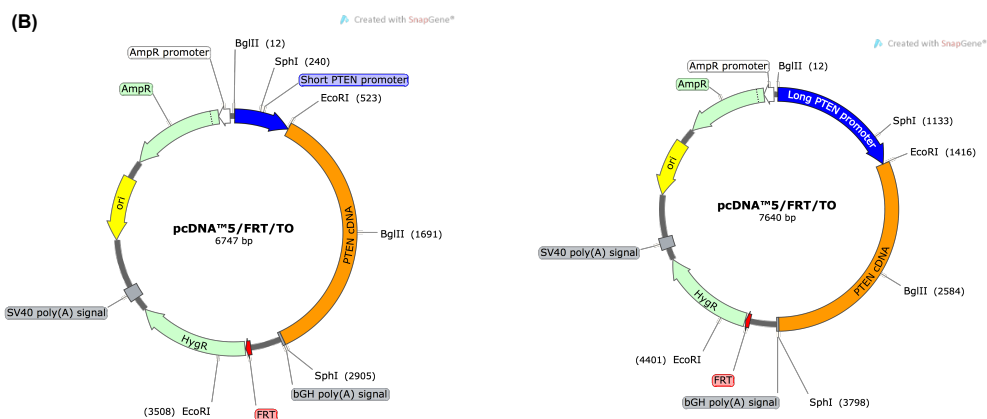
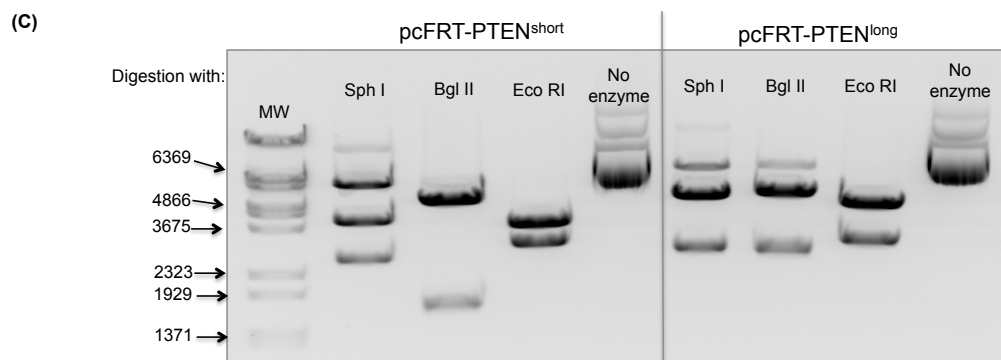


Figure 5.12. pFRT-PTEN cloning scheme (A) The pFRT-PTEN plasmid was cloned from three fragments: 1.) Short or Long PTEN promoter between BglII and EcoRI restriction sites (digested from the pGEM-T-vector with the corresponding enzymes); 2.) PTEN cDNA between EcoRI and SphI sites (digested from the pOTB7 expression vector); 3.) pcDNA5/FRT/TO

plasmid between SphI and BglII restriction sites. **(B)** Plasmid maps of pcFRT-PTEN^{short} and pcFRT-PTEN^{long}. PTEN promoter regions are flanked with BglII/EcoRI; PTEN cDNA fragment containing polyadenylation (polyA) region and part of promoter region is surrounded with EcoRI/SphI. FRT and hygromycin resistance sites are highlighted in red and green respectively **(C)** 1% Agarose gel with pcFRT-PTEN plasmids digested with SphI, BglII or Eco RI restriction enzymes. The sizes of the bands are consistent with the expected sizes: pcFRT-PTEN^{short} plasmid is 6747 bp long; Sph I cuts the plasmid onto 2665 and 4082 fragments; BglII cuts the plasmid onto 1680 and 5067 fragments; Eco RI cuts the plasmid onto 2985 and 3762 fragments. pcFRT-PTEN^{long} plasmid is 7640 bp long; SphI cuts the plasmid onto 2573 and 5067 fragments; BglII cuts the plasmid onto 2665 and 4975 fragments; EcoRI cuts the plasmid onto 2985 and 4655 fragments. All bands on the gel approximately correspond to these fragments. MW – DNA ladder

These two versions of pcFRT-PTEN plasmid were designed for further transfection into the Flp-In cells and effect from each promoter on the gene expression would be compared and assessed later (in order to determine the promoter giving the closest protein expression levels to the PTEN levels in isogenic heterozygous cells).

5.2.4. Attempt of PTEN integration in HCT116 Host Flp-In cells candidates by Flp-mediated recombination

Single integrant host cell lines (Flp-In host cell lines N95, 97, 100 and 102) were then used for PTEN integration. Cells were co-transfected with (i) pcFRT-PTEN containing the PTEN minigene (with either the short or long promoter); and (ii) pOG44 encoding Flp recombinase using Lipofectamine 2000 reagent (Figure 5.13(A)). According to the manufacturer's instructions, a ratio of 9:1 (w/w) pOG44: pcFRT-PTEN was optimal for this system (Flp-In System, Invitrogen). Cells were transfected with total concentration of 24 µg DNA per 10 cm dish. The ratio of DNA:Lipofectamine 2000 which yielded the highest transfection efficiency was determined as 1:3 (w/w). Clones that successfully underwent a Flp-FRT recombination should carry hygromycin B resistance. The drug concentration (100 µg/ml for N95, 100 and 102 colonies and 50 µg/ml for N97) was determined on the basis of the kill-curve assay by selecting the lowest concentration of the drug showing all non-transfected cell dead after 6-9 days (data not shown). After drug selection no single hygromycin B resistant colony was observed in all Flp-In Host cells analysed. The transfection/selection experiment was repeated as above but using a reduced concentration of hygromycin B (25 µg/ml) and an increased ratio of pcFRT-PTEN:pOG44 (4:1) resulted in the growth of several colonies. However, none of these colonies expressed PTEN driven by either the short or the long promoter as judged by immunoblotting (10-15 individual clones were screened for each cell line, representative blots are shown in Figure 5.13(B,C)).

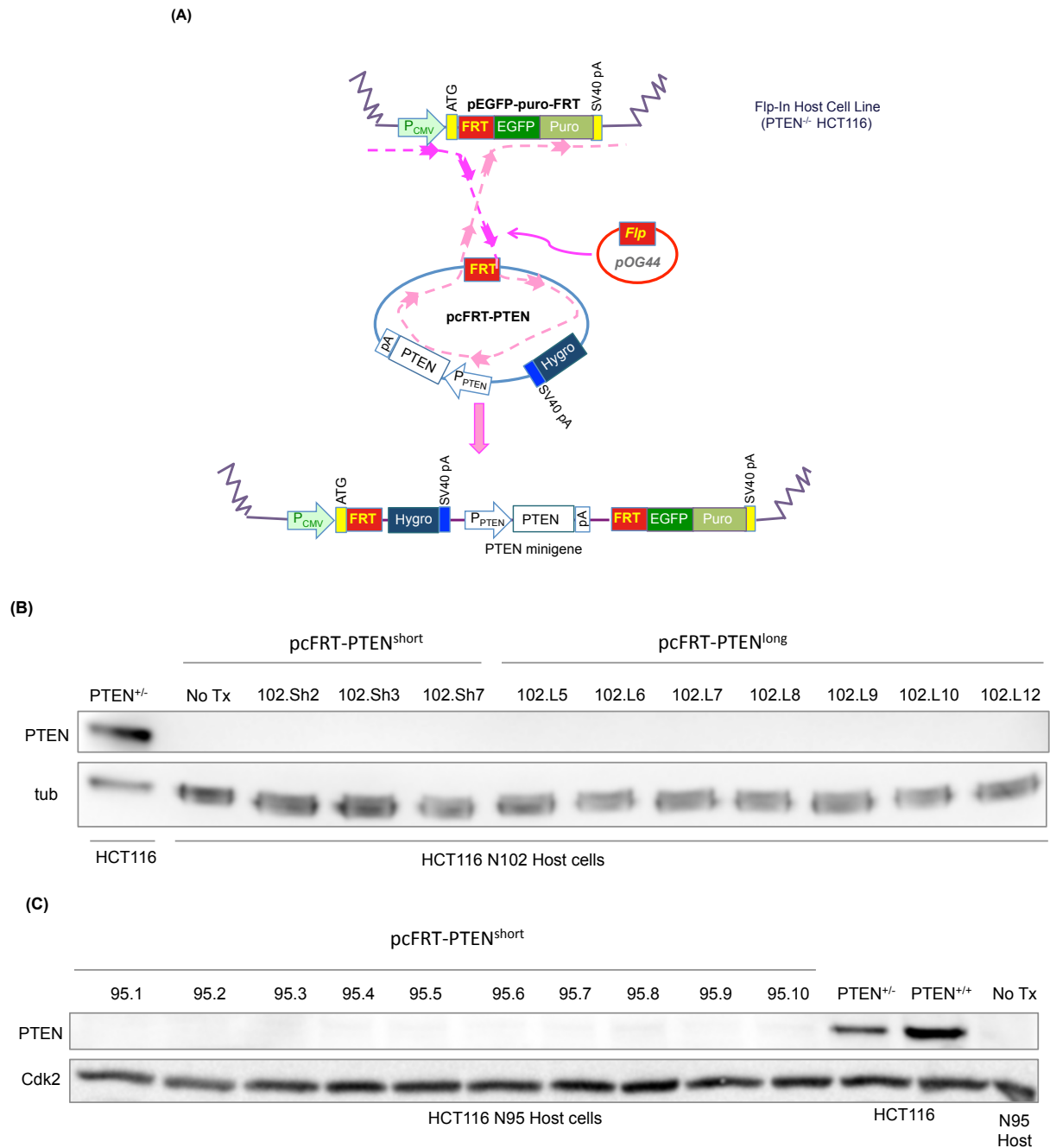


Figure 5.13. **(A)** Schematic illustration of Flp-FRT recombination in Flp-In Host cells based on pEGFP-puro-FRT single integrants. **(B), (C)** PTEN expression in Host Flp-In cell lines co-transfected with pcFRT-PTEN^{short} or pcFRT-PTEN^{long} and pOG44 plasmids. After transfection cells were screened for gain of PTEN expression by immunoblotting. Several hygromycin resistant colonies on the basis of **(B)** N102 and **(C)** N95 Host cells are shown as representative examples. Individual colonies are numbered such that the first digits indicate the parental and the digits after the point indicate the individual clone number. Sh- short promoter, L – long promoter-based cells. PTEN^{+/-} and PTEN^{+/+} cells were loaded as a positive control. Tubulin and Cdk2 signals were measured as total protein level controls. Tx – transfection.

In order to discover why the final recombination step did not give any PTEN positive colonies, the following components were checked individually: (i) pOG44 and recombination; (ii) pFRT-PTEN and (iii) Flp-In Host cells.

- (i) For pOG44 and Flp recombinase expression testing, the commercial Flp-In T-Rex-293 cell line and pcDNA5/FRT/CAT/TO positive control plasmid (Invitrogen, kindly provided by Dr Lorna R Fiedler) were used. The Invitrogen-produced FRT-containing Flp-In Host cell line was co-transfected with pOG44:pcDNA5/FRT/CAT/TO in ratio 9:1 (w/w) according to a manufacturer's protocol. Several hygromycin B resistant colonies (150 ug/ml hygromycin B in media) were isolated and chloramphenicol acetyl transferase (CAT) expression was induced by treatment with 1 µg/ml Doxycycline (pcDNA5/FRT/CAT/TO plasmid expresses TetR, the expression repressor. Tetracycline or its derivative doxycycline bind a TetR protein, which removes it from the DNA hence relieving the repression (Flp-In T-Rex Core Kit, Invitrogen)). CAT expression was assessed by Western blotting (Figure 5.14(A)). All colonies expressed high levels of CAT upon doxycycline treatment indicating that recombination had been successful and that the pOG44 plasmid was able to supply the recombinase to mediate recombination.

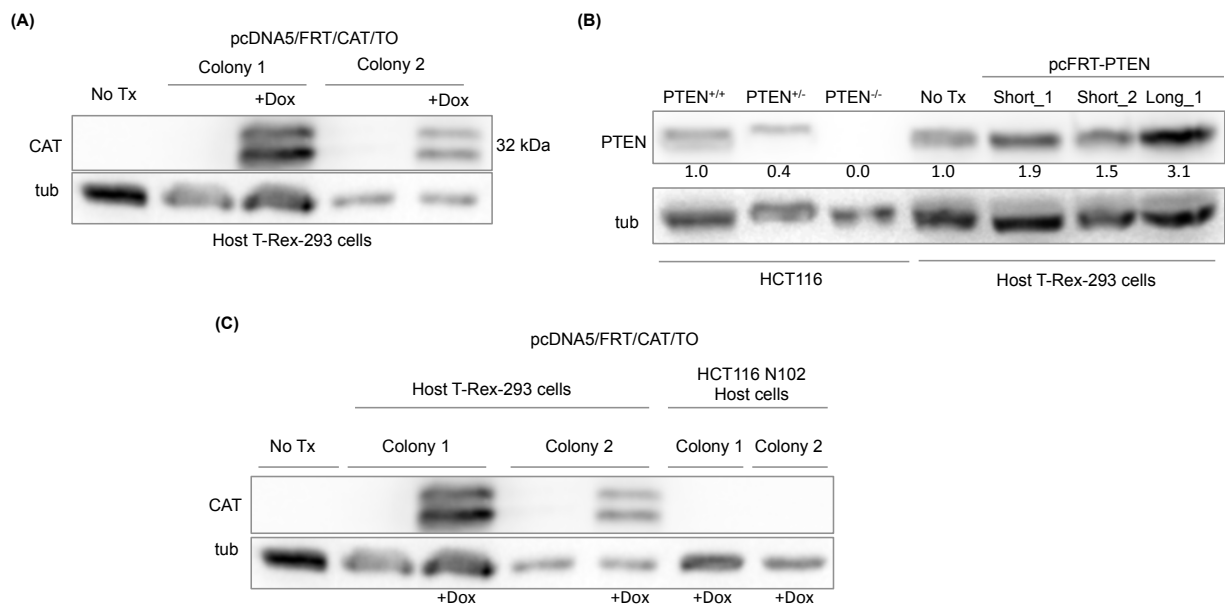


Figure 5.14. CAT and PTEN expression in Flp-In T-Rex-293 cells and Host Flp-In HCT116 cells. **(A)** Individual colonies of hygromycin B resistant Flp-In T-Rex-293 cells co-transfected with pcDNA5/FRT/CAT/TO and pOG44 were immunoblotted to determine CAT expression (32 kDa). Doxycycline (Dox) was used as an inducing agent in the Flp-In T-Rex system according to the manufacturer's manual. **(B)** Individual colonies of hygromycin B resistant Flp-In T-Rex-293 cells co-transfected with either pcFRT-PTEN^{short} (two clones) and pcFRT-PTEN^{long} (one clone) plasmids and pOG44 were immunoblotted to determine PTEN expression compared to non-transfected T-Rex cells. These representative clones using each PTEN plasmid are shown as example. HCT116 cells with different levels of PTEN were included for control. The numbers under the bands represent the ratio of protein expression with control (non-transfected Host T-Rex-293 cells, No Tx) samples arbitrary set as 1.0 and protein determined by ImageStudioLite

(LI-COR Biosciences). **(C)** Individual colonies of hygromycin B resistant Flp-In HCT116 N102 cells or T-Rex-293 cells co-transfected with pcDNA5/FRT/CAT/TO and pOG44 were immunoblotted for CAT. All samples were incubated with Dox to induce CAT expression. Lysates were loaded on SDS-PAGE according to total protein levels calculated by Bradford assay (BioRad), then analysed by Western blotting. Tubulin levels were measured in all samples as a loading control. Tx- transfection.

- (ii) The pcFRT-PTEN plasmids were also tested in Flp-In T-Rex-293 cell line. The same transfection-recombination protocol resulted in growth of several hygromycin B resistant colonies (150 ug/ml hygromycin B in media). PTEN expression was assessed by Western blotting (Figure 5.14(B)). Colonies transfected with either pcFRT-PTEN^{short} (Colony Short_1) or pcFRT-PTEN^{long} (Colony Long_1) demonstrated a clearly detectable increase in PTEN expression compared to non-transfected Host T-Rex cells (with a slightly greater increase in expression using pcFRT-PTEN^{long}). This small rise in the signal detected for PTEN was encouraging given that HEK-293 cells are naturally hypotriploid containing 3 copies of chromosome 10, i.e. 3 copies of PTEN gene. This means that an addition of one PTEN copy by Flp-FRT recombination provides only a 1/3 increase in gene expression.
- (iii) Finally, the host cells constructed here (N95, 97, 100, 102) were tested to determine if they could mediate recombination with pOG44 and the commercial pcDNA5/FRT/CAT/TO plasmid co-transfected in ratios 9:1 and 15:1 (w/w). None of the cells or conditions gave hygromycin B resistant colonies. When the hygromycin B concentration was decreased (down to 25 µg/ml), several resistant colonies were isolated and analysed but did not express CAT. (Figure 5.14C)). This implied that the Host Flp-In cell lines were defective.

5.2.5. Conclusions

The plasmid containing both FRT-site and wild type PTEN mini-gene was generated. In parallel another FRT-site containing plasmid pEGFP-Puro-FRT was generated and introduced into PTEN(-/-) HCT116 cells. About 200 cell clones carrying at least one integrated plasmid were selected on the basis of puromycin resistance and then screened by flow cytometry using GFP fluorescence as a read-out. Around 50 GFP-positive colonies were selected for genomic analysis. Single integrated clones were selected by Southern Blotting and were subject for co-transfection with PTEN-FRT-containing plasmid and Flp recombinase. Unfortunately this step was not successful and recombination did not happen.

The plasmid that was used for generation of Flp-In Host cells (pEGFP-puro-FRT) was designed slightly differently from the plasmid provided by Invitrogen (pFRT/lacZeo) (see below). Even though pEGFP-puro-FRT was sequenced for the FRT site it still seemed to be not enough for

the Flp-mediated recombination to happen. The reason can arise from the small mismatch between FRT sites in pEGFP-puro-FRT and pFRT/lacZeo plasmids:

pEGFP-puro-FRT:

ATG__GAAGTTCCTATTCCGAAGTTCCTATTCTctagaaaGtATAGGAACTTC

pFRT/lacZeo:

ATGGAAGTTACTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC

The FRT site within the pEGFP-puro-FRT plasmid was designed identically to the manual sequence (Figure 5.1(B)), however it was two bases shorter than the FRT site in the commercial pFRT/lacZeo plasmid. This mismatch doesn't affect two essential 13 bp FRT repeats sequence (capital letters, **a** and **b** on Figure 5.1(B), (Andrews et al. 1985, Turan, Bode 2011), but creates a frame shift between ATG-FRT and Hygro sites. This could be the main reason for getting no colonies with recombination upon Hygro treatment.

Unfortunately changing such a mismatch would require performing all the experiments again and would be very time consuming. Thus, it was decided to use an alternative technique to introduce a single PTEN copy into PTEN^{-/-} cells. Whilst we were working with the FRT system, the development of an alternative system, CRISPR/Cas9 was moving on rapidly and this seemed to offer an alternative approach to genome editing.

5.3. PTEN knock-in via CRISPR/Cas9

CRISPR/Cas9 knock-in system consists of three components: (i) Cas9 endonuclease (in the form of a protein or protein – encoding plasmid); (ii) guide RNA (gRNA) that contains a sequence that is complimentary to the sequence of interest in the genome (can be also encoded by a DNA plasmid); and (iii) donor DNA (PTEN in this case). At first, gRNA forms a complex with Cas9 and guides it to the target DNA. Then, Cas9 induces double-strand break (DSB) in target sequence that is complimentary to gRNA, forcing the cell to repair the DSB either by non-homologous end joining (NHEJ) or homology-directed repair (HDR). HDR can be used to knock-in an alteration of interest, for this donor DNA has to be introduced (Figure 5.15). Three components of CRISPR/Cas9 system used within this study:

- (i) The hCas9 plasmid encoding human codon-optimised version of the Cas9 protein was obtained from Addgene (Mali et al. 2013b).
- (ii) gRNA was chosen to guide Cas9 to the predefined human AAVS1 locus in the PPP1R12C gene on the long arm of human chromosome 19. Because one single integrant required for this study we needed to identify a genomic location that is suitable: (i) allows site specific integration, (ii) does not disrupt any essential loci in genome; and (iii) has been previously shown to allow integration and expression of the integrated construct. One site that fits all these requirements is that targeted by adeno-associated virus. This site is widely used for stable and long-term expression of genes

to the AAVS1 safe harbour location via the gRNA which is designed to be complimentary to a 5'-GN₂₀GG-3' T2 target site within the AAVS1 target sequence. Then, Cas9 creates a double strand break (DSB), which can be repaired by Homology Dependent Repair using a PTEN donor sequence to fill the DSB gap.

- (iii) Finally, the third component (donor DNA plasmid) was constructed (see detailed discussion in the next Chapter 5.3.1). Schematically the CRISPR-Cas9 knock-in method is described on Figure 5.15(B).

5.3.1. pcPTEN-AAV generation

pGEM-T_AAV construct

Donor plasmid was designed to contain a PTEN minigene flanked by two homology arms that would direct donor DNA to the AAVS1 target locus to repair Cas9 mediated DSB via homology recombination. Initially, two AAV homology arms (476 bp and 576 bp each) flanking T2 targeting sequence were ordered from IDT and both cloned into the pGEM-T vector. In order to incorporate donor PTEN (WT or mut) sequence, a short multiple cloning site was inserted between these two homology arms as shown on Figure 5.16 (this work was done by Ms Sara Bik).

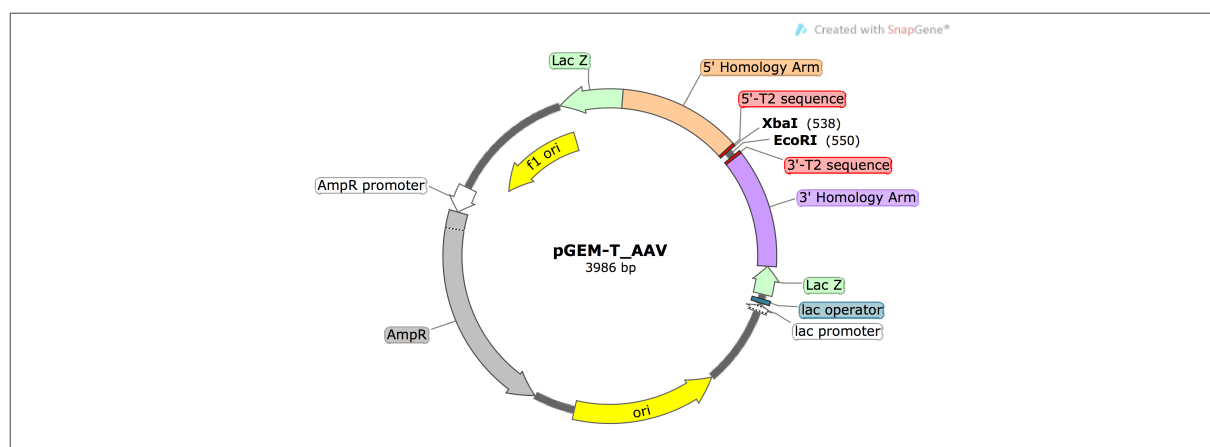


Figure 5.16. Plasmid map of pGEM-T_AAV. 5' (475 bp) and 3' (576 bp) homology arms consist of AAVS1 sequence surrounding T2 targeting fragment. XbaI and EcoRI restriction sites were used for PTEN cloning.

PTEN promoter

Expression levels of PTEN are very important for this study as they can affect phenotype so it was critical to assess promoters of differing strengths. Ideally would be use of the native PTEN promoter but it was unclear if the short and long variants tested so far would give native levels of expression. Therefore we used those two promoters and compared them to the commonly used CMV promoter

For the next stage PTEN minigene had to be cloned between homology arms using XbaI and EcoRI restriction sites. For this, PTEN promoter was picked out of human cytomegalovirus (CMV) and native PTEN promoters (short or long). It was decided to compare promoters strength at this stage in order to reduce amount of CRISPR knock-in conditions at the next more time consuming stages.

Originally, pcDNA3-PTEN (containing CMV promoter, PTEN cDNA and bGH polyA sites), pcFRT-PTEN^{short} or pcFRT-PTEN^{long} (with native promoters and native polyA sites) were cotransfected together with pBabeHygro (5:1 ratio) into PTEN^{-/-} HCT116 cells. The resulting hygromycin resistant colonies were analysed by Western blotting for PTEN expression. Unfortunately, no PTEN expression was detected (Figure 5.17(A)), perhaps suggesting that the growth suppressive properties of ectopic PTEN provided powerful selection for cells that selectively lost the ability to express the PTEN cDNA. A simple means of circumventing this problem would be to link the selectable marker and PTEN expression directly.

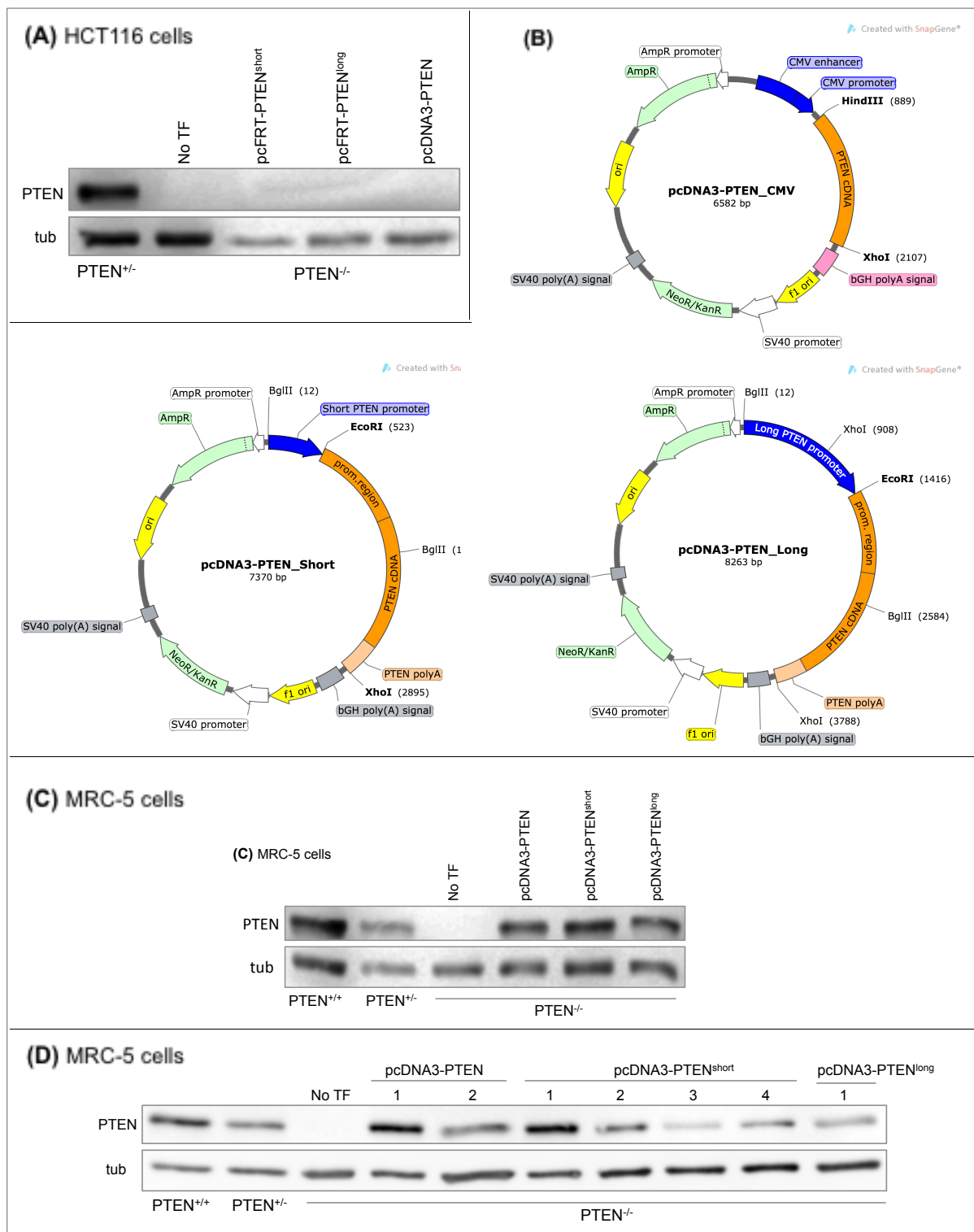


Figure 5.17. Comparison of PTEN promoters. **(A)** PTEN levels in HCT116 $PTEN^{-/-}$ cells cotransfected with pBabeHygro and either pcFRT- $PTEN^{short}$, pcFRT- $PTEN^{long}$ or pcDNA3-PTEN plasmids. $PTEN^{+/-}$ HCT116 cells were included as a positive control. Tubulin levels were measured in all samples as a loading control. **(B)** Plasmid maps for pcDNA3-PTEN, pcDNA3- $PTEN^{short}$ and pcDNA3- $PTEN^{long}$. Promoter regions are highlighted in blue. pcDNA3-PTEN contains bGH polyA site, while pcDNA3- $PTEN^{short}$ and pcDNA3- $PTEN^{long}$ plasmids utilise native PTEN polyA signal. **(C)-(D)** PTEN levels in MRC-5 $PTEN^{-/-}$ cells stably transfected with pcDNA3-PTEN, pcDNA3- $PTEN^{short}$ or pcDNA3- $PTEN^{long}$. Total transfectants (pooled cells) are

shown in C whilst D shows results from isolated colonies. PTEN^{+/+} and PTEN^{+/-} MRC-5 cells were included as controls. Tubulin levels were measured in all samples as loading controls. TF-transfection.

The vector backbone used for the CMV construct was pcDNA3 which encodes *Tn5* gene enabling selection for neomycin. Consequently, the PTEN minigenes with native PTEN promoters were introduced into pcDNA3, deleting the CMV promoter simultaneously generating the pcDNA3-PTEN^{short} and pcDNA3-PTEN^{long} constructs (Figure 5.17(B)). To achieve this construction, PTEN promoter fragments from pcFRT-PTEN plasmids (*Bgl* II- pcFRT-PTEN^{short} -EcoRI or *Bgl*III- pcFRT-PTEN^{long} -EcoRI) and PTEN cDNA containing PTEN prom region and polyA site from pOTB7-PTEN (EcoRI- PTEN-PolyA- XhoI) were co-ligated into pcDNA3 vector digested with *Bgl*III/XhoI. Resulting constructs were characterised by restriction digestion and sequencing (Appendix 12.2).

These plasmids were then transfected into MRC-5 PTEN^{-/-} cells (HCT116 PTEN^{-/-} cells appeared to become resistant to neomycin and thus could not be used for this experiment). After transfection, stable MRC-5 clones and pools resistant to neomycin were analysed via immunoblotting to determine PTEN expression levels. Comparison of pools of transfected cells and individually selected clones demonstrated clear ectopic PTEN expression (Figure 5.17(C,D)). There was variation in the level of PTEN expression amongst clones isolated from transfected cells both between promoters and when using the same promoter; this is likely to arise from promoter strength, integration position and the number of plasmids integrated. Each promoter yielded clones that gave levels of PTEN expression similar to that in PTEN^{+/-} and PTEN^{+/+} MRC-5 cells; for example, clone 2 (in pcDNA3-PTEN transfected cells), clone 2 (in pcDNA3-PTEN^{short} transfected cells) and clone 1 (in pcDNA3-PTEN^{long} transfected cells) displayed PTEN levels comparable with a single copy of endogenous PTEN (as in MRC-5 heterozygous PTEN^{+/-} cells). Thus, there seemed to be no benefit in using PTEN promoters over the CMV promoter for driving expression of PTEN. Moreover, the CMV promoter is very well characterised and has a shorter sequence, which could be beneficial for future CRSIPR/Cas9 HDR step. Thus, the CMV promoter (with the CMV enhancer) was chosen over native promoters for further PTEN knock-in experiments.

pcPTEN-AAV construct

After finalising the promoter for driving PTEN expression, the minigene containing the wild type PTEN cDNA under control of the CMV promoter was introduced into the pGEM-T_AAV vector. To achieve this, the *Nru*I/*Xba*I fragment containing PTEN cDNA and CMV promoter was cut from pcDNA3-PTEN. The pGEM-T_AAV construct was digested with *Eco*RI and the overhanging end was filled with dNTPs using the Klenow fragment of DNA polymerase I to yield a blunt end. The Klenow fragment was inactivated by heat treatment and this linearised

vector was then digested with XbaI and ligated with the CMV-PTEN insert in reverse complement orientation (3'-XbaI –CMV – PTEN- NruI-5'): the blunt end produced by NruI digestion was ligated to the filled in EcoRI site. This intermediate plasmid pGEM-PTEN-AAV-noPolyA was purified and characterised by restriction digestion and sequencing (Appendix 12.2).

To complete construction of the minigene, the bovine growth hormone (bGH) polyA site flanked with XhoI/XbaI was inserted from the pIRES-Puro2 vector. Primarily, pIRES-Puro2 was digested with XhoI and 5'- XhoI overhang end was filled by Klenow to create a 5' blunt end, following by XbaI digestion. pGEM-PTEN-AAV-noPolyA was digested with XbaI and dephosphorylated. Then, 5'- XbaI was ligated to XbaI site within pIRES-Puro2 fragment, while 3'- XbaI site was filled with Klenow to yield 3'- XbaI blunt end that was ligated to 5'- XhoI blunt end within pIRES-Puro2 fragment. The correct orientation of insert incorporation was verified by restriction digestion and sequencing (Appendix 12.2). The plasmid map of resulting pcPTEN-AAV construct is shown on Figure 5.18(A).

Subsequently, these new plasmids were transiently transfected into HCT116 PTEN^{-/-} cells to determine if they drive ectopic PTEN expression. As expected, PTEN was successfully detected in cells transfected with pcPTEN-AAV but not with pGEM-PTEN-AAV-noPolyA (Figure 5.18(B)).

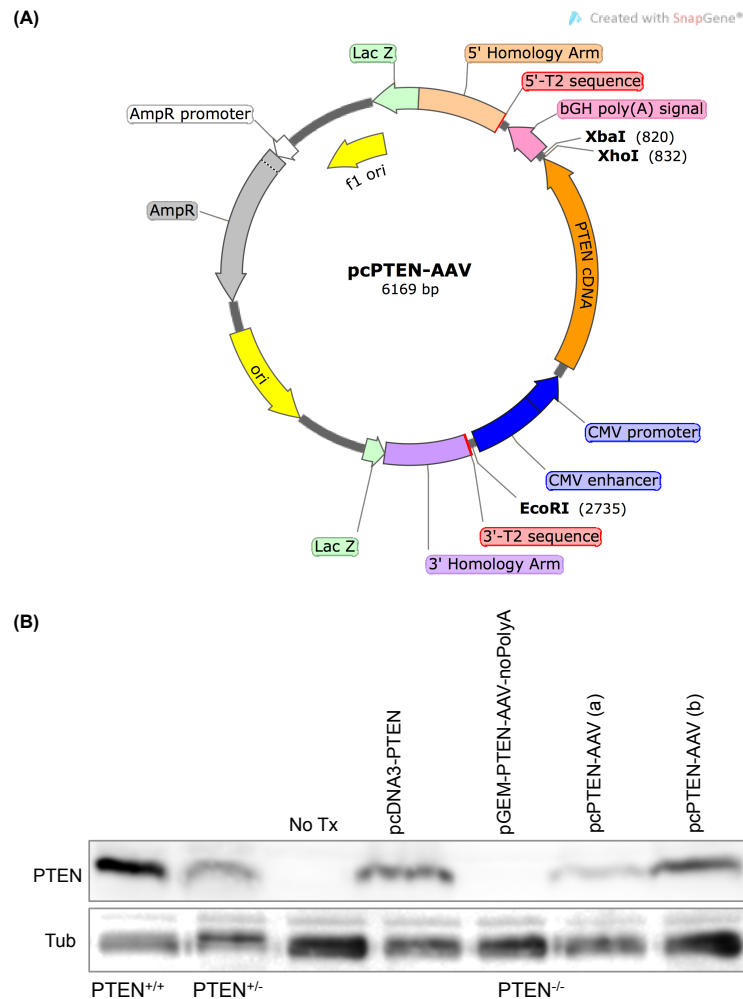


Figure 5.18. **(A)** Plasmid map of pcPTEN-AAV. **(B)** Immunoblot showing PTEN expression levels in HCT116 PTEN^{-/-} cells transiently transfected with either pcDNA3-PTEN, pGEM-PTEN-AAV-noPolyA, pcPTEN-AAV (two preparations labelled a and b). PTEN^{+/+} and PTEN^{+/-} HCT116 cells were included as a positive PTEN control. Tubulin levels were measured in all samples as a loading control. Tx- transfection.

Finally four PTEN mutants were cloned into the pcPTEN-AAV vector from pGEM-T-PTEN constructs using HindIII & XhoI sites yielding pcPTEN_C124S-AAV, pcPTEN_G129E-AAV, pcPTEN_H93A-AAV and pcPTEN_V403A-AAV constructs. Since XhoI enzyme cuts K13E&K289E mutant twice, it was cloned into HindIII & ClaI sites resulting in pcPTEN_K13E&K289E-AAV. Insert junctions with the vector backbone and the presence of the mutation were verified by sequencing (Appendix 12.2).

5.3.2. CRISPR/Cas 9 transfection

When all CRISPR components were ready, they were co-transfected into HCT116 PTEN^{-/-Φ}, HCT116 PTEN^{-/-Γ}, MRC-5 and PNT1A PTEN^{-/-} cells lines.

Initially, Lipofectamine 2000 mediated transfection was optimised for all cells (as explained in earlier experiments). Plasmids encoding Cas 9, gRNA-AAVS1 and donor PTEN-AAV (WT or one of five PTEN mutants) components were co-transfected as described in Table 5.3.

Table 5.3. Composition of Lipofectamine 2000 based transfection mixtures for CRISPR/Cas9 knock-in via HDR with PTEN donor plasmid (pcPTEN-AAV). Amounts are shown for cells cultured in 10 cm dishes.

	HCT116	MRC-5	PNT1A
DNA:Lipofectamine 2000 ratio	1:3	1:5	1:5
Total DNA, μ g	24	24	12
DNA mix			
hCas9	5 parts	5 parts	5 parts
gRNA-AAVS1	2.5 parts	2.5 parts	2.5 parts
pcPTEN-AAV	2.5 parts	2.5 parts	2.5 parts
pBabeHygro	1 part	1 part	
pBabePuro			1 part
Hygro selection	600 μ g/ml	400 μ g/ml	
Puro selection			0.5 μ g/ml
Neo selection			600 μ g/ml

1/11 part of pBabeHygro or pBabePuro was utilised in order to allow hygromycin/puromycin selection respectively to eliminate non-transfected cells. Hygromycin selection was employed for HCT116 PTEN^{-/-} and MRC-5 PTEN^{-/-} cells as they were generated using puromycin selection (Chapter 4). While PNT1A PTEN^{-/-} cells were created under both puromycin and hygromycin selection, dose response curves indicated that they retained sensitivity to puromycin at a concentration 0.5 μ g/ml. Thus, puromycin or neomycin selections were used for this cell line (puromycin was used when cells were co-transfected with pBabePuro and neomycin could be used because the hCas9 plasmid contains a neomycin resistance gene). Drugs were added to transfected cells 24 hours after transfection and were used until all non-transfected cells on a control plate were dead (about 7-10 days). Then, colonies were allowed to grow in media without drugs.

Several tens of resistant colonies were selected in the HCT116 PTEN^{-/-} cell background but none were recovered when using MRC-5 PTEN^{-/-} or PNT1A PTEN^{-/-} cells. Therefore, electroporation was utilised as an alternative transfection method for these two cell lines, which demonstrated better transfection efficiency for these lines compared to Lipofectamine 2000 transfection (Table 5.4).

Table 5.4. Composition of electroporation DNA mixtures for MRC-5 PTEN^{-/-} and PNT1A PTEN^{-/-} cell transfection. Total DNA amount: 10 µg per 10 cm dish.

DNA mix	MRC-5	PNT1A
hCas9	5 parts	5 parts
gRNA-AAVS1	1.25 parts	1.25 parts
pcPTEN-AAV	3.75 parts	3.75 parts
pBabeHygro	1 part	
pBabePuro		1 part
Hygro selection	400 µg/ml	
Puro selection		0.5 µg/ml
Neo selection		600 µg/ml

Cells were electroporated and selected with the appropriate antibiotic as described above and several tens of colonies were grown for each cell line in all drug-selection conditions. Resistant cell colonies were analysed using PCR and Western blotting.

5.3.2.1. PCR based analysis of knock-in clones

In order to determine if the CRISPR-Cas9 knock-in was successful and verify that the PTEN minigene was successfully incorporated into the pre-determined AAVS1 locus, a PCR screening method was developed. For this, two sets of primers were designed to allow the amplification from the genomic DNA with the AAVS1 region contained in the targeting vector to the PTEN donor insert (Figure 5.19).

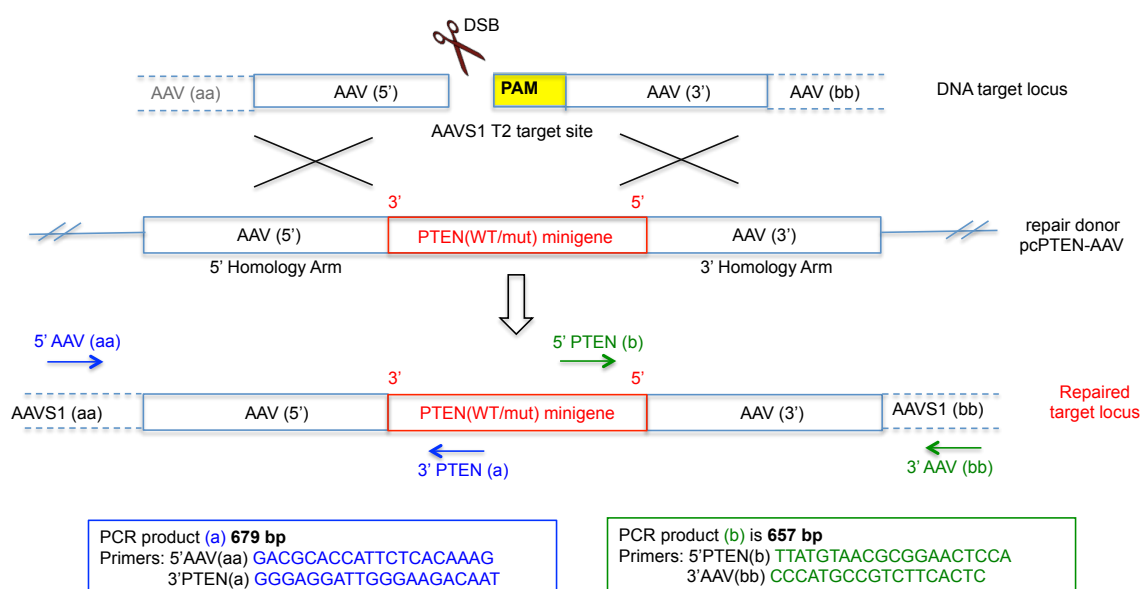


Figure 5.19. PCR scheme for determining integration of the PTEN minigene into the AAVS1 locus. (a) primer set consists of 5'AAV(aa) primer from the endogenous AAVS1 gDNA locus outside of 5' homology arm and 3'PTEN(a) primer from the PTEN donor pcPTEN-AAV plasmid (highlighted in blue). (b) primer set consists of 5'PTEN(b) primer from the PTEN donor and 3'AAV(bb) primer from the AAVS1 locus outside of 3' homology arm (highlighted in green). With correct integration, (a) primers should amplify 679 bp PCR product and (b) primers – 657 bp.

The Homo sapiens chromosome 19 GRCh38.p2 primary assembly (GeneBank accession number NC_000019.10) was used as a template for primer design in the gDNA AAVS1 region outside the homology arms contained in the targeting vector pcPTEN AAV. The CMV promoter region within donor plasmid was used for 5'PTEN(b) primer design and bGH polyA region served as a template for 3'PTEN(a) primer (because the PTEN minigene was incorporated in the reverse complement orientation within the pcPTEN-AAV plasmid). Primers were designed using Primer3 (Rozen, Skaletsky 1998). As a positive control, TS2^{PTEN} PCR primer covering the PTEN deletion in the genomic DNA (Chapter 3) was used. This primer would verify the gDNA quality and the PCR reagents for all samples as the amplified target sequence should be present in all cells.

gDNA from each cell colony was extracted via the short purification protocol(Chapter 2.1.3.1). PCR reactions were carried out with GoTaq G2 polymerase according to the conditions in Table 5.5.

Table 5.5. PCR conditions and two primer sets for PCR screening of knock-in cell colonies. The TS2^{PTEN} primer was used as a positive control.

	Specific primers		T melting, °C	PCR product
(a) primers	5'AAV(aa): GACGCACCATTCTCACAAAG	3'PTEN(a): GGGAGGATTGGGAAGACAAT	63.3 63.9	679 bp
(b) primers	5'PTEN(b): TTATGTAACGCGGAAGCTCCA	3'AAV(bb): CCCATGCCGTCTTCACTC	63 64.5	657 bp
TS2 ^{PTEN} primers (control)	5': TTTGCTTGAGATCAAGATTGCAGA	3': TCCTTATTTGGATATTCTCCCAATG	66.8 66.2	350 bp

Polymerase	GoTaq G2			
	T, °C	Duration	No of cycles	
Initial denaturation	95	2 min	1	
Denaturation	95	30 s	25	
Annealing	59	1 min		
Extension	72	1 min		
Final extension	72	5 min	1	
Hold	4	Indefinite	-	

PCR composition (20 µl)
2 µl gDNA
1x Reaction Buffer
80 ng each primer
200 µM each dNTP
1 M betaine
0.1 µl polymerase

All knock-in resistant colonies from four cell lines and six different donor DNAs (at least 20 of each condition) were screened with (b) primer set and TS2^{PTEN} primer set yielding only four colonies with the successful (b) PCR products, while TS2^{PTEN} PCR product was present in all samples. Typical examples of PCR reactions resolved on a 2% agarose gel are shown in Figure 5.20(A).

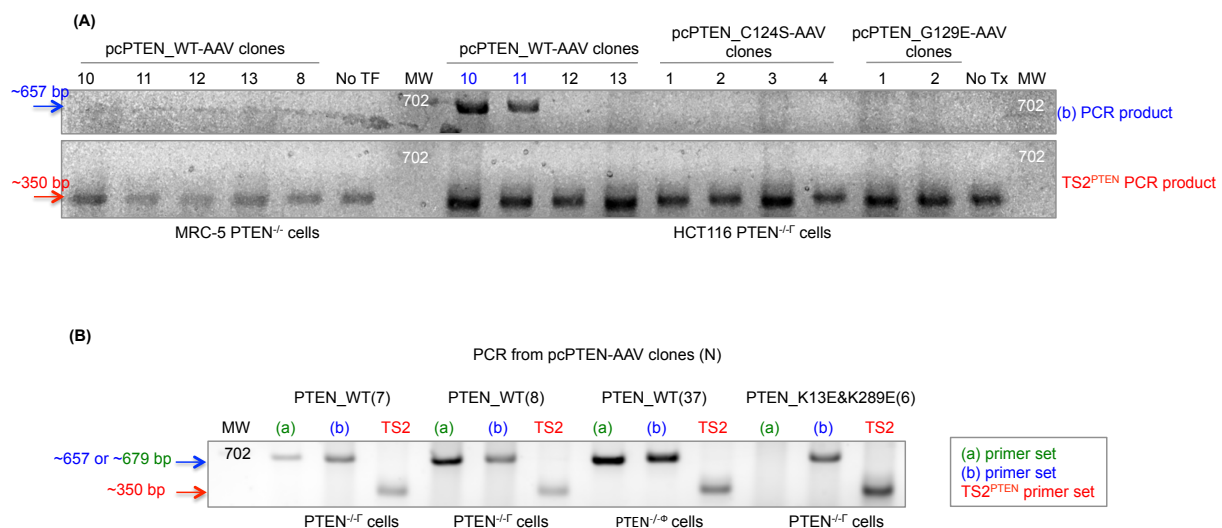


Figure 5.20. PCR screening of PTEN knock-in colonies. **(A)** Representative agarose gel with PCR reactions from several gDNA samples amplified with (b) primer set. (b) PCR product (657 bp) was detected only in 2 wells (highlighted with blue arrows), while control TS2^{PTEN} PCR product (~350 bp) was present in all samples (red arrow). gDNA samples were purified from MRC-5 PTEN^{-/-} or HCT116 PTEN^{-/-} cells co-transfected with WT, C124S or G129E PTEN donor DNA. **(B)** Comparing (a) and (b) PCR primer sets in HCT116 PTEN^{-/-} or PTEN^{-/-} cells. TS2^{PTEN} set of primers was used as a positive control for gDNA quality demonstrating a 350 bp band in all samples. (a), (b) and TS2^{PTEN} PCR products are highlighted in green, blue and red respectively. MW - DNA ladder. Tx- transfection.

Out of these four positive colonies three clones (N 7, 8 and 37) generated with WT PTEN donor, resulted in both (a) and (b) PCR products (679 and 657 bp respectively). Whereas PTEN^{K13E&K289E} Clone N 6 contained only (b) PCR product but not (a) (Figure 5.20(B)).

This low percentage of successful colonies was expected because the repair of Cas9-mediated DSB via homology recombination is known to be a relatively rare event (Lin et al. 2014a). NHEJ is a much more preferable process by cells, hence several changes in transfection protocol were applied in order to increase the HDR rate. For this, a higher concentration of donor plasmid and lower concentration of the drug resistance plasmid to enforce a greater likelihood of only selecting co-transfected cells were tested according to conditions in Table 5.6. All changes were initially applied to HCT116 PTEN^{-/-} cells due to its higher growth speed compared to MRC-5 and PNT1A cells.

Table 5.6. Alternative compositions of Lipofectamine 2000 based transfection DNA mixtures for HCT116 PTEN^{-/-} cells knock-in. **(A)** Composition utilised higher pcPTEN-AAV donor concentration and **(B)** composition consists of less pBabeHygro resistant plasmid. DNA amount: 24 µg per 10 cm dish.

DNA mix	(A)	(B)
hCas9	5 parts	10 parts
gRNA-AAVS1	1.25 parts	2.5 parts
pcPTEN-AAV	3.75 parts	7.5 parts
pBabeHygro	1 part	1 part

Another alteration tested was replacing hCas9 plasmid with the mutant hCas9_D10A, which functions as a nickase and performs a single strand DNA break in the complementary target strand instead of DSB. This mutant can induce HDR with reduced levels of NHEJ-induced indels at some sites (Mali et al. 2013b, Sander, Joung 2014).

Lastly, cell cycle synchronisation of transfected cells was optimised in order to deliver Cas 9-gRNA complex to M-phase synchronised cells. It was reported that HDR rates could be improved up to 31% with nocodazole synchronisation in comparison with 9% in unsynchronised HEK293T cells (Lin et al. 2014a). For this, 200 ng/ml nocodazole was added to cells 7 hours after transfection and 17 hours prior cell splitting and drug treatment. This timing was based on the assumption that plasmids take about 24 hours to incorporate into gDNA and express their encoded proteins or RNA, and cells take about 17 hours to be synchronised by nocodazole.

Unfortunately, all these methodological alterations did not improve the HDR rate. In total, out of more than 500 screened colonies in all four cell lines (HCT116 PTEN^{-/-Φ}, HCT116 PTEN^{-/-Γ}, MRC-5 and PNT1A PTEN^{-/-} cells) only 10 were positive when assessed with PCR using the (b) primer set. Each of these 10 colonies were based on HCT116 PTEN^{-/-} cells (out of which six were derived from PTEN^{-/-Γ} and 4 – from PTEN^{-/-Φ} cell lines). When these 10 positive colonies were further screened by PCR using the (a) primer set, only 6 provided a positive PCR product indicating only partial integration of the donor PTEN DNA in the remaining 4 colonies. Four of those six fully positive colonies represent a successful integration of WT PTEN from pcPTEN-AAV and the other two contained the K13E&K289E mutant from pcPTEN_K13E-K289E-AAV.

5.3.2.2. Western blotting characterisation of knock-in clones

The six successful knock-in colonies that were characterised via PCR screening were analysed via western blotting. PTEN expression was detected in five out of six clones, pointing out that 1 colony probably harboured a random expression-affecting mutation or a frame shift within a donor PTEN insert (Figure 5.21). PTEN^{-/-Φ} Clone N 6 (red font) did not express PTEN even though it was positive in PCR screening (with both (a) and (b) primer sets). PTEN levels expressed in all other clones were similar to PTEN^{+/-} endogenous PTEN levels.

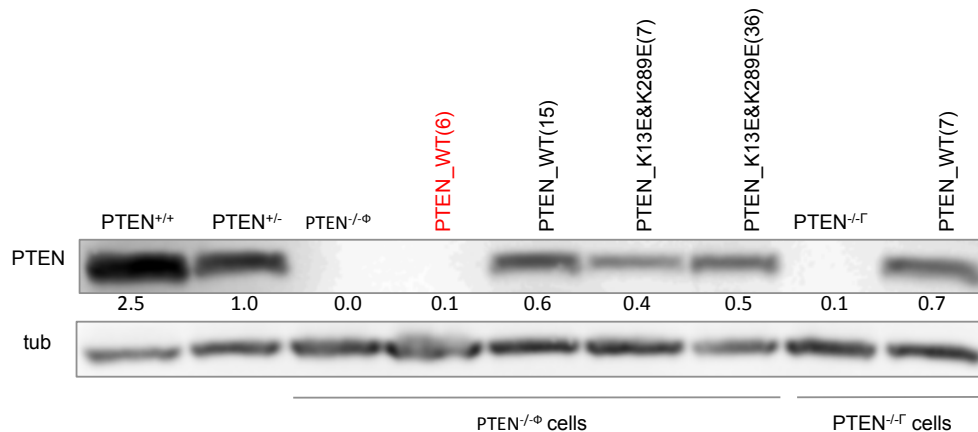


Figure 5.21. western blotting analysis of successful CRISPR-Cas9 PTEN knock-in clones on the basis of HCT116 PTEN^{-/-} or PTEN^{-/-}Φ cells. PTEN^{+/+} and PTEN^{+/-} cell samples were run as a positive control of PTEN levels. Tubulin levels were assessed as a loading control. The numbers under the bands represent the ratio of protein expression with PTEN^{+/+} samples arbitrary set as 1.0 and determined by ImageStudioLite (LI-COR Biosciences).

As expected transgene PTEN levels in CRISPR-Cas9 knock-in cells were comparable to the endogenous PTEN levels in PTEN^{+/-} cells indicating a likelihood of a single integration of the PTEN donor plasmid inside of cellular genome (however, only Southern Blotting could completely confirm this statement). Levels of exogenous PTEN expression were slightly lower than endogenous PTEN indicating that CMV promoter could be slightly weaker than native PTEN promoter in the genome.

5.3.3. Conclusions

The use of the CRISPR/Cas9 system lead to the successful integration of wild type and the K13E&K289E mutant into the genomes of HCT116 PTEN null cells. However, integrants housing the other PTEN mutants in HCT116 cells or integrants derived from MRC-5 or PNT1A cells were not isolated, limiting the use of this system. In order to attempt to generate the whole range of the integrants desired, a simplified system for introducing minigenes into cells was assessed. This being based on the Sleeping Beauty transposon.

5.4. PTEN knock-in via Sleeping Beauty Transposons

The Sleeping beauty system (SB) utilises transposable elements for transgene integration into the genome (Geurts et al. 2003, Dupuy et al. 2005, Mátés et al. 2009, Kowarz, Löscher & Marschalek 2015). This system is as easy and fast as simple plasmid transfection but has certain advantages over it: (i) target site specificity: while transfected plasmids can integrate into start codon or PolyA sequence of endogenous genes, integration of SB transposons is limited to TA-dinucleotides (near 30,000 potential sites in the human genome); and (ii)

insulation from surrounding genetic elements: the insulator function of Inverted Terminal Repeats (ITRs) flanking the gene of interest in transposon fragment can allow robust long-term gene expression even if integration occurs at sites that if a standard plasmid integrated would yield no expression (Kowarz, Löscher & Marschalek 2015). The main drawback of this system is that there is no way of controlling the copy numbers of integrated transgene. Usually, 6-10 integrations ± 2 copies are expected per cell using SB. This may cause overexpression of the gene of interest (PTEN in this case) in comparison with a single integration that was planned originally. However, given the time constraints resulting from pursuing the other targeted integration systems, the SB system was chosen as a quick way of introducing PTEN WT or PTEN mutants in PTEN^{-/-} cells at least with the comparable exogenous PTEN expression levels between each other (but possibly not with endogenous levels in PTEN^{+/-} or PTEN^{+/+} cells).

The SB method is based on a simple co-transfection of a plasmid containing the gene of interest flanked with ITRs (transposon) together with the SB100X transposase-encoding plasmid into cells resulting in integration of the sequence flanked by the ITRs (Figure 5.22(A)). The plasmids encoding the SB100X transposase (pCMV(CAT)T7-SB100, (Mátés et al. 2009)) and the transposon backbone (pSBbi-GB, (Kowarz, Löscher & Marschalek 2015), Figure 5.22(B)) were obtained from Addgene. Initially, the transposon construct encoding PTEN was constructed as described below.

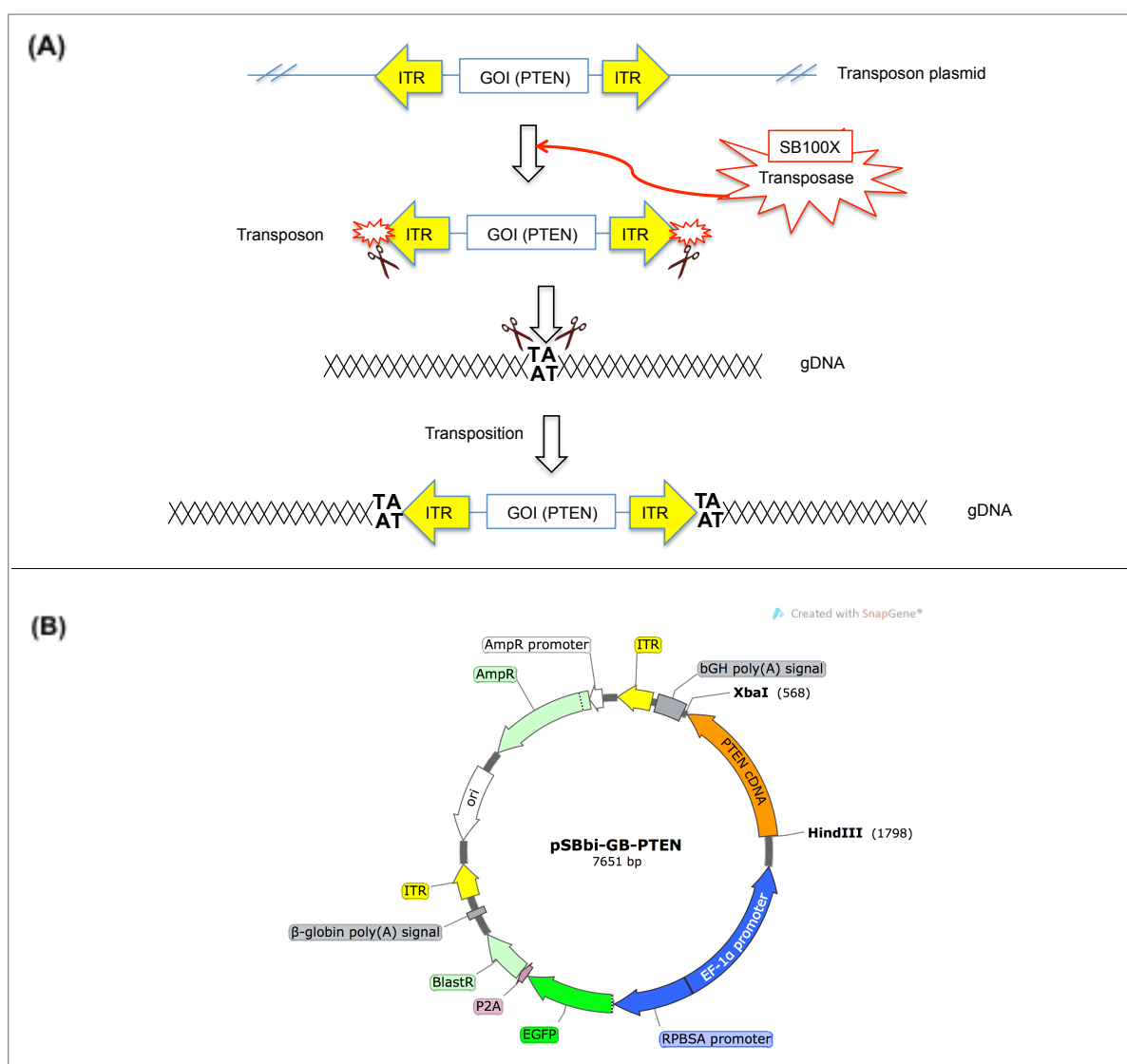


Figure 5.22. **(A)** Schematic showing integration of a Sleeping Beauty encoded gene of interest (GOI). The SB100X transposase binds to the inverted repeats (ITRs) flanking the gene of interest and cuts the plasmid DNA via endonuclease activity. The released transposon binds a strand of gDNA with a TA dinucleotide. At the next stage, the transposase generates a double strand break in the genomic DNA and allows the artificial transposon to integrate. **(B)** Plasmid map of pSBbi-GB-PTEN. EF1α/RPBSA bi-directional promoter regulates (i) PTEN expression in the 3' to 5' direction; and (ii) GFP and blasticidin (blast) selectable markers separated by 2A peptide in the 5' to 3' direction. The two inverted ITR fragments indicate sites where the transposase would bind and cut.

5.4.1. pSBbi-GB-PTEN construction and transfection

PTEN_{WT}/mut cDNAs were subcloned from appropriate pcPTEN-AAV vectors in reverse orientation into HindIII/XbaI sites within pSBbi-GB. The resulting pSBbi-GB-PTEN constructs were characterised via restriction digestion and sequencing of insert junctions with the vector backbone. Each PTEN mutation was also confirmed. (Appendix 12.2).

All pSBbi-GB-PTEN plasmids were co-transfected with the pCMV(CAT)T7-SB100 plasmid encoding SB100X transposase into HCT116 PTEN^{-/-} cells using Lipofectamine 2000 transfection and into MRC-5 cells using electroporation as shown in Table 5.7.

Table 5.7. The composition of SB transfection mixture for PTEN knock-in: **(A)** Lipofectamine 2000 transfection (for HCT116 cells); **(B)** Electroporation (for MRC-5 cells).

(A)				
	DNA	Lipofectamine 2000	pSBbi-GB-PTEN	pCMV(CAT)T7-SB100
ratio	1	3	19	1
Amounts, μg	4	12	3.8	0.2

(B)			
	DNA	pSBbi-GB-PTEN	pCMV(CAT)T7-SB100
ratio		19	1
Amounts, μg	10	9.5	0.5

PNT1A cells were not studied in this experiment due to their very low growth rate and limiting time remaining).

Pools and ~ six clones for each condition with successful transposon integration were selected with blasticidin (10 $\mu\text{g}/\text{ml}$), and resistant colonies were analysed with flow cytometry to detect the GFP and Western blotting for ectopic PTEN.

5.4.2. Analysing PTEN transposon integration via flow cytometry

All pSBbi-GB-PTEN plasmids express GFP, which could be used for the initial screening of the resistant cell colonies and could potentially provide a means of quantifying expression from the integrated plasmid. Fluorescence was compared for all clones and those emitting GFP signals of a similar intensity were selected for further experiments; it was envisaged that clones expressing similar levels of GFP would express similar levels of PTEN, an essential prerequisite for comparing the effects of PTEN mutations on phenotypes such as synthetic lethality and senescence. GFP intensity plots determined by flow cytometry for total transfected cells (pools) and for isolated clones transfected with pSBbi-GB-PTEN_{WT/mut} using PTEN^{-/-} HCT116 or PTEN^{-/-} MRC-5 parental cells are presented in Figure 5.23 and Figure 5.24 respectively.

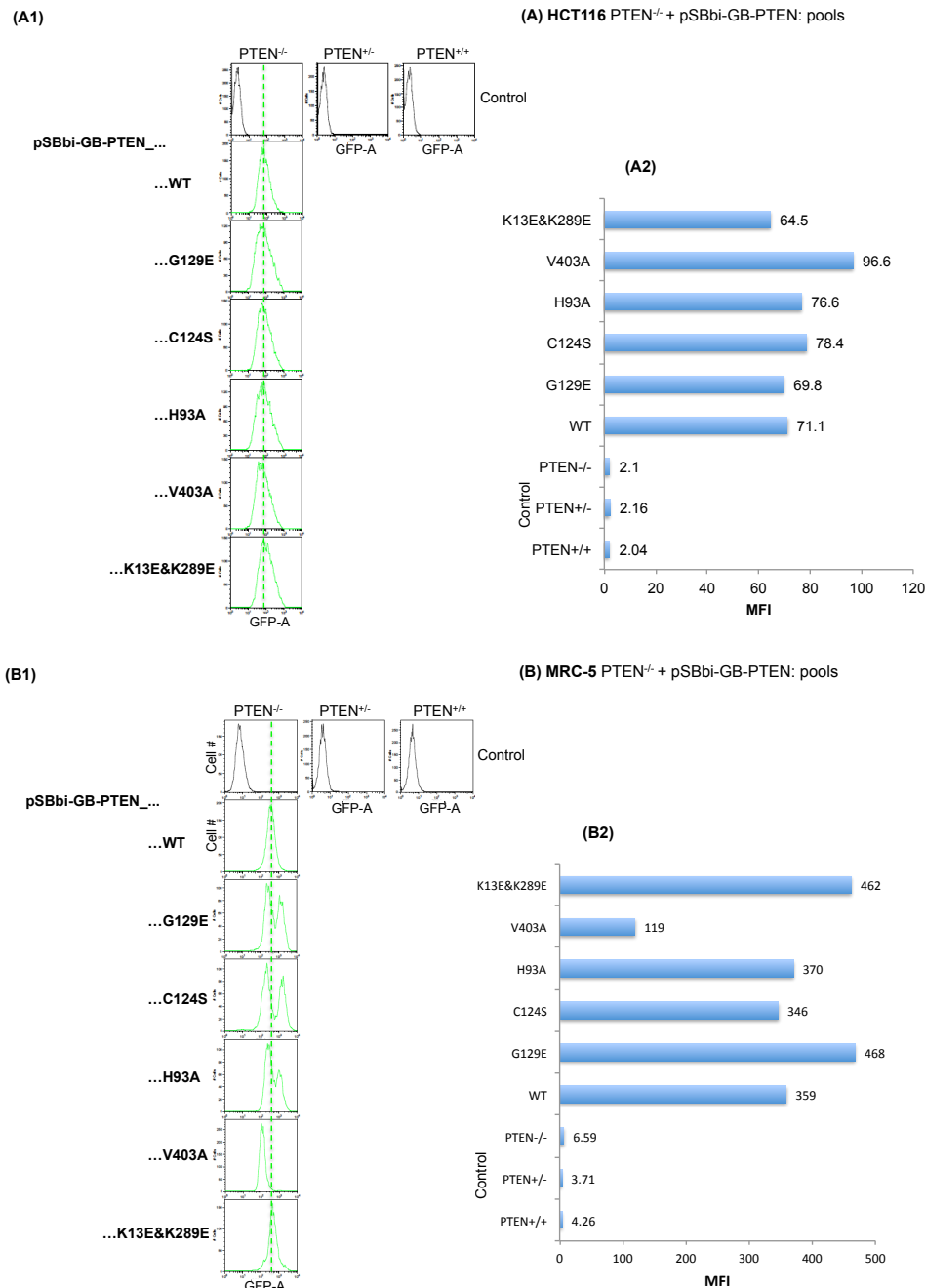
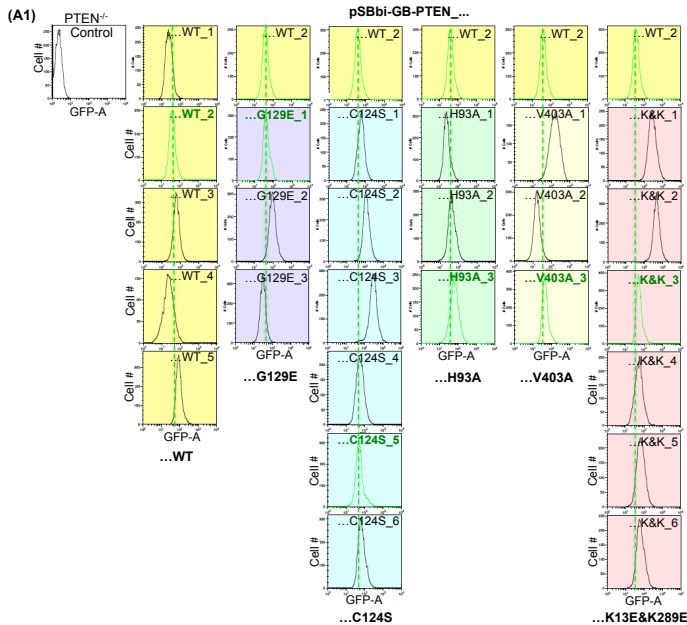
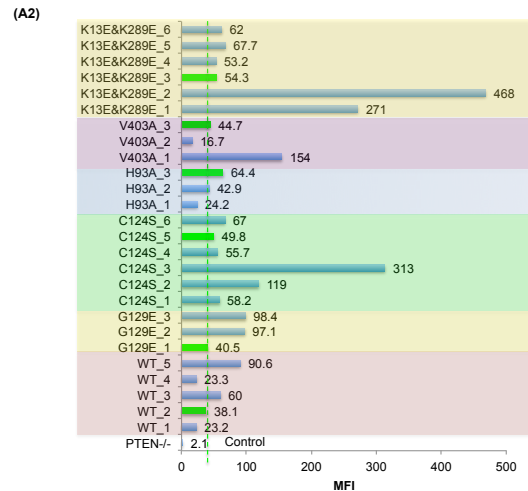


Figure 5.23. GFP intensity in cellular pools transfected with PTEN_{WT/mut} Sleeping Beauty constructs: **(A)** HCT116 PTEN^{-/-} pools; **(B)** MRC-5 pools. Fluorescence was measured with flow cytometry. Data are shown as histogram plots **(A1 and B1)** or Median Fluorescence Intensity (MFI) bar plots **(A2 and B2)**. Autofluorescence was measured in the absence of transfection in parental cells (labelled as control).

(A) HCT116 PTEN^{-/-} + pSBbi-GB-PTEN: clones



(B) MRC-5 PTEN^{-/-} + pSBbi-GB-PTEN: clones

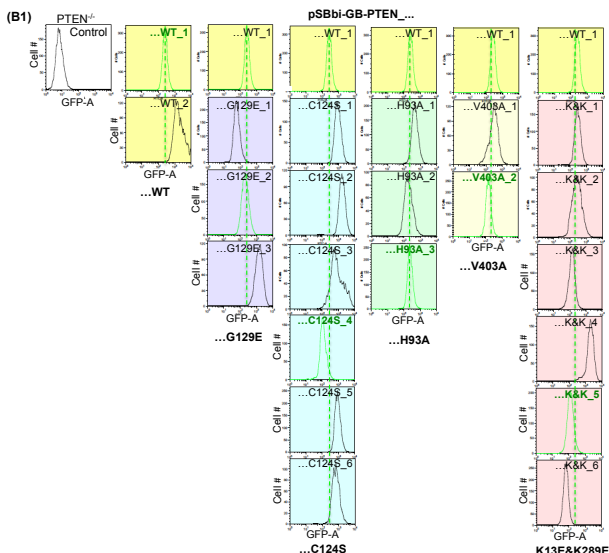
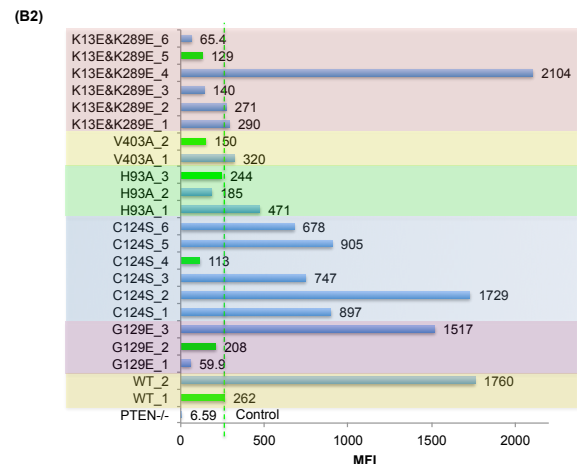


Figure 5.24. GFP intensity in cellular clones transfected with PTEN_WT/mut Sleeping Beauty constructs: (A) HCT116 PTEN^{-/-} clones; (B) MRC-5 clones. Fluorescence was measured with flow cytometry. Data are shown as histogram plots (A1 and B1) or Median Fluorescence Intensity (MFI) bar plots (A2 and B2). Those clones that have the closest to chosen WT-clone (green plot) GFP intensity are highlighted with green font. Autofluorescence was measured in the absence of transfection in HCT116 cells (labelled as control). K&K = K13E&K289E.

The flow cytometry data demonstrated very close GFP intensity for all HCT116 cellular pools suggesting that transfection was generally quite consistent. On the contrary, the MRC-5 pools displayed substantially more variation in GFP expression, perhaps indicating more variable transfection efficiencies for different cells in the parental population. The pools gave a rapid indication that the system was functional and on the strength of this, clones resistant to

blastidicin were isolated, amplified and screened for GFP fluorescence intensity. Several clones for each mutant and WT PTEN transfection were scanned in each cell line and chosen for further analysis.

5.4.3. Analysing PTEN transposon integration via western blotting

After choosing the clones based on similarity of the GFP fluorescence intensity to the PTEN_WT clones, the selected clones were analysed by western blotting for PTEN. When normalised to tubulin levels, PTEN levels were mostly higher in the transfected cells (both pools and clones) when compared to PTEN^{+/-} and even PTEN^{+/+} cells in both HCT116 and MRC-5 cell lines (Figure 5.25).

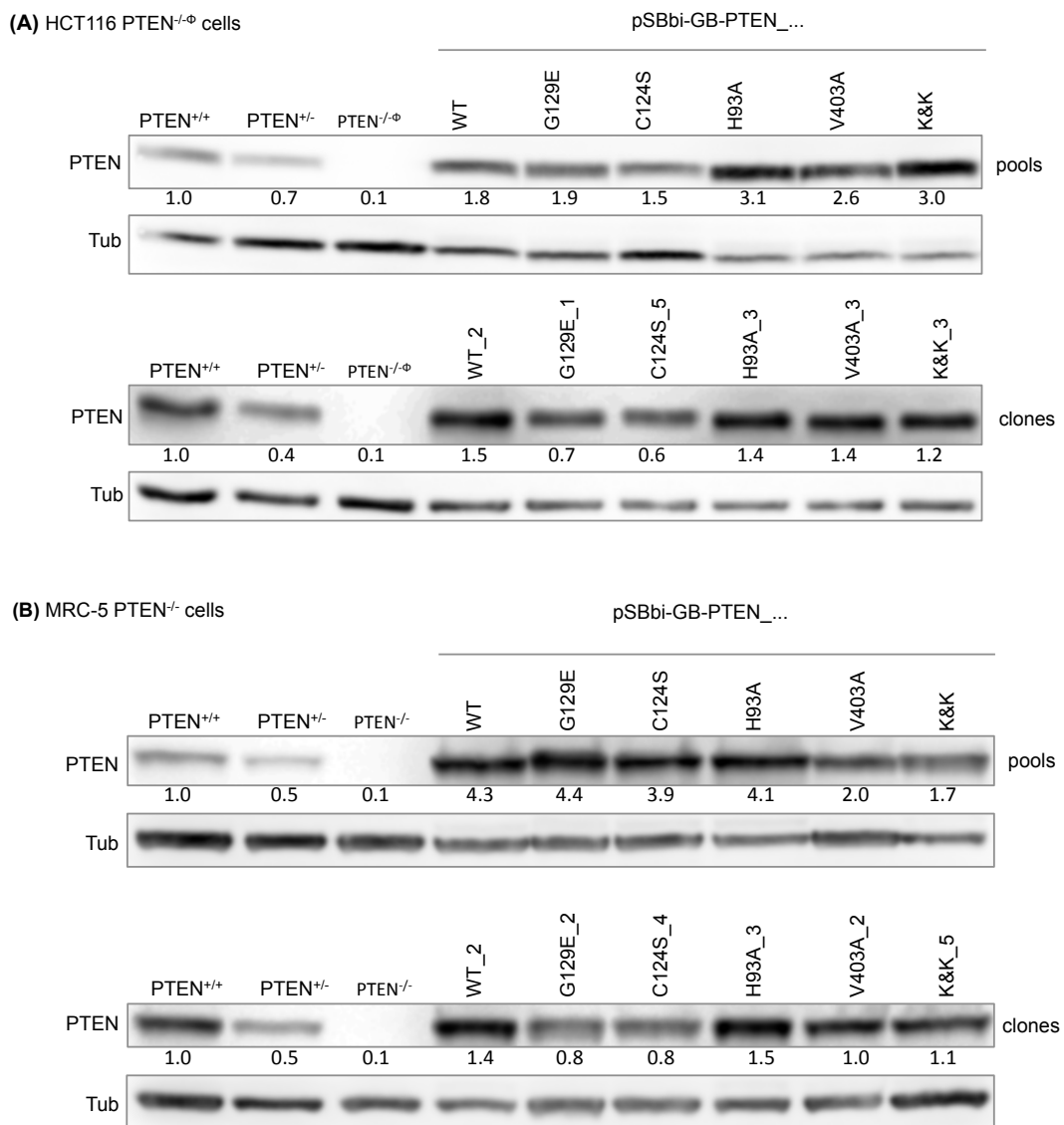


Figure 5.25. PTEN expression levels in cells transfected with pSBbi-GB-PTEN_WT/mut: **(A)** HCT116 PTEN^{-/-} cells; **(B)** MRC-5 PTEN^{-/-} cells. PTEN^{+/+} and PTEN^{+/-} cell samples were run as a positive controls for PTEN levels. Tubulin levels were determined as loading controls. K&K

= K13E&K289E. The numbers under the bands represent the ratio of protein expression with PTEN^{+/+} samples arbitrary set as 1.0 for each cell line and determined by ImageStudioLite (LI-COR Biosciences). The figure contains a representative blot from three independent experiments.

Interestingly, the PTEN_G129E and PTEN_C124S mutants demonstrated the lowest PTEN levels in clonal samples derived from both HCT116 and MRC-5 cell lines. These two mutations disrupt PTEN catalytic function. Considering that GFP levels were comparable between all the clones, this result appears to indicate that the steady state levels of PTEN are reduced when its catalytic function is disrupted. This may be due to many factors including altered protein stability. In contrast, cells carrying the PTEN_V403A mutant (which is within the PDZ domain) express similar steady state levels of PTEN to the wild type clones in both cell types constructed. This mutation has been previously reported to reduce PTEN stability in Cos-7 cells (Valiente et al. 2005), perhaps indicating different mechanisms of turnover between these cell types.

5.4.4. Conclusions

Sleeping Beauty-based integration allowed a quick and functional integration of PTEN (WT and all mutants) into PTEN^{-/-} HCT116 and MRC-5 cells. Analysis of GFP fluorescence by flow cytometry allowed rapid identification of cell clones that could be normalised for GFP expression and this was largely reflected by similar expression levels of PTEN. Unfortunately this method does not allow isolation of single integrants as was originally planned for this project. Therefore, the PTEN levels in these knock-in clones could not be compared to the endogenous PTEN^{+/+} levels for accurate investigation in functional experiments with PTEN. However, the isolation of these cells would enable comparative experiments to be performed, enabling comparison of PTEN-mut expressing cells with cells expressing wild type PTEN, especially as PTEN protein levels were shown to be broadly similar across all clones used.

5.5. Functional characterisation of PTEN Knock-in cell lines

5.5.1. Introduction

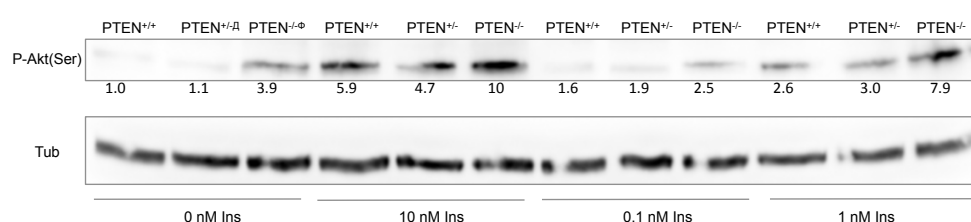
Akt phosphorylation at Ser473 and Thr308 is dependent on PtdIns(3,4,5)P₃ (further referred as PIP3) generated by activated PI3K and thus is proportional to the cellular levels of this lipid (Maehama, Dixon 1998). Since PIP3 is a major PTEN substrate *in vivo*, measuring P-Akt levels is often used as a surrogate measure of PTEN activity, particularly when PTEN expression has been altered in some way, as for instance it has in the knock-in cell lines compared to the parental PTEN null cells. However, as PTEN is downstream of PI3K, it is important to pre-stimulate quiescent cells with low amounts of insulin in order to slightly increase the activity of

PI3K above its basal levels and sequentially. This would minimally stimulate PI3K so that there is chance that PTEN can produce a noticeable suppression of Akt phosphorylation (Schmid et al. 2004, Rosivatz et al. 2006, Georgiades et al. 2010, Nguyen et al. 2011).

5.5.2. P-Akt assay

Initially, the optimal low insulin concentration which caused a slight increase in Akt phosphorylation was determined using both HCT116 and MRC-5 cells. For this, the cells were cultured in starvation media (without FBS) for 20 hours prior stimulation with various insulin concentrations. 15 minutes after insulin treatment, cells were lysed and subject to immunoblotting for phospho-Akt and tubulin (Figure 5.26).

(A) HCT116 cells



(B) MRC-5 cells

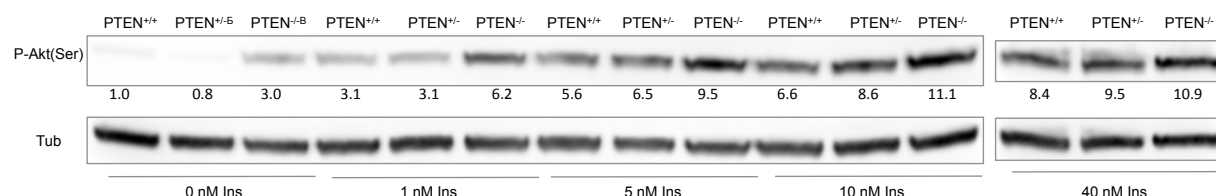
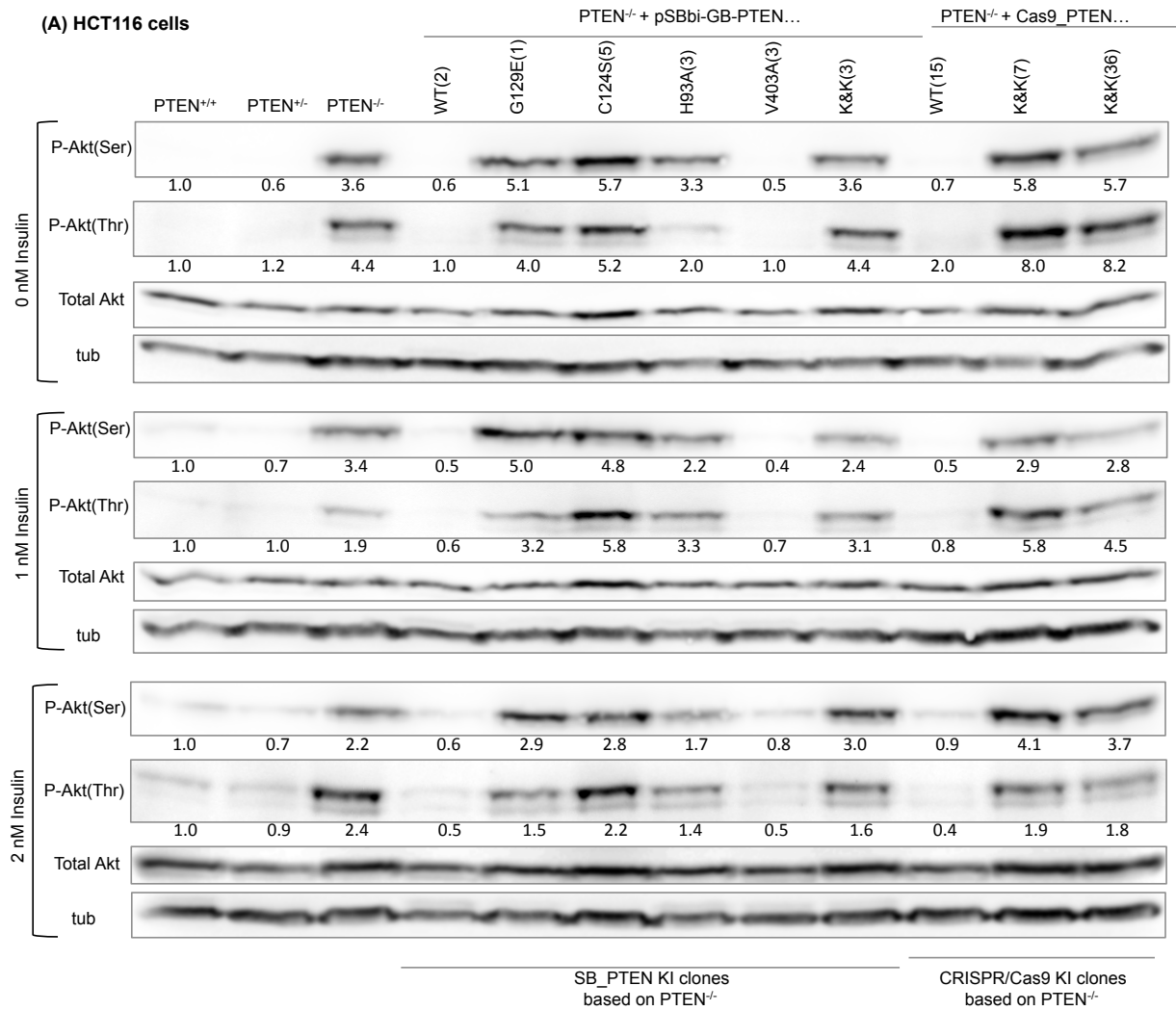


Figure 5.26. P-Akt(Ser473) protein levels in PTEN^{-/-}, PTEN^{+/-} and PTEN^{+/+} cells stimulated with various insulin concentrations. (A) HCT116 and (B) MRC-5 cells. Tubulin was used as a loading control. The numbers under the bands represent the ratio of protein expression with non-treated PTEN^{+/+} samples arbitrary set as 1.0 for each cell line and determined by ImageStudioLite (LI-COR Biosciences).

1 nM insulin demonstrated the lowest detectable increase in phospho-Akt in both cell lines. As expected P-Akt levels were higher in PTEN^{-/-} cells compared to PTEN^{+/-} and PTEN^{+/+} cells, as PTEN acts as a suppressor of Akt phosphorylation. This effect is detectable in both cell lines. At higher insulin concentrations additional PI3K stimulation appears to generate sufficient PIP3 to cause generation of P-Akt irrespective of PTEN status. Interestingly, in PTEN^{-/-} knock-out clones derived from both HCT116 and MRC-5 cells detectable P-Akt levels remained in cells cultured for 20h without serum indicating the importance of PTEN in allowing P-Akt dephosphorylation. Prolongation of starvation time lead to an unhealthy cellular phenotype and cells were not judged fit for further use (data not shown). Thus, non-stimulated cells were considered as low-stimulated cells in the next experiments.

It is noteworthy that this PTEN functionality assay resolved some of the uncertainty regarding HCT116 Clone D (PTEN^{+/-}) that was discussed in Chapter 3.2.2.2. Akt phosphorylation is inhibited in this clone to comparable levels as found in WT PTEN^{+/+} cells. Also one copy of PTEN is enough for cells to behave like WT with respect to P-Akt (see Figure 5.26(A)). This indicates that the clone D is effectively PTEN^{+/-} in terms of catalytic activity^{###}. Next P-Akt levels in all knock-in clones were monitored upon activation with insulin (0, 1, 2 nM). Akt phosphorylation was investigated in cells with mutant PTEN KIs (both via CRISPR/Cas9 and SB methods) and sequentially compared to the endogenous PTEN^{-/-}, PTEN^{+/-} and PTEN^{+/+} cells (Figure 5.27).



^{###} See Chapter 3.2.2.2 for more details. Briefly, HCT116 KO Clone D harboured two indels from two gRNAs: (i) a 17 bp gap at the TS1 sequence and (ii) R233 del. at the TS2 sequence. It was not clear if R233 del. happened in the same chromosome as the 17 bp gap and also if this would cause complete loss of PTEN function, i.e. PTEN^{-/-} genotype. This PTEN assay and P-Akt inhibition (in combination with PCR analysis) indicated that actually Clone D expresses one copy of PTEN with intact lipid phosphatase function.

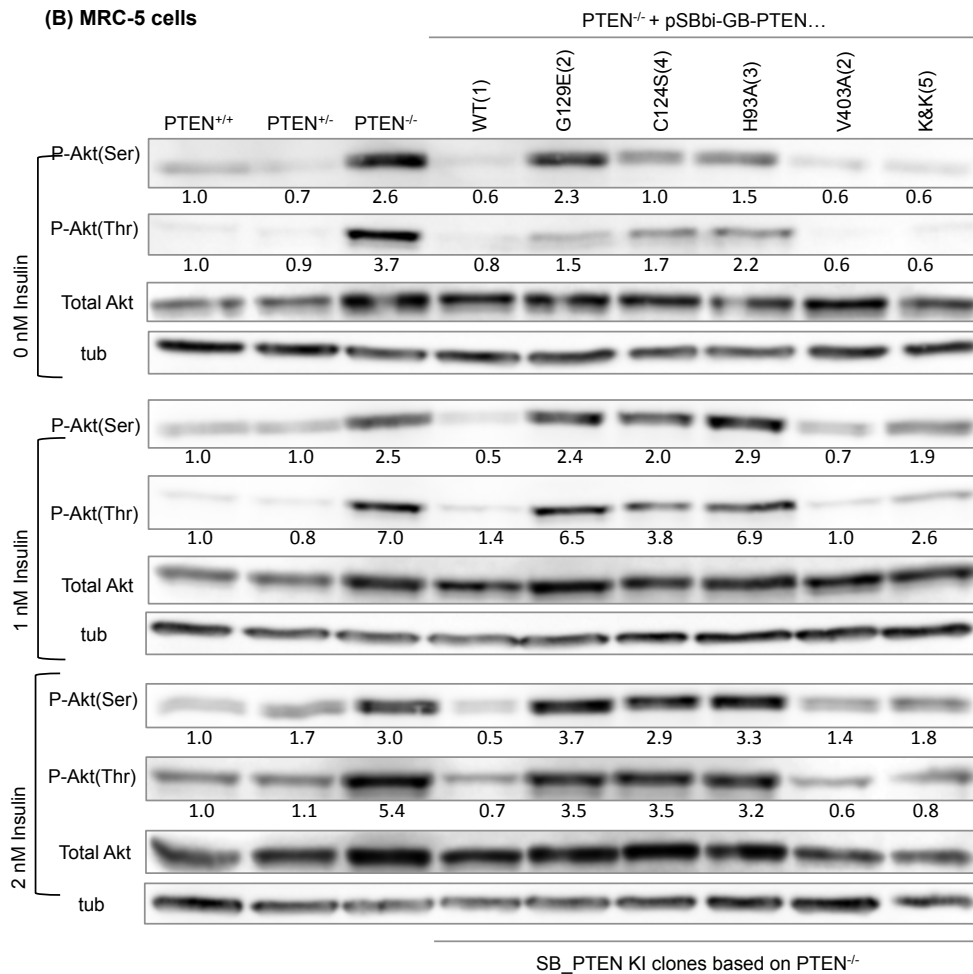


Figure 5.27. P-Akt(Ser473) and P-Akt(Thr308) levels in PTEN knock-in **(A)** HCT116 and **(B)** MRC-5 cells. P-Akt levels upon activation with 0, 1 or 2 nM insulin for 15 min in PTEN^{-/-}, PTEN^{+/-} and PTEN^{+/+} cells were compared with P-Akt levels in PTEN knock-in clones (numbered in brackets) generated from PTEN^{-/-} cells. Total Akt and tubulin were used as loading controls. K&K = K13E&K289E. The numbers under the bands represent the ratio of protein expression with PTEN^{+/+} samples arbitrary set as 1.0 for each each insulin treatment condition and determined by ImageStudioLite (LI-COR Biosciences).

Similar to the control experiment (Figure 5.26), Akt phosphorylation (on both Ser473 and Thr308 sites) was blocked in PTEN^{+/+} and PTEN^{+/-} cells, as well as in the knock-in clones expressing (i) WT PTEN and (ii) V403A PTEN mutants (both CRISPR and SB knock-in). (i) Indeed, WT PTEN should restore P-Akt inhibition similar to PTEN^{+/-} or PTEN^{+/+} cells. (ii) PTEN-V403A mutation within the PDZ-binding domain is known to disrupt PTEN binding to PDZ domains of other proteins affecting PTEN stability in Cos-7 cells (Valiente et al. 2005) although apparently not in HCT116 or MRC5 cells (Figure 5.27). It was also reported that complete removal of the PDZ domain from PTEN noticeably decreased its ability to inhibit Akt activity in NIH3T3 cells (Wu et al. 2000). However, the P-Akt results in Figure 5.27 suggest that the V403A mutation does not disrupt lipid phosphatase PTEN function in HCT116 and MRC-5 cells.

On the contrary, knock-in clones with G129E and C124S PTEN mutations within the phosphatase motif did not inhibit P-Akt levels in both cell lines. This is expected as the C124S mutation generates a catalytically dead protein lacking both lipid and protein phosphatase activity (Maehama, Dixon 1998, Myers et al. 1998), whereas G129E mutant retains activity towards phospho-peptides but loses the PIP3 phosphatase function (Vivanco, Sawyers 2002, Myers et al. 1998). These P-Akt results are also consistent with previously reported data (Li, Sun 1998, Radu et al. 2003, He et al. 2012, Papa et al. 2014).

The cells carrying H93A PTEN mutation also failed to suppress Akt phosphorylation on Ser473 site in both cell lines. Interestingly, Thr308 site was still phosphorylated in MRC-5 knock-in cells but not in HCT116 cells. This suggested that this mutation disrupts PTEN phosphatase activity towards PIP3 substrate only partially in HCT116 cells in comparison with total loss of lipid activity in MRC-5 cells. These findings may be explained by the nature of H93A mutation. This mutation within the “WPD” loop of the phosphatase domain reduces the PIP3 phosphatase activity by ~75% (Lee et al. 1999). The residual 25% of PTEN activity appears to be sufficient to suppress Akt phosphorylation partially on Thr308 site in HCT116 cells but not in MRC-5 cells. This also could be linked to the PI3K mutation, which is carried by HCT116 cells (Wang et al. 2007).

The K13E&K289E double mutant impairs PTEN trafficking to the nucleus because ubiquitinylation at these two lysine residues regulates PTEN nuclear-cytoplasmic shuttling (Song et al. 2008). It is known that the K289E mutation alone does not disrupt the PTEN activity *in vitro* and *in vivo* (Song et al. 2008) : this mutant still dephosphorylates the PIP3 substrate, inhibits tumour growth in soft agar assay and has only slightly higher proliferation advantage in U87-MG transfected cells compared to PTEN_WT (Georgescu et al. 2000). However, the K13E mutant displays wild-type activity only *in vitro* by retaining robust phosphatase activity against PIP3 substrate (Walker et al. 2004). In contrast, *in vivo* this mutant fails either to prevent Akt phosphorylation, or inhibit cell proliferation when expressed in U87-MG cells (Walker et al. 2004). The defect in the K13E mutant activity was demonstrated to be due primarily to failure to target PTEN to the plasma membrane (Walker et al. 2004). These data suggest that the K13E&K289E double mutant should also behave as catalytically dead mutant *in vivo*.

This hypothesis was confirmed on the basis of HCT116 cells expressing the double mutant via both SB and CRISPR/Cas9 knock-in methods: P-Akt levels were comparable to those in cells expressing catalytically dead PTEN mutants. Surprisingly, the MRC-5 knock-in clone expressing K13E&K289E mutant still demonstrated strong suppression of Akt phosphorylation (on both Ser473 and Thr308 sites) comparable to cells expressing PTEN_WT. This result indicates that the effect of the K13E mutation is cell line dependent and that the MCR-5 clone with the K13E&K289E PTEN mutation either overcomes the failure to target this protein to the

plasma membrane, or cytoplasmic PTEN is enough for suppression of Akt phosphorylation. Further work is required to follow up these observations, investigating the nuclear and cytoplasmic protein fractions and their activities. However, these experiments were not performed within this project due to lack of time.

Finally, two WT PTEN knock-in derived from HCT116 PTEN^{-/-} cells via CRISPR/Cas9 method were analysed to confirm that only one of two knock-in clones was expressing functional and active PTEN. Even though the PCR assay indicated that both Clone 6 and Clone 15 contained *PTEN* cDNA only one of them (Clone 15) expressed PTEN protein on western blot assay (Figure 5.21). In agreement with these western blot data, the P-Akt assay also demonstrated that Akt phosphorylation was suppressed only in Clone 15 but not in Clone 6 (Figure 5.28).

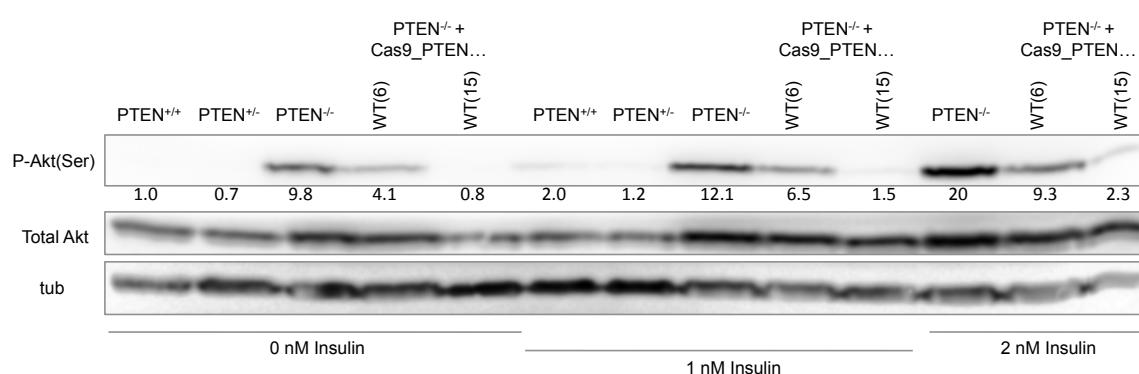


Figure 5.28. PTEN activity in knock-in Clone 6 and Clone 15 based on HCT116 PTEN^{-/-} cells. Quiescent cells were activated with 0-2 nM insulin and P-Akt levels were compared to P-Akt levels in PTEN^{-/-}, PTEN^{+/-} and PTEN^{+/+} cells. Total Akt and tubulin were used as loading controls. The numbers under the bands represent the ratio of protein expression with PTEN^{+/+} samples arbitrary set as 1.0 for each cell line and determined by ImageStudioLite (LI-COR Biosciences).

5.5.3. Conclusions

The determination of the levels of P-Akt in cells allowed assessment of the lipid phosphatase activity of PTEN in all PTEN knock-in clones *in vivo* as summarised in Table 5.8.

Table 5.8. PTEN activity in knock-in cells based on P-Akt assay. A- active, NA- Non-Active.

		PTEN activity			
		HCT116 cells		MRC-5 cells	
		Ser473	Thr308	Ser473	Thr308
PTEN ^{+/+}		A	A	A	A
PTEN ^{+/-}		A	A	A	A
PTEN ^{-/-}		NA	NA	NA	NA
PTEN ^{-/-} +	WT	A	A	A	A
	G129E	NA	NA	NA	NA
	C124S	NA	NA	NA	NA
	H93A	NA	A	NA	NA
	V403A	A	A	A	A
	K13E&K289E	NA	NA	A	A

6. Results and discussion: PICS in PTEN^{+/-} cells

6.1. Introduction

It was previously demonstrated that PTEN associated cellular senescence (PICS) can be observed in PTEN^{+/-} primary mouse embryonic fibroblasts (MEFs) upon either VO-OHpic treatment (500 nM) or knock-down of PTEN with RNAi (Alimonti et al. 2010). In their paper, Alimonti *et al.* assessed senescence by measuring senescence-associated β -galactosidase (SA- β -Gal) activity.

There are a number of parameters besides SA- β -Gal activity that can be assessed in senescent cells, for instance permanent withdrawal from the cell cycle (compared to reversibly withdrawal found in quiescent cells), morphological senescence footprints (increase in size, flattened or thin cytoplasmic appearance), up-regulation of some anti-proliferative proteins (such as p16, p27, p53), senescence-associated heterochromatic foci (SAHF) and elevated levels of DNA damage response proteins (such as ATM, CHK2 and γ -H2AX) (reviewed in (Muller 2009)). SA- β -Gal is one of the most common and convenient intracellular markers associated with senescence and can be relatively easy detected via staining with one of the β -Gal substrates: (i) chromogenic substrate 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal (Dimri et al. 1995, Kurz et al. 2000, Alimonti et al. 2010)); fluorescent substrates (ii) fluorescein- β -D-galactopyranoside (FDG (Nolan et al. 1988, Yang, Hu 2004)), (iii) 5-dodecanoylamino fluorescein di- β -D-galactopyranoside (FDG-C12 (Kurz et al. 2000, Debacq-Chainiaux et al. 2009, Noppe et al. 2009)), (iv) 4-methylumbelliferyl- β -D-galactopyranoside (MUG (Gary, Kindell 2005)); and (v) chemiluminescent substrate 3-(4-methoxyspiro(1,2-dioxetane-3,2'-(5'-chloro)-tricyclo-[3.3.1.1^{3,7}]decan)-4-yl)phenyl- β -D-galactopyranoside (Galacton (Bassaneze, Miyakawa & Krieger 2008)). Originally SA- β -Gal activity should be detected at pH 6.0 in order to distinct it from the lysosomal β -Gal that is present in most mammalian cells with maximal activity at pH 3.0-5.0 (Dimri et al. 1995, Kurz et al. 2000) and from the *LacZ*-encoded β -Gal that might be expressed as a reporter gene from the plasmids used for cell transfection at pH 7.3 (Nolan et al. 1988, Fiering et al. 1991).

In this study, the VO-OHpic-mediated PICS phenotype was assessed in PTEN^{+/-} cell lines derived from immortalised non-tumourigenic MRC-5 and PNT1A cells and colon cancer HCT116 cells. Finding PICS in cancer cells would be an advance and therapeutically relevant.

6.2. SA- β -Gal activity detection: assay optimisation

Initially, in this project the SA- β -Gal activity was measured via staining with X-Gal as the most commonly reported in the senescence field (Dimri et al. 1995, Kurz et al. 2000, Alimonti et al. 2010). The method is based on formation of the insoluble blue compound as a product of X-Gal cleavage by β -Gal yielding blue coloration for SA- β -Gal-positive cells. The cells with various PTEN genotypes were treated with or without VO-OHpic and subsequently SA- β -Gal activity was assessed by X-Gal staining. Unfortunately similar numbers of blue cells were observed in control (DMSO) or VO-OHpic treated cells regardless of the PTEN genotype. Changing ratio and components of staining solution did not alter the results (data not shown)^{§§§§}. One of the key drawbacks of the X-Gal-based assay is its potential subjectivity and the fact that it is only semiquantitative (Yang, Hu 2004, Gary, Kindell 2005). The intensity of staining can vary significantly even in homogenous population of cells (Gary, Kindell 2005). Sometimes even quiescent cells can reveal SA- β -Gal if cells become densely confluent (Bassaneze, Miyakawa & Krieger 2008). Thus, locally confluent regions of the dish may stain brighter than other areas, making the selection of field used for counting an important factor affecting the percentage of positively-stained cells. Furthermore, cells that appear as negative in the beginning may give rise to visible staining upon longer incubation. These difficulties are surmountable when there is a dramatic difference in signal between positive and negative cell populations. Unfortunately, this was not the case with the cells studied within this project and large variations within the same cell population were observed for both DMSO and VO-OHpic treated cells making it difficult to quantify SA- β -Gal activity objectively.

An improvement of the X-Gal method for determining SA- β -Gal activity in cells is to use a fluorescent β -Gal substrate and determine the fluorescence in the entire experimental population of cells (Noppe et al. 2009, Kikuchi et al. 2010, Nolan et al. 1988, Kurz et al. 2000, Debacq-Chainiaux et al. 2009, Gary, Kindell 2005, Bassaneze, Miyakawa & Krieger 2008). Therefore, such approaches were explored here.

6.2.1. Measuring SA- β -Gal using FDG substrate

FDG is a non-fluorescent β -Gal substrate (Nolan et al. 1988, Yang, Hu 2004) that upon cleavage by β -Gal yields a fluorescein molecule (Figure 6.1(A)) that can be detected by flow cytometry (Nolan et al. 1988). FDG is unable to enter cells therefore a fixation and permeabilisation step is required prior staining. In order to extend the PICS findings of Alimonti *et al.* to other human cancer cells, HCT116 PTEN^{+/-} cells were incubated with or without VO-OHpic (500 nM) for 3 days, then fixed and stained with FDG according to the protocol in

^{§§§§} Results are not shown as no images were taken from the microscope due to lack of the camera at the stage of the experiment was carried out.

(Kikuchi et al. 2010). Unfortunately, both vehicle and VO-OHpic treatment resulted in equally high fluorescence intensity (Figure 6.1(B)).

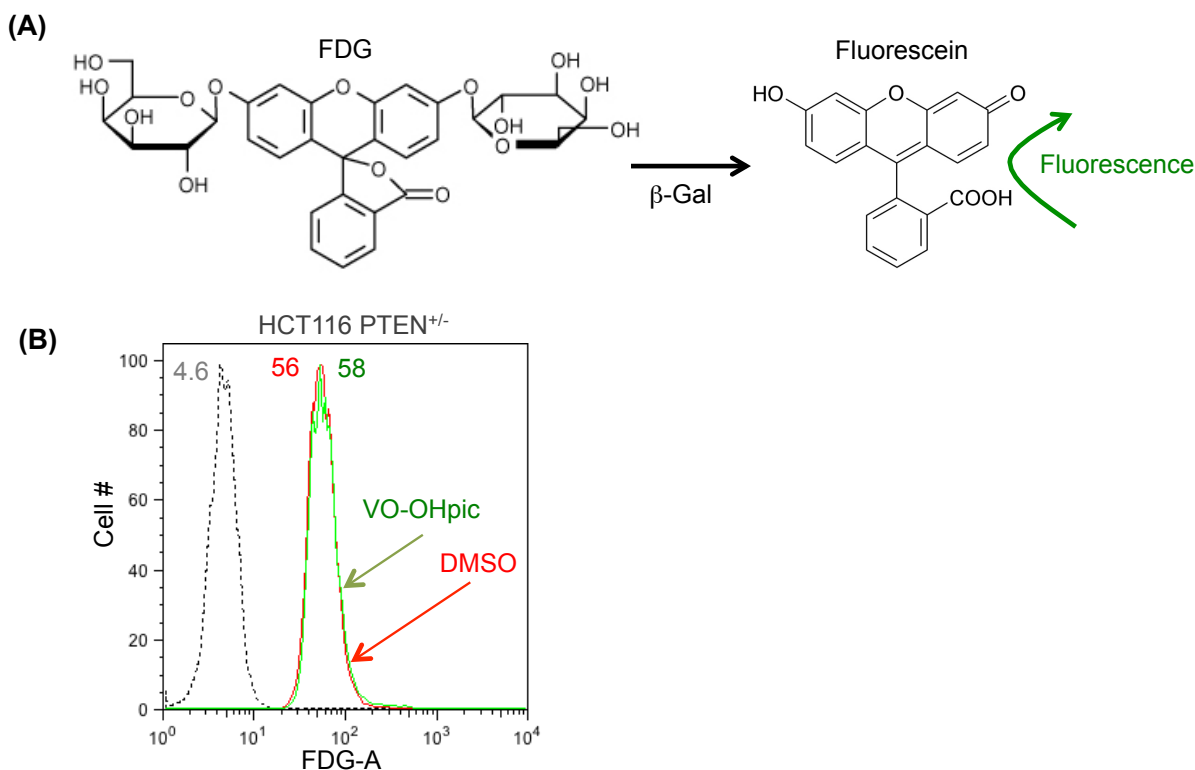


Figure 6.1. **(A)** FDG structure and its cleavage by $\beta\text{-Gal}$. **(B)** SA- $\beta\text{-Gal}$ activity assessment in HCT116 PTEN^{+/-} cells upon VO-OHpic treatment (500 nM) using FDG substrate via flow cytometry. Cells were incubated with VO-OHpic for 3 days following by 15 min fixation with 4% formaldehyde and 2 hours incubation with 20 μM of FDG. Fluorescence intensity was plotted on a histogram after excluding all aggregates and non-single cells. Cells without substrate were used as a negative control for measuring autofluorescence (grey dotted peak). The values near the peaks represent the median fluorescence intensity (MFI) of the representative population (DMSO – red, VO-OHpic – green). The figure contains a representative hystogram from three independent experiments.

No improvement was achieved upon increasing incubation time (up to 12 days), pH changing (from pH 7.0 that recommended in the protocol by Kikuchi *et al.* (Kikuchi et al. 2010) to pH 6.0 that is reported to be optimal pH for SA- $\beta\text{-Gal}$ detection (Dimri et al. 1995, Kurz et al. 2000)) or using alternate batches of VO-OHpic (data not shown).

In order to understand why there was no difference between treated and non-treated cells, it was decided to carry out the control senescence experiment with conditions that are known to induce senescence. This would enable determination of whether the problem was in the FDG assay or in the induction of senescence itself. For this, the FDG assay was applied to cells treated with the small molecule pyridostatin (Figure 6.2(A)) that is known to induce dysfunctional telomeres and a replication-dependent DNA damage response, resulting in cell cycle arrest and appearance of senescent cells (Rodriguez et al. 2012, Müller et al. 2012).

Rodriguez *et al.* demonstrated that U251 and A549 cells were the most sensitive to treatment with pyridostatin in a panel of 60 different cancer cell lines. Therefore, the human lung carcinoma epithelial cells A549 was chosen for this control FDG senescence experiment.

A549 cells (30×10^4 cells per 10 cm dish) were treated with 500 nM of pyridostatin. Every 3 days cells were diluted to the original seeding density in a fresh media supplemented with the compound. The FDG assay was carried out on 6, 9, 12 and 15 days after treatment. Even though pyridostatin-treated cells demonstrated fewer division (there were considerably less cell number compared to mock-treated cells) and underwent dramatic changes in morphology (many cells were notably bigger in size and looked more flat) visible after 6 days of treatment (data not shown), the FDG assay still showed no substantial difference in fluorescence intensity between pyridostatin-treated cells and control cells at all time points. The representative histogram for 12 days-treated cells is shown in Figure 6.2(B).

Further, it was decided to test an additional of washing step after the substrate incubation in order to check if such a high fluorescence signal in cells including the DMSO-treated cells could be due to the non-specific substrate binding. Unfortunately this step did not improve the difference in fluorescence signal, but instead the whole signal was washed out down to the autofluorescence level (Figure 6.2 (C)).

These results indicated that despite the senescent appearance of the A549 cells after pyridostatin treatment the FDG assay did not indicate senescence, with high fluorescence levels in control and experimental cell populations. This may have been due to some proportion of the substrate being already cleaved to release fluorescein and yielding high background levels. This hypothesis was assessed by measuring the fluorescence of the intact FDG substrate (20 μ M in PBS) using a fluorimeter (Figure 6.2(D)). The substrate alone emitted very high fluorescein fluorescence without exposure to β -Gal. A fresh batch of FDG was purchased but it also demonstrated the same results (data not shown), perhaps indicating instability in the compound upon storage or during use.

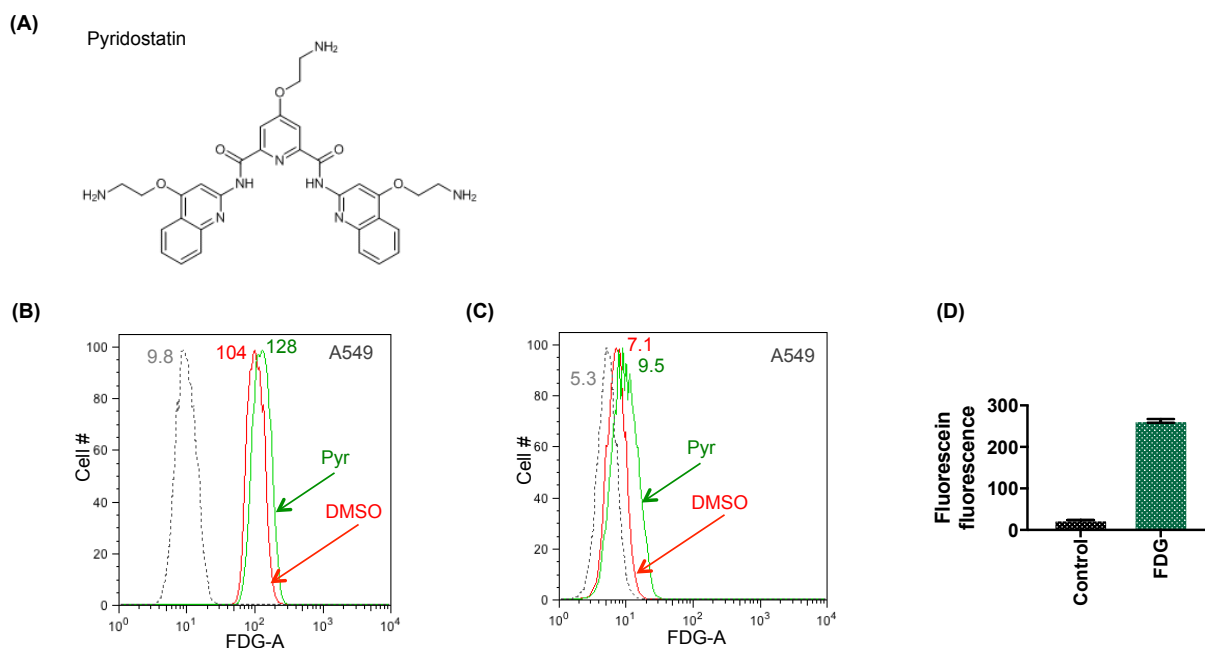


Figure 6.2. **(A)** Pyridostatin structure. **(B), (C)** SA- β -Gal activity detection in A549 cells upon pyridostatin treatment (500 nM) for 12 days **(B)** and 15 days **(C)**. Treated cells were fixed by 4% formaldehyde, incubated with FDG for 2 hours and directly analysed via flow cytometry **(B)**. A washing step after substrate incubation and prior flow cytometry detection was tested on cells pre-treated with pyridostatin for 15 days **(C)**. Fluorescence intensity from FDG substrate was plotted on a histogram after excluding all aggregates and non-single cells. The numbers near the peaks represent the MFI values of populations treated with DMSO (red font) and pyridostatin (green font). Cells without substrate were used as a negative control for measuring autofluorescence (grey dotted peak). Pyr. – pyridostatin. **(D)** Fluorescence measurement of the FDG working solution dissolved in PBS. The release of fluorescein signal was assessed upon laser excitation at 488 nm. Control – PBS. **(B)-(D)** The figures contain representative data from three independent experiments.

6.2.2. Measuring SA- β -Gal using FDG-C12 substrate

An alternative to the FDG substrate for measuring SA- β -Gal activity was to use the related FDG-C12 substrate that can be used in live cells (Figure 6.3(A)). Unlike FDG, this compound is membrane-permeable and is non-fluorescent before hydrolysis via β -Gal action. When cleaved by β -Gal, the compound emits green fluorescence and remains confined within cells. This substrate and flow cytometry analysis of intact cells should also improve the assay sensitivity (Kurz et al. 2000). To decrease the interference of the lysosomal β -Gal on SA- β -Gal activity, some studies modulate intracellular pH (lysosomal alkalinisation) using chloroquine or bafilomycin A1. These compounds increase lysosomal pH from 4.0 (optimal for lysosomal β -Gal) to pH 6.0 (optimal for SA- β -Gal) (Kurz et al. 2000, Noppe et al. 2009, Debacq-Chainiaux et al. 2009). Chloroquine is a weak base that after concentrating in lysosomes, raises their pH

(Poole, Ohkuma 1981, Zhu et al. 2012). Bafilomycin A1 specifically inhibits vacuolar-type H⁺-ATPase and thus inhibits lysosomal acidification (Yoshimori et al. 1991). However, the effect of lysosomal inhibitors was shown to be cell line dependent. Thus, Noppe *et al.* (Noppe et al. 2009) showed that Bafilomycin A1 provides with a significant fluorescence shift in fibroblasts after induction of stress-induced premature senescence compared with no shift at all when treated with chloroquine. In contrast, Kurz *et al.* (Kurz et al. 2000) demonstrated that these both inhibitors could be used to detect SA-β-Gal activity of human umbilical vein endothelial cells (HUVEC) at different replicative ages. Interestingly, these two studies also show that pH modulators appeared not to be essential for SA-β-Gal activity quantification via flow cytometry: actually, the increase in fluorescence intensity from the SA-β-Gal cells was equally well detected without using the inhibitors of lysosomal β-Gal. This was explained by the fact that SA-β-Gal activity was caused by lysosomal β-Gal and that the characterised specificity of SA-β-Gal for senescence arises from the residual activity of lysosomal β-Gal when detected at a sub-optimal pH in senescent cells that have accumulated more β-Gal as a consequence of increase in lysosomal mass. It was also shown that lysosomal accumulation was characteristic for senescent cells (Kurz et al. 2000, Lee et al. 2006). Thus, there are no requirements to use pH modulators at all but in the presence of lysosomal inhibitors the absolute level of β-Gal activity was shown to be reduced by more than 50-fold, which could be important for SA-β-Gal detection sensitivity.

Since Bafilomycin A1 was shown to be non-toxic for fibroblasts whereas chloroquine was (Noppe et al. 2009), Bafilomycin A1 was first tested as a lysosomal pH modulator.

A549 cells pre-treated with pyridostatin for 6-12 days (as for the FDG assay) were sequentially incubated with Bafilomycin A1 (100 nM) and FDG-C12 (33 μM) and subject to flow cytometry. The flow cytometry data with this substrate were markedly improved when compared to the data obtained with the FDG substrate, with 6 and 9 days incubation time demonstrating higher fluorescence levels for pyridostatin-treated cells indicating the onset of senescence (Figure 6.3(B)). However, 12 days incubation was not consistent with shorter periods, suggesting that this method could potentially detect SA-β-Gal inconsistently. Thus, more experiments and optimisation steps were performed to evaluate this detection method.

Initially, the effects of the different lysosomal inhibitor, chloroquine, or no inhibitor at all, were assessed. For this, A549 cells were pre-treated with pyridostatin for 9 days and then incubated with Bafilomycin A1, Chloroquine or no pH modulator (Figure 6.3(C)). Unfortunately, the results were not consistent with the previous experiment. Pyridostatin-treated cells incubated with Bafilomycin A1 demonstrated no difference in fluorescence in comparison with the control cells. The same result was for cells treated with no lysosomal inhibitors. Chloroquine treatment

allowed detecting SA- β -Gal positive cells but it also caused an increase of propidium iodide (PI) positive cells (data not shown) indicating its cytotoxicity.

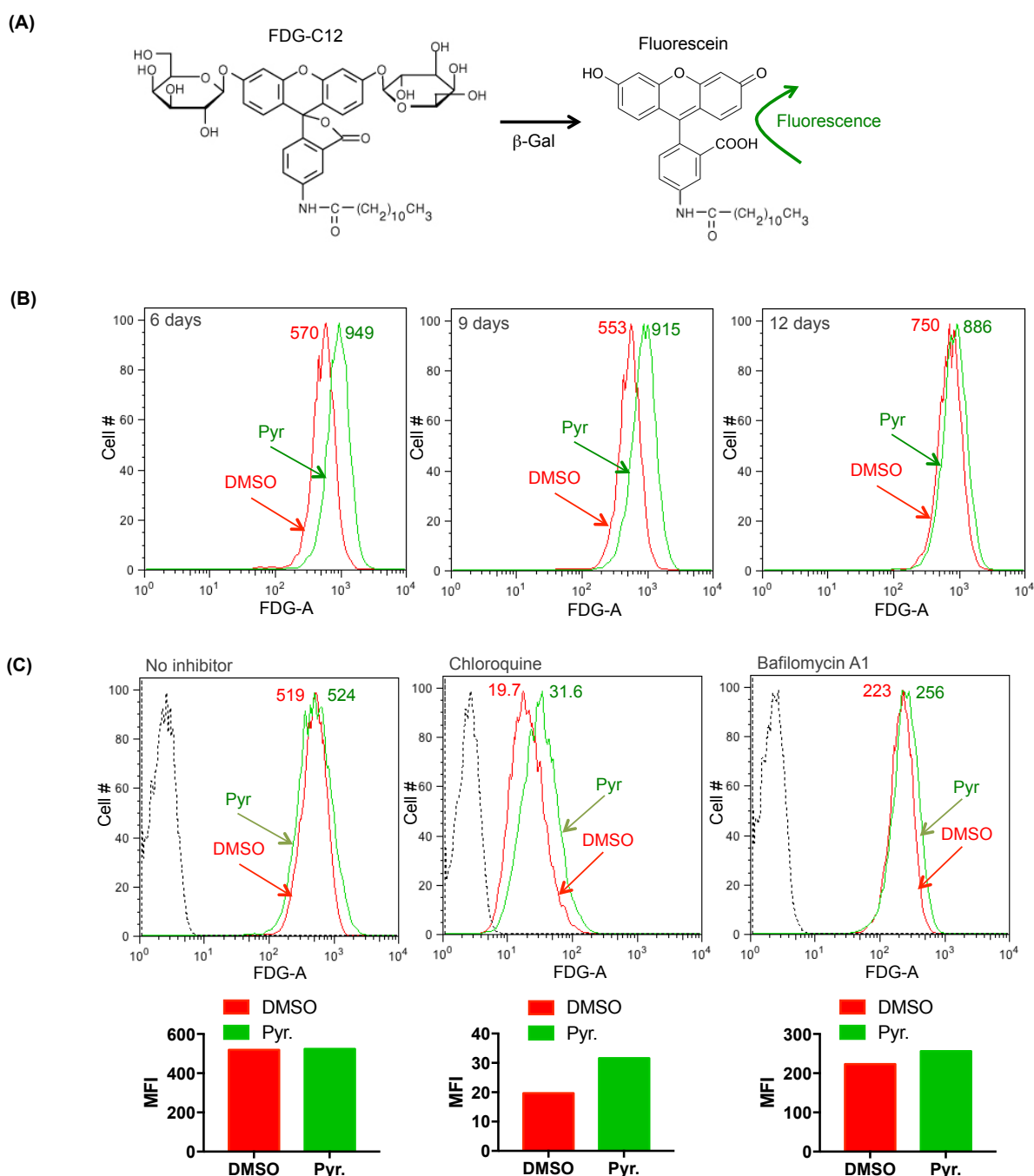


Figure 6.3. **(A)** FDG-C12 structure and its cleavage by β -Gal. **(B)** SA- β -Gal activity assessment in A549 cells upon pyridostatin treatment (500 nM) for 6, 9 and 12 days, following by sequential incubation with 100 nM of bafilomycin A1 and 33 μ M of FDG-C12 substrate for 1 hour each. Fluorescence intensity was measured via flow cytometry and plotted on a histogram after excluding dead cells (PI-positive), subcellular debris and aggregates. The values near the peaks represent the median fluorescence intensity (MFI) of the representative population (DMSO – red, pyridostatin – green). **(C)** Comparison of pH modulators influence on SA- β -Gal detection. A549 cells were pre-treated with pyridostatin (500 nM) for 9 days and then incubated with (i) no lysosomal inhibitors (left); (ii) 300 μ M chloroquine for 2 hours (middle); or (iii) 100 nM

of bafilomycin A1 for 1 hour, following by staining with FDG-C12 (33 μ M) for 1 hour. Histograms (top row) and MFI values plotted on bar graphs (bottom row) demonstrated increase in fluorescence upon pyridostatin treatment only in chloroquine-incubated cells. Cells without substrate were used as a negative control for measuring cells autofluorescence (grey dotted peak). Pyr. – pyridostatin. **(B)-(C)** The figures contain representative data from three independent experiments.

Having such inconsistent results, it was necessary to check reproducibility of this method for cells treated with pyridostatin for different time. Several independent experiments were carried out in A549 cells as well as in MRC-5, PNT1A and HCT116 cells. Bafilomycin A1 was used as a pH modulator. MRC-5, PNT1A and HCT116 cells did not demonstrate any increase in SA- β -Gal activity. Whereas, A549 cells exhibited an increase in medium fluorescence intensity (MFI) levels in pyridostatin-treated cells compared to mock-treated cells for all incubation time points (Figure 6.4).

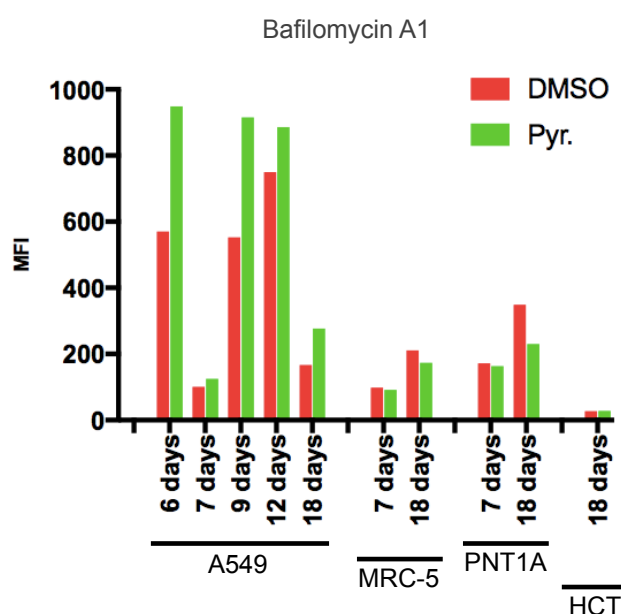


Figure 6.4. SA- β -Gal activity in A549, MRC-5, PNT1A and HCT116 cells upon pyridostatin treatment (500 nM) for 6-18 days, followed by sequential incubation with bafilomycin A1 (100 nM) and FDG-C12 (33 μ M) for 1 hour each. Fluorescence intensity was measured via flow cytometry and MFI values were plotted on a bar graph after excluding dead cells, subcellular debris and aggregates. Pyr. – pyridostatin.

However, this increase was not steady. Moreover, the basic levels of SA- β -Gal activity in these cells varied a lot suggesting that this FDG-C12 assay and flow cytometry detection was not very reliable for SA- β -Gal detection in experiments tested. Using higher Bafilomycin A1 concentration did not improve the assay as well, rather caused more cellular death. Considering that pyridostatin-mediated senescence was a positive control experiment these results meant that the FDG-C12 assay was not working consistently rather than the senescence induction.

6.2.3. Measuring SA- β -Gal using MUG substrate

Given the inconsistency of the fluorescein-based assays, another fluorescent β -Gal substrate in an alternative detection method was tested in the control senescence experiment with pyridostatin. This time an average SA- β -Gal activity was determined in cell extracts by the conversion of 4-methylumbelliferyl- β -D-galactopyranoside (MUG) to the fluorescent hydrolysis product 4-methylumbelliferone (4-MU) (Figure 6.5(A)) at pH 6.0 according to the protocol in (Gary, Kindell 2005). Cellular lysates from control and pyridostatin-treated A549 cells were incubated with MUG and the fluorescence in supernatants was measured via a fluorometer. This method also allowed comparison of normalised SA- β -Gal activity in cellular samples incubated with pyridostatin for different time points via dividing observed fluorescence values by the total amount of protein in each sample.

The results demonstrated consistent and a statistically significant increase in cellular SA- β -Gal fluorescence after incubation with pyridostatin for different time points (Figure 6.5(B)).

In order to confirm that this assay would work with different cell lines and different senescent inducers, one more control experiment was performed and analysed using this MUG-based assay. To this end, human primary IMR90-ER:Ras cells were used as an inducible system of oncogene-induced senescence. This system is based on IMR90 human diploid fibroblasts stably expressing 4-hydroxytamoxifen (4-OHT)-inducible H-RasV12 (ER:Ras) (Barradas et al. 2009, Young et al. 2009, Narita et al. 2011, Takahashi et al. 2012, Acosta et al. 2013). The original study demonstrated that oncogene-induced senescence and growth arrest can be enforced in IMR90 cells by retroviral-mediated expression of oncogenic *Ras* (H-RasV12) (Serrano et al. 1997, Ferbeyre et al. 2000, Narita et al. 2006). Later Barradas *et al.* (Barradas et al. 2009) simplified this system by stably expressing a 4-OHT-inducible ER:Ras fusion protein in IMR90 cells. This protein contains a mutant form of the estrogen receptor (ER) ligand-binding domain and H-RasV12. It was shown that senescence onset in this system occurs after around 5-6 days after 4-OHT induction (Barradas et al. 2009, Young et al. 2009). Therefore, the MUG assay was tested on IMR90-ER:Ras cells (kind gift from Dr Gordon Peters's laboratory, Cancer Research UK London Research Institute, London, UK) pre-treated with 4-OHT (VWR, UK) for 5-6.5 days and a statistically valid increase in fluorescence was detected in senescence-induced cells compared to control cells (Figure 6.5(C)).

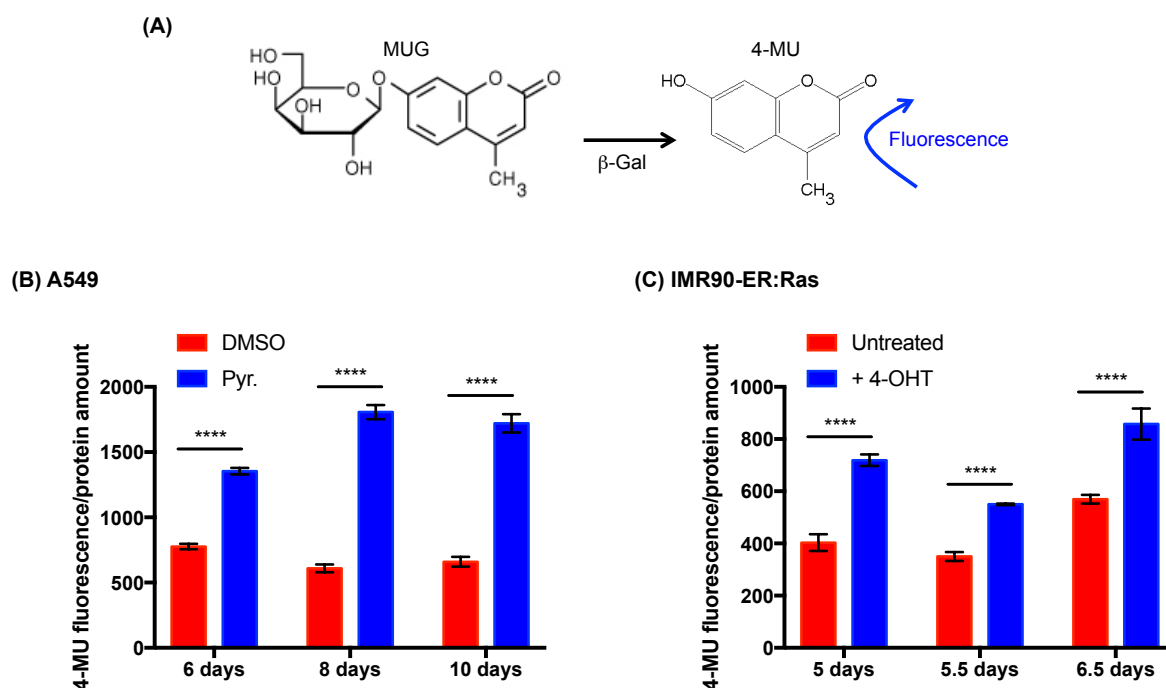


Figure 6.5. (A) MUG structure and its cleavage by β -Gal. (B), (C) SA- β -Gal activity assessment in cells upon senescence induction using MUG substrate (0.85 μ M): (B) A549 cells treated with 500 nM pyridostatin (Pyr.); and (C) IMR90-ER:Ras cells treated with 100 nM 4-hydroxytamoxifen (4-OHT). Control or induced cells were lysed and stained with MUG. Fluorescence intensity was measured in a 96-well plate and normalised by the total protein levels. MUG solution without cells was used as a background control. Data are reported as mean $\pm$ standard deviation (SD) of three independent experiments. Significant differences (5% cut-off for significance performed by two-way ANOVA test using GraphPad Prism 7) to the control samples are indicated by stars (**** $p < 0.0001$).

In conclusion, the MUG-based SA- β -Gal detection method proved being simple and reliable for senescence detection in two independent cell systems. This method was then applied to estimate PICS via SA- β -Gal activity in PTEN^{+/-} cells pre-treated with VO-OHpic.

6.3. PICS assessment in PTEN^{+/-} cells

The MUG assay was utilised to measure SA- β -Gal in PTEN^{+/-} cells compared to PTEN^{+/+} and PTEN^{-/-} cells after VO-OHpic treatment. For this, HCT116 and MRC-5 cells were incubated with this compound (1 μ M, based on VO-OHpic concentration used in PICS experiments on MEFs (Alimonti et al. 2010) and in other *in vivo* experiments (Silva et al. 2011, Tzenaki et al. 2012, Tzenaki, Aivaliotis & Papakonstanti 2015)) for 3-12 days (cells were split and replated every 3 days with the fresh media containing VO-OHpic), then lysed and treated with MUG substrate similar to the control experiments. No significant increase in fluorescence enhancement was detected in cells treated with VO-OHpic. Similar SA- β -Gal levels were detected in both HCT116 and MRC-5 cells regardless their PTEN status or time of incubation with VO-OHpic. A representative 12 days experiment is shown on Figure 6.6.

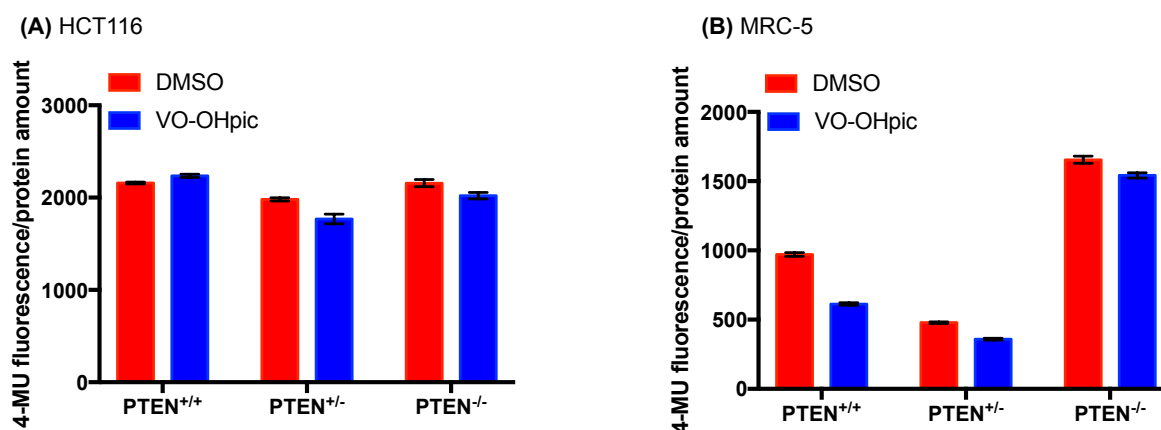


Figure 6.6. SA-β-Gal activity in cells upon VO-OHPic treatment using the MUG substrate: **(A)** HCT116 cells; **(B)** MRC-5 cells. Cells were treated with DMSO (0.5%) or VO-OHPic (1 μM) for 12 days, then lysed and incubated with MUG substrate (0.85 μM). Histograms show means and error bars show SD from three independent experiments.

The lack of SA-β-Gal activity was consistent with other observations of the cells treated with VO-OHPic such as lack of growth arrest and change in morphology in treated cells (data not shown). In order to further validate these results, VO-OHPic activity and stability was verified *in vitro*. Firstly, the full chemical characterisation (ESI⁺-MS, elemental analysis, IR analysis and UV-VIS analysis – see Chapter 2.2.2. Materials and Methods) was performed with the complex. The analysis results were consistent with the literature (Yano et al. 2002, Nakai et al. 2004).

Further, VO-OHPic inhibitory potency was tested by phosphatase assay. For this, VO-OHPic-mediated inhibition of PTPMT1 phosphatase activity was monitored by measuring hydrolysis of an artificial phosphatase substrate 3-O-methylfluorescein phosphate (OMFP)^{*****} (Georgiades et al. 2010, Mak, Vilar & Woscholski 2010). PTPMT1 phosphatase was kindly provided by Dr Rudiger Woscholski's laboratory and chosen for this experiment due to its PTEN-like active site (Pagliarini, Worby & Dixon 2004, Xiao et al. 2011) and expression efficiency. It was also previously demonstrated that this PTP strongly binds VO-OHPic (Woscholski *et al.*, unpublished) and therefore can be used as a positive control in VO-OHPic inhibition experiment. The *in vitro* phosphatase assay was performed with PTPMT1 incubated with different concentrations of VO-OHPic for 15 min followed by addition of OMFP. VO-OHPic inhibition was determined based on its inhibition activity towards OMFP hydrolysis into OMF catalysed by PTPMT1. Increase in fluorescence signal was measured and plotted as slope from kinetic curves against VO-OHPic concentration. The results demonstrated that VO-OHPic

^{*****} The work on VO-OHPic characterisation published by Mak *et al.* (Mak, Vilar & Woscholski 2010) demonstrated a possibility of using OMFP as an artificial substrate in the phosphatase inhibition assay instead of PIP3. OMFP was used as a common substrate for a range of phosphatases as well as PTEN (Gottlin et al. 1996, Li et al. 1997, Wang et al. 2004, Sun et al. 2005) and Mak *et al.* showed its suitability for testing VO-OHPic as a PTEN inhibitor. In their report, the VO-OHPic inhibition activity on PTEN in OMFP assay (IC₅₀, 46 nM ± 10 nM) was found to be similar to the PIP3 assay (IC₅₀, 35 nM ± 2 nM).

still was a potent phosphatase inhibitor ($IC_{50} = 24.3 \text{ nM} \pm 9.8$) (Figure 6.7(A)), which was consistent with VO-OHpic IC_{50} inhibiting in a PTEN/OMFP assay (IC_{50} , $46 \text{ nM} \pm 10 \text{ nM}$).

Next, the compound stability under the tissue culture conditions was assessed by measuring the UV-VIS spectra of cellular supernatants containing different concentrations of VO-OHpic before and after incubation in the tissue culture incubator (at 37°C in the atmosphere of 5% CO_2). The characteristic absorption peak for VO-OHpic at $\lambda_{\text{max}} = 305 \text{ nm}$ (Nakai et al. 2004) was used as a point of comparison for VO-OHpic stability measurements. The height and the shape of this peak did not change after incubation of the compound with cells for 3 days (Figure 6.7(B)) indicating that VO-OHpic does not undergo degradation under tissue culture conditions (at least not detectable by electron absorption spectra).

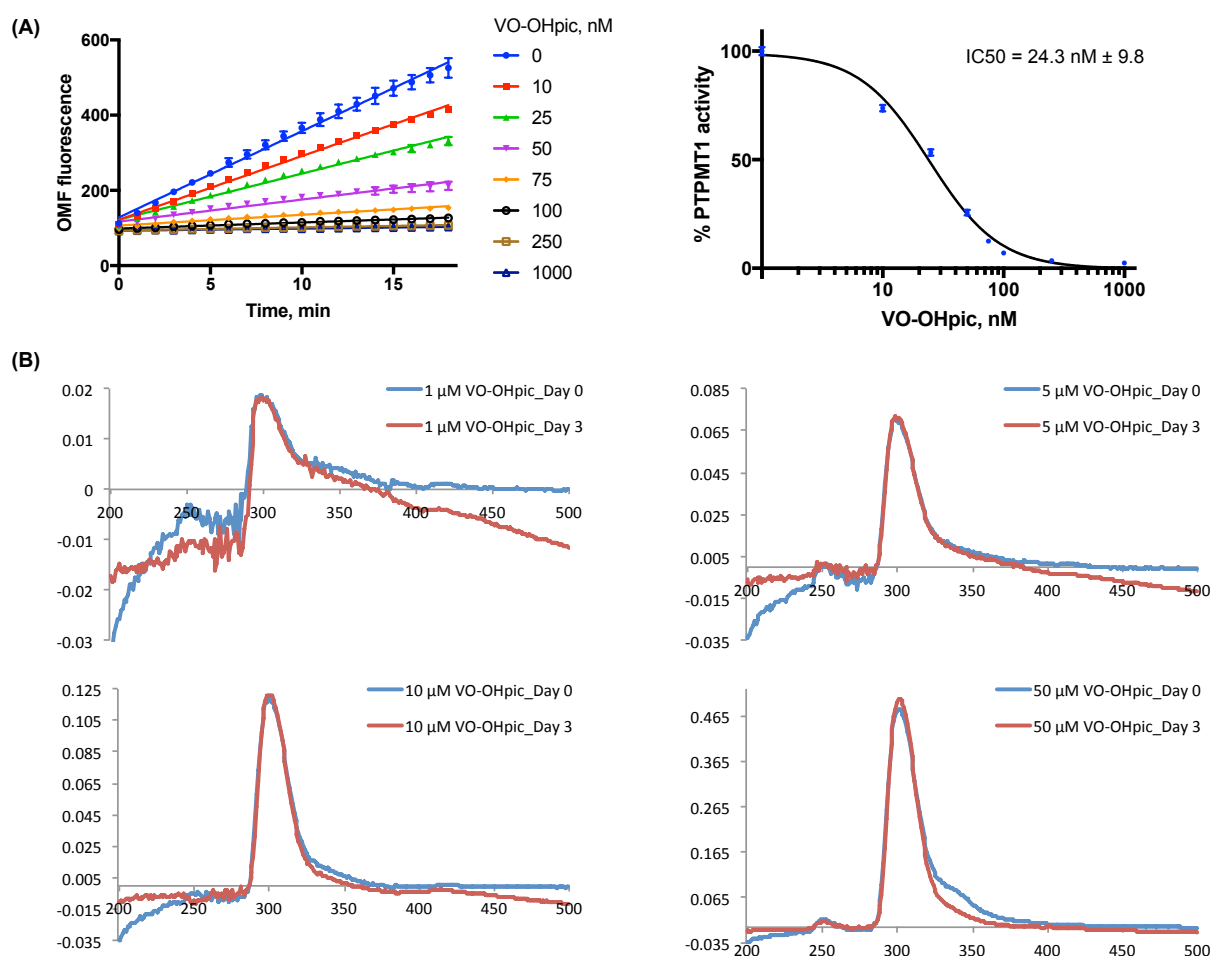


Figure 6.7. **(A)** Inhibition of recombinant PTPMT1 by VO-OHpic *in vitro*. Recombinant phosphatase was pre-incubated with between 10 and 1000 nM VO-OHpic for 15 min prior addition of OMFP substrate (15 μM). PTPMT1 activity was detected by measuring OMFP fluorescence kinetics over time (left graph). Slope values for different VO-OHpic concentrations were plotted to calculate IC_{50} ($24.3 \text{ nM} \pm 9.8$, right graph). The results are presented as the mean $\pm$ SD of three independent experiments. **(B)** UV-VIS spectra of VO-OHpic incubated under tissue culture conditions. HCT116 cells were incubated in growth media supplemented with VO-OHpic in concentration 1 – 50 μM . Absorbance spectra after 3 days incubation with VO-OHpic were compared with day 0. No difference in VO-OHpic absorbance was detected due to incubation conditions.

Further, the cell lines were checked for the presence of mycoplasma contamination, as this could cause some changes in cellular metabolism and proliferation (Drexler, Uphoff 2002) and subsequently interfere with the cellular response to PTEN inhibition and/or senescence. However, all cell lines screened with PCR Mycoplasma kit (ThermoFisher) did not exhibit any signs of mycoplasma (data not shown). These experiments confirmed that VO-OHpic was still intact and active throughout the cellular assays. However, the VO-OHpic still did not induce PICS phenotype.

At the next stage another PTEN inhibitor, bisperoxovanadium-based (BpV(HOpic), Santa Cruz, USA) was tested as an alternative senescence mediator in PTEN^{+/-} cells. BpV(HOpic) (Figure 6.8(A)) is a known potent inhibitor of PTPs that binds to a range of PTPs in micromolar and nanomolar concentrations and inhibits PTEN in a nanomolar concentration (IC₅₀ = 14 nM ± 2.3) (Schmid et al. 2004, Rosivatz et al. 2006). However, up to 12 days incubation with this phosphatase also did not lead to the SA-β-Gal response in PTEN^{+/-} HCT116 and MRC-5 cells (Figure 6.8(B)).

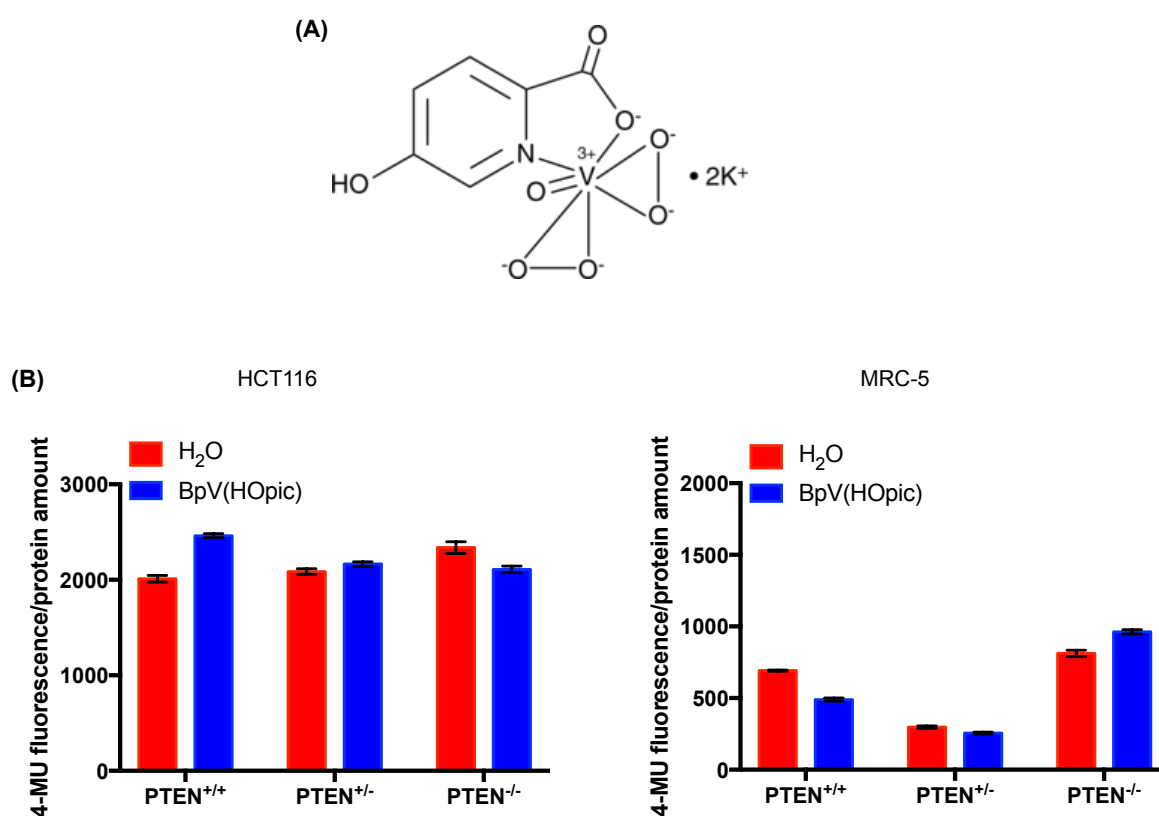


Figure 6.8. **(A)** Structure of BpV(HOpic). **(B)** SA-β-Gal activity in HCT116 (left) and MRC-5 (right) cells of the indicated genotype upon BpV(HOpic) treatment using MUG substrate. Cells were treated with H₂O or VO-OHpic (1 μM) for 12 days, then lysed and incubated with MUG (0.85 μM). Error bars show SD from three independent experiments.

Thus, the SA-β-Gal response in PTEN^{+/-} cells was not detected upon treatment with PTEN inhibitors. However, as discussed previously, SA-β-Gal activity is not the only senescence

marker (Cristofano et al. 1998, Wei, Sedivy 1999, Krishna et al. 1999, Severino et al. 2000, Coates 2002, Going et al. 2002, Lee et al. 2006). SA- β -Gal activity is associated with cellular senescence but is not highly specific: strong SA- β -Gal signal was observed in proliferating cell populations in dysplastic epithelia (Going et al. 2002) ; in human fibroblasts expressing viral oncogene SV40 large T antigen at crisis (mortality stage 2) state (Wei, Sedivy 1999) ; in confluent non-transformed fibroblasts, in immortal fibroblasts at high cell density and in low density normal fibroblasts upon oxidation with H₂O₂ (Severino et al. 2000) ; finally Krishna *et al.* reported SA- β -Gal activity in immortalised A549, promyelocytic leukemia (HL60), hepatoma (HepG2), and colon carcinoma (Caco-TC7) cells. With quantitative HPLC methods, β -Gal was detected at both pH 4.5 and pH 6.0 in all cell lines, irrespective of senescence status (Krishna et al. 1999). In conclusion, SA- β -Gal activity appearance can depend on cell culture conditions, cell type, quiescence, abundance and alternative splicing mechanisms (Cristofano et al. 1998, Going et al. 2002, Severino et al. 2000). However it is still useful to measure and is widely used.

Unfortunately, there are no completely specific senescence markers known yet as they all can be associated or caused by other cellular processes, therefore senescent cells should be identified by more than one method (Muller 2009).

The apparent lack of senescence may be due to lack of VO-OHpic response so we needed to characterise cells treated with it further. We did this by looking at P-Akt levels, cell proliferation and metabolite levels. Herein, three more *in vivo* experiments were carried out with PTEN inhibitors: cellular viability assay, P-Akt assay and metabolomics to assess PTEN inhibition *in vivo*.

6.4. P-Akt assay in cells upon PTEN inhibition

In order to determine if VO-OHpic and BpV(HOpic) inhibited PTEN *in vivo* as previously shown (Schmid et al. 2004, Rosivatz et al. 2006, Alimonti et al. 2010), a P-Akt assay was performed. Alimonti *et al.* confirmed PTEN inhibition by western blot analysis showing an increase in P-Akt(Ser 473) levels in MEFs treated with VO-OHpic (Alimonti et al. 2010). Rosivatz *et al.* and Schmid *et al.* confirmed that Akt was phosphorylated on both the Ser 473 and Thr 308 sites in NIH 3T3 cells (Schmid et al. 2004, Rosivatz et al. 2006) and L1 fibroblasts (Rosivatz et al. 2006) upon treatment with VO-OHpic and BpV(HOpic) respectively. These protocols were applied to the human cells in this project.

Initially the optimal insulin concentration increasing the PIP3 levels slightly above its basal levels was determined. MRC-5 and HCT116 cells were analysed in the Chapter 5.5.4 and 1 nM insulin was chosen as the optimal value for these two cell lines. PNT1A cells were analysed in a similar manner and 10 nM of insulin demonstrated the lowest detectable Akt phosphorylation

levels (Figure 6.9). Surprisingly, not only expression of P-Akt was gradually elevated upon genetic reduction in PTEN levels but also expression of total Akt was altered in the sequence $PTEN^{+/+} < PTEN^{+/-} < PTEN^{-/-}$ irrespective of insulin stimulation. This effect was only observed in PNT1A cells and not in HCT116 or MRC-5 (Figure 6.10), suggesting that Akt transcription, translation, stability and/or degradation could be affected in PNT1A cells upon PTEN KO.

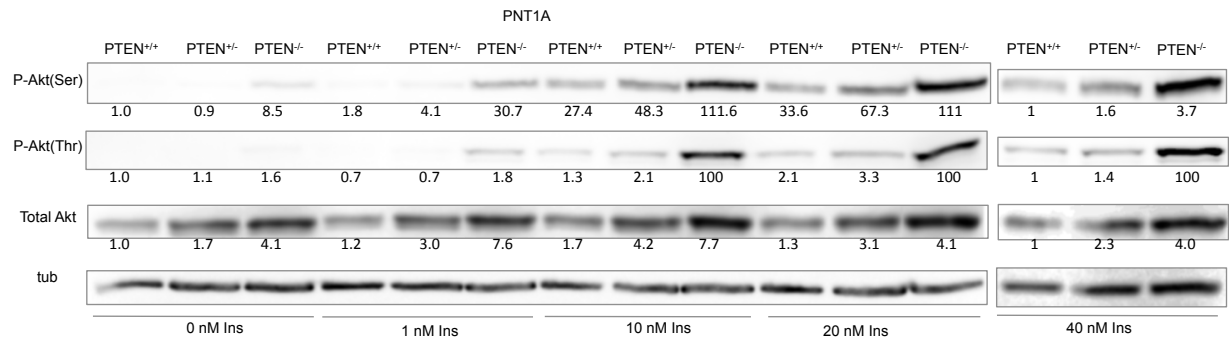


Figure 6.9. P-Akt(Ser473) and P-Akt(Thr308) protein levels in PNT1A $PTEN^{-/-}$, $PTEN^{+/-}$ and $PTEN^{+/+}$ quiescent cells stimulated with various insulin concentrations. Tubulin and total Akt were used as a loading control. The numbers under the bands represent the ratio of protein expression with $PTEN^{+/+}$ samples arbitrary set as 1.0 for each protein and determined by ImageStudioLite (LI-COR Biosciences).

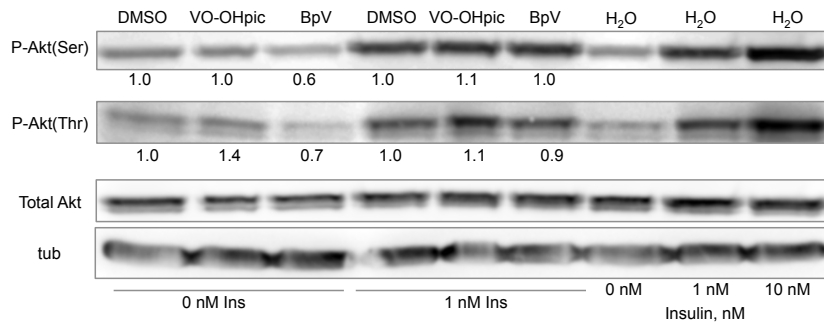
The PTEN inhibitors were then tested on cells treated with the appropriate insulin concentrations in the P-Akt immunoblot assay. Following the protocol from (Rosivatz et al. 2006), quiescent cells (cultured in starvation media for 20 hours) were pre-incubated with 500 nM of VO-OHPic or BpV(HOPic) for 30 min, following by insulin stimulation for 15 min. Primarily, cell lines with WT PTEN levels were analysed. Unfortunately, no effect on Akt phosphorylation was observed in MRC-5 and PNT1A cells (Figure 6.10(A,B)).

However, HCT116 $PTEN^{+/+}$ cells demonstrated an increase of P-Akt levels upon treatment with both VO-OHPic and BpV(HOPic) (Figure 6.10(C)). An elevated phosphorylation of Akt were observed for both Ser 473 and Thr 403 sites in cells treated with a low insulin concentration (0.01 nM), whereas this result was inconsistent upon stimulation with 1 nM insulin. Further, heterozygous $PTEN^{+/-}$ HCT116 cells were analysed in parallel and exactly the same trend was observed for this cell line as for wild type cells.

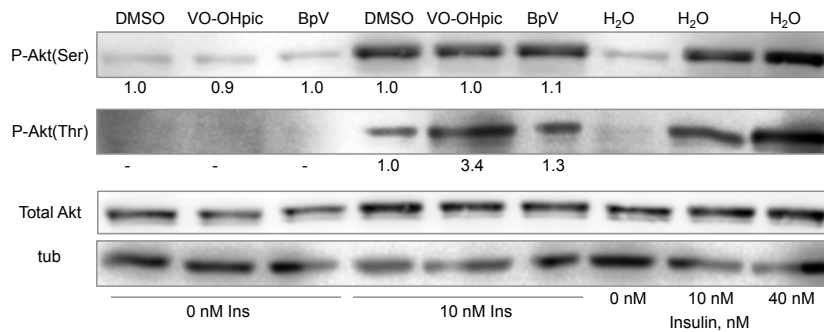
This result is in contrast to other studies that showed that heterozygous *PTEN* MEFs (Alimonti et al. 2010) or cells with medium PTEN levels (Hep3B) (Augello et al. 2016) demonstrated a stronger response and higher P-Akt levels upon PTEN inhibition with VO-OHPic when compared to cells with higher endogenous PTEN levels. Moreover, Augello *et al.* did not detect an increase in P-Akt levels in PLC/PRF/5 cells that express the highest PTEN levels. In our hands, PTEN expression levels did not affect the P-Akt response to VO-OHPic and BpV(HOPic) in HCT116 cells (Figure 6.10(C)).

HCT116 PTEN null cells were analysed as a negative control. *A priori*, one would expect, PTEN^{-/-} cells to give no P-Akt response upon further PTEN inhibition. It was previously confirmed in PTEN negative UM-UC-3 bladder carcinoma cell line (Schmid et al. 2004, Rosivatz et al. 2006) and in hepatocellular carcinoma SNU475 cells (Augello et al. 2016). Unfortunately, HCT116 cells demonstrated a slight P-Akt increase upon further PTEN inhibition (Figure 6.10(D)).

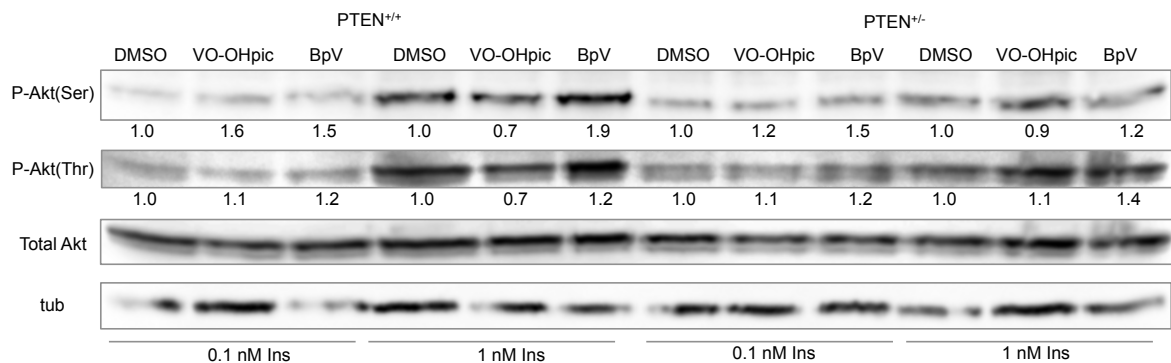
(A) MRC-5 (PTEN^{+/+})



(B) PNT1A (PTEN^{+/+})



(C) HCT116



(D) HCT116 PTEN^{-/-}

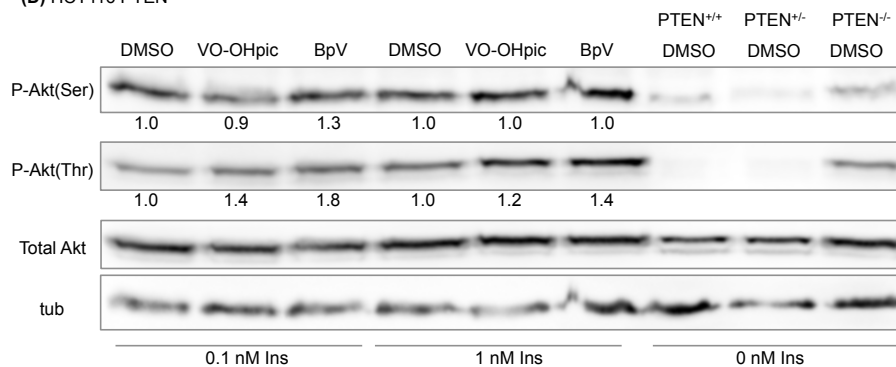


Figure 6.10. Assessment of Akt phosphorylation upon treatment with VO-OHpic or BpV(HOpic). Starved **(A)** MRC-5 PTEN^{+/+}, **(B)** PNT1A PTEN^{+/+}, **(C)** HCT116 PTEN^{+/+} and PTEN^{+/-} and **(D)** HCT116 PTEN^{-/-} cells were incubated with 500 nM of VO-OHpic or BpV(HOpic) for 30 min, followed by insulin stimulation at indicated concentrations for 15 min. P-Akt levels on both Ser and Thr sites were compared to the mock-treated cells. No stimulation and full stimulation samples were used as controls. The numbers represent the ratio of P-Akt

expression, with control (vehicle-treated) samples arbitrary set at 1.0 for each cell line protein and insulin concentration.

Perhaps, both VO-OHPic and BpV(HOPic) targeted other phosphatases that could compensate for the lack of PTEN activity in reducing Akt phosphorylation, suggesting possible off target effects and indicating that VO-OHPic (like BpV(HOPic)) is not entirely specific as a PTEN inhibitor. Although, the majority of papers reported that VO-OHPic is a specific and potent PTEN inhibitor *in vivo* (Alimonti et al. 2010, Augello et al. 2016) and *in vitro* when compared against other CBPs (cysteine-based phosphatases) such as PTP- β , Sac 1 and myotubularin (MTM1) (Rosivatz et al. 2006, Mak, Vilar & Woscholski 2010), Spinelli *et al.* had raised concerns about its specificity (Spinelli, Lindsay & Leslie 2015) and demonstrated that VO-OHPic inhibited tyrosine phosphatase SHP1 (Src homology region 2 domain-containing phosphatase 1) with a similar potency to PTEN *in vitro*. Moreover, at least one more phosphatase, PHLPP1 (pleckstrin-homology domain leucine-rich repeat protein phosphatase 1), is known to directly dephosphorylate Akt *in vitro* and *in vivo* (Gao, Furnari & Newton 2005, Chen et al. 2011, Molina et al. 2012). It was reported that PTEN, PHLPP1 and PTEN co-suppressor and stabiliser NHERF1 (Na⁺/H⁺ exchanger regulatory factor 1) can be connected in an inhibitory network with synergistic effects on the control of the Akt pathway in glioblastoma and prostate cancer cells (Molina et al. 2012). For instance, Molina *et al.* demonstrated that knockdown of PHLPP1 in the glioblastoma LN229 PTEN-depleted cells strongly activated Akt by increasing phosphorylation on both Ser 473 and Thr 308 sites, while single PHLPP1 depletion by itself was not sufficient to elevate P-Akt levels. Similarly, double depletion of PTEN and PHLPP1 or PTEN and NHERF1 in prostate cancer DU145 cells elevated Akt phosphorylation, whereas single depletion of these proteins had no significant effect on Akt phosphorylation. Inhibition of both of these phosphatases by VO-OHPic/BpV(HOPic) could be one of the reasons for the effects seen in HCT116 PTEN^{-/-} cells. And their synergetic effect could be the reason for MRC-5/PNT1A negative data.

Given the weak increase in Akt phosphorylation upon VO-OHPic/BpV(HOPic) treatment of HCT116 cells at 500 nM, a VO-OHPic dose response experiment was performed using HCT116 PTEN^{+/+} and PTEN^{+/-} cells.

Following serum deprivation, cells were treated with VO-OHPic at various concentrations (similar to the protocol in (Rosivatz et al. 2006)) for longer time (1 hour). Following compound treatment, cells were stimulated with 0.1 nM insulin for 15 min. Cells non-treated with VO-OHPic or with insulin were included as negative controls. (Figure 6.11). Repeating this experiment gave highly variable results (data not shown) and attempts were then made to standardise this assay.

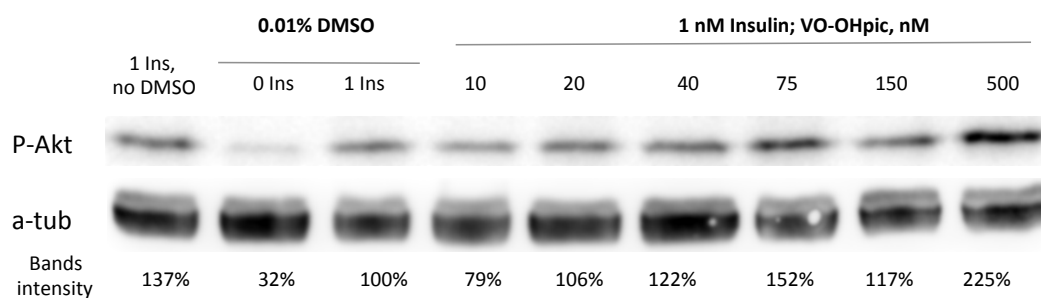


Figure 6.11. Dose response of Akt phosphorylation (Ser473) to VO-OHpic. Akt activation upon VO-OHpic treatment in concentration 10-500 nM for 1 hour. Cells were stimulated with 1 nM Insulin after 19 hours starvation in FBS-free media. Tubulin was loaded to indicate total protein levels. The numbers under the bands represent the percentage of protein expression with control (DMSO-treated, stimulated with 1 nM Insulin) samples arbitrary set as 100% and determined by ImageJ.

Various preparations of VO-OHpic were assessed and compound prepared from the higher concentration stock showed greater levels of P-Akt indicating a higher potency (Figure 6.12).

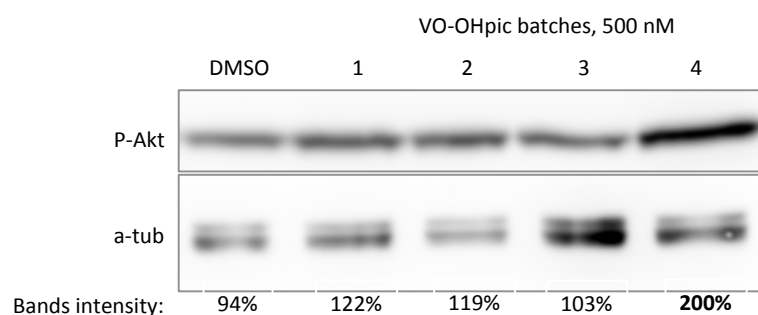


Figure 6.12. Determining batch to batch response variation in preparations of VO-OHpic in HCT116s. Akt activation (Ser473) upon 1 hour treatment with various batches of VO-OHpic compound in concentration 500 nM: 1) from 1mM stock; 2) from 5 mM stock; 3) from 10 mM stock; 4) from 25 mM stock. Cells were stimulated with 15 nM insulin after 19 hours starvation in FBS-free growing media. Lysates were loaded on SDS-PAGE normalised for total protein levels calculated by Bio-Rad DC protein assay, then analysed by Western blotting. Tubulin was blotted as a loading control.

However, performing a second dose response experiment with this higher concentration stock solution of VO-OHpic did not yield expected results (Figure 6.13).

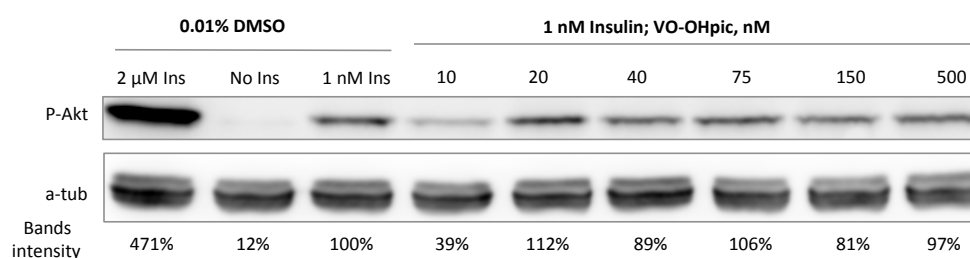


Figure 6.13. Dose response of Akt phosphorylation (Ser473) to VO-OHpic from the 25 mM stock. Akt phosphorylation after 1 hour of treatment with VO-OHpic at concentrations from 10-500 nM. Cells were stimulated with 1 nM insulin after 19 hours starvation in FBS-free growing

media. Lysates were loaded on SDS-PAGE normalised for total protein levels calculated by Bio-Rad DC protein assay, then analysed by Western blotting. Tubulin was blotted as a loading control. The numbers under the bands represent the percentage of protein expression with control (DMSO-treated, stimulated with 1 nM Insulin) samples arbitrary set as 100% and determined by ImageJ.

Given the difference observed between the same stock in two sequential experiments (Figure 6.12 vs Figure 6.13) it appeared that the VO-OHpic had lost potency over time (data in Figure 6.13 were gathered 14 days after that in Fig 6.12). Therefore a fresh batch of VO-OHpic was synthesised according to the procedure in Chapter 2.2 and this was immediately tested in the P-Akt experiment. Cells treated with the VO-OHpic batch prepared from newly-synthesised solid showed a far greater increase in P-Akt signal after 1 hour treatment (Figure 6.14).

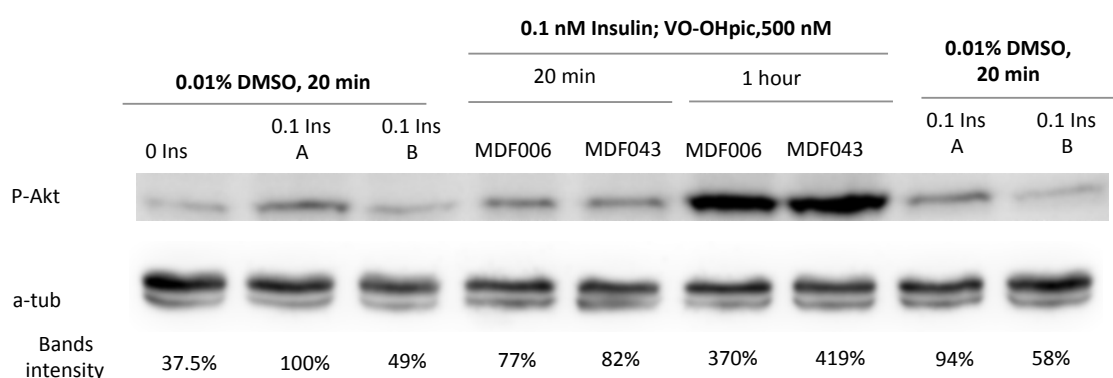


Figure 6.14. Dose response of Akt phosphorylation to freshly prepared VO-OHpic. Old (MDF006) and new (MDF043) preparations of VO-OHpic were used at 500nM for 20 min or 1 hour on HCT116 cells that had been cultured in serum free conditions for 19h. Cells were then stimulated with 0.1 nM Insulin and lysed and analysed by immunoblotting. Tubulin was used as a loading control.

These results demonstrated that the compound was not stable in the DMSO solution (even when kept at -20°C) and should be prepared fresh from solid every time.

To further optimise the assay in HCT116 cells, a longer starvation period was employed (40 hours) after which cells were treated with 0.01-1nM insulin with and without prior treatment for 15 mins with 500 nM VO-OHpic. This regime yielded an increase in the P-Akt signal in the presence of VO-OHpic compared to DMSO (Figure 6.15(A)). Next, Akt phosphorylation upon longer VO-OHpic treatment (up to 22 hours) manner was assessed. Unfortunately, only 500 nM VO-OHpic demonstrated a significant increase in P-Akt levels (1.69 ± 0.09 fold increase compared to DMSO treatment). Lower VO-OHpic concentrations did not demonstrate any rise in P-Akt signal (Figure 6.15(B)). A 1.69 fold increase correlates well with the data in (Alimonti et al. 2010) where MEFs were treated with 500 nM VO-OHpic. However, there are no published data regarding treatment with sub micromolar VO-OHpic concentrations for the human cells. Indeed, only higher concentrations were tested in a recent study on hepatocellular carcinoma cells (Augello et al. 2016). Rosivatz *et al.* published data with

submicromolar VO-OHpic concentrations for murine L1 and NIH 3T3 cells. Testing NIH 3T3 cells with the reagents in use here demonstrated that these mouse derived cells were about 10 fold more sensitive to insulin stimulation than HCT116 cells (Figure 6.15(C)). A 1.52 fold growth was observed in P-Akt levels upon 500 nM VO-OHpic treatment and 0.01 nM insulin stimulation of NIH 3T3 cells. This result was in line with the data reported by Rosivatz *et al.* (Rosivatz *et al.* 2006).

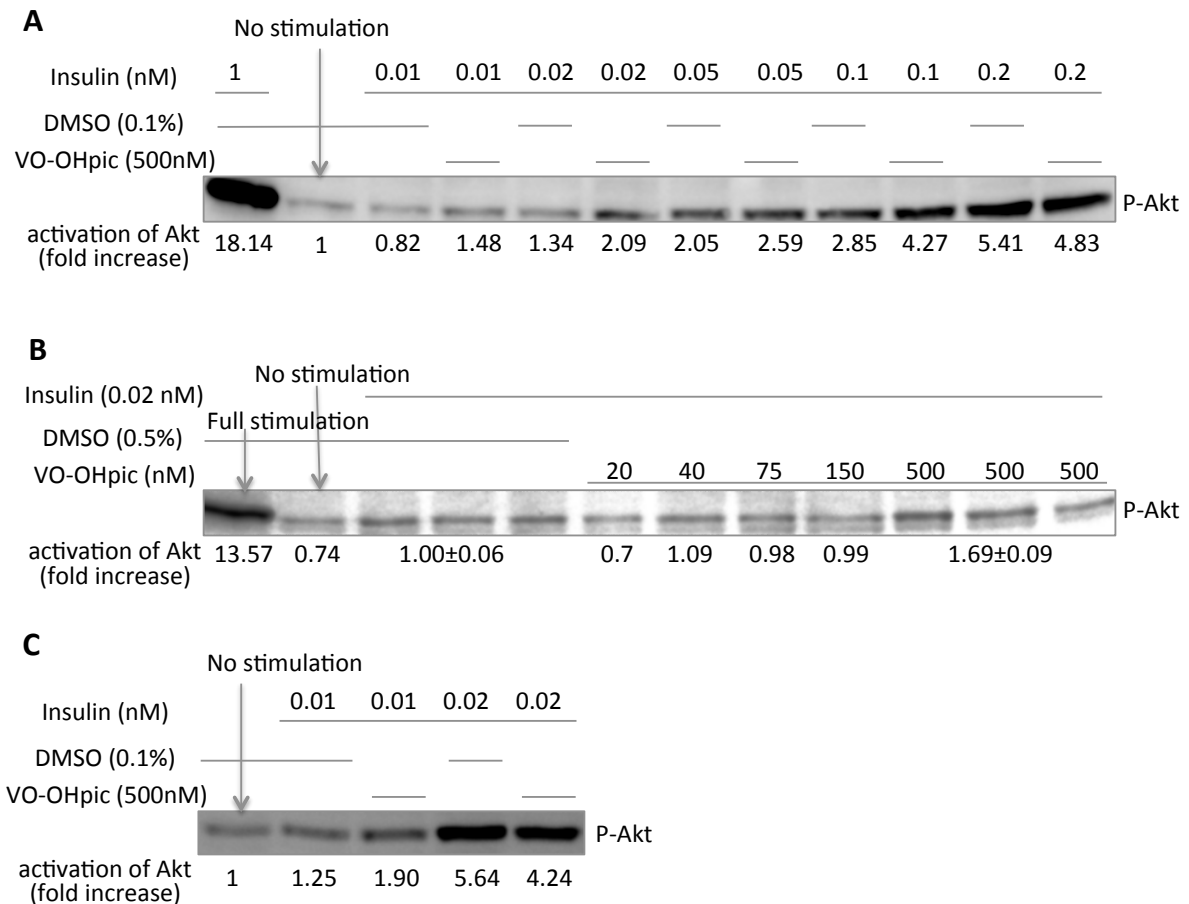


Figure 6.15. Akt activation upon VO-OHpic treatment at low insulin concentration. Akt activation upon VO-OHpic treatment at low insulin concentration was quantified. **(A)** HCT116 cells were treated with 500 nM VO-OHpic or DMSO (1 hour) after 40 hours starvation in FBS-free growing media, and then were stimulated with 0.01 – 0.2 nM insulin. High insulin concentration (1 nM) was used as full stimulation. **(B)** Assessment of the dose dependence of Akt activation in HCT116 cells upon VO-OHpic treatment. After 43 hours starvation and 22 hours VO-OHpic treatment cells were stimulated with 0.02 nM insulin. High insulin concentration (1 nM) was used as full stimulation. **(C)** NIH 3T3 cells were treated with 500 nM VO-OHpic or DMSO (1 hour) after 40 hours starvation in NCS-free growing media, and then were stimulated with 1 nM Insulin. High insulin concentration (10 nM) was used as full stimulation. Total protein levels were calculated and loading normalised according to Bio-Rad DC protein assay, then analysed by Western blotting. The numbers under the bands represent the ratio of protein expression with control (DMSO-treated, stimulated with 1 nM Insulin) samples arbitrary set as 1.0 and determined by ImageJ

In order to check what is the reason for the lower phosphorylation levels, reagents stocks were checked and longer starvation and incubation time was tested. For this, fresh insulin stock was prepared and used for stimulation of HCT116 and NIH 3T3 cells. Unfortunately, none of the insulin concentrations used demonstrated a significant difference in Akt phosphorylation levels with VO-OHpic treated samples compared to the DMSO control samples (Figure 6.16(A, B)). Thus, preparing fresh insulin did not improve the results.

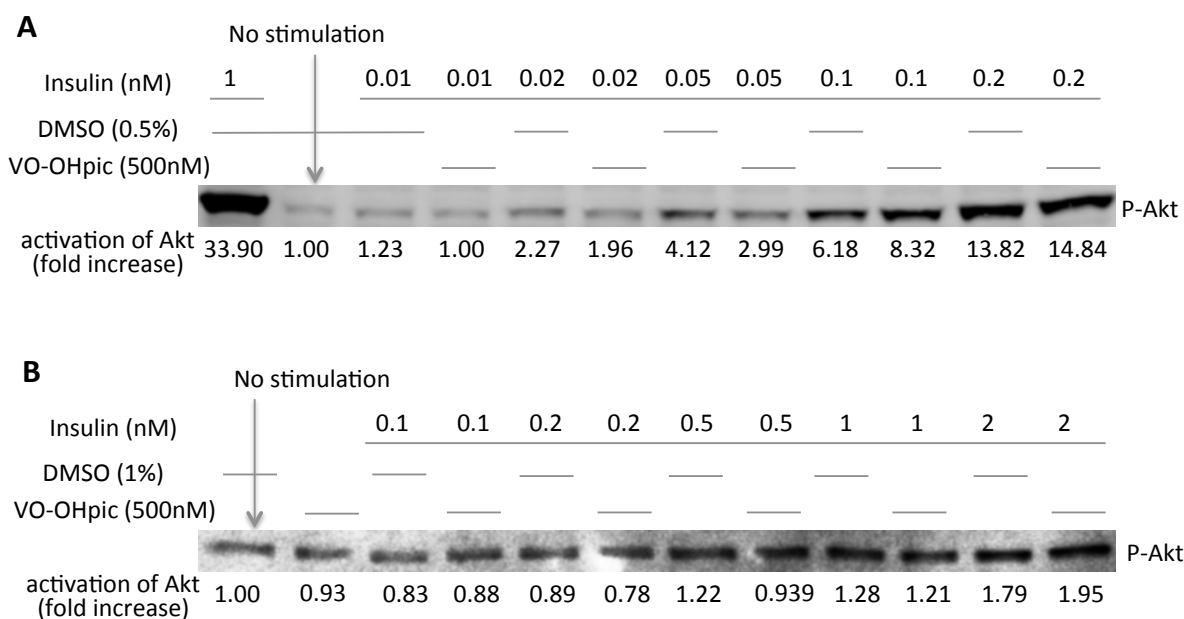


Figure 6.16. Akt activation upon VO-OHpic treatment in fresh insulin. Akt activation upon VO-OHpic treatment using fresh insulin stimulation was quantified. **(A)** HCT116 cells were treated with 500 nM VO-OHpic or DMSO (1.25 hours) after 43 hours starvation in FBS-free growing media, and then were stimulated with 0.01 – 0.2 nM fresh insulin for 15 min. High insulin concentration (1 nM) was used as full stimulation. **(B)** NIH 3T3 cells were treated with 500 nM VO-OHpic or DMSO (23 hours) after 75 hours starvation in NCS-free growing media, and then were stimulated with 1 nM fresh insulin for 15 min. High insulin concentration (10 nM) was used as full stimulation. Total protein levels were calculated and loaded according to Bio-Rad DC protein assay, then analysed by Western blotting. Tubulin levels were used as a loading control.

Following that, VO-OHpic was checked. Various batches (synthesised at different time, prepared fresh from solid at different concentrations in DMSO) and different incubation time (15 min-23 hours) were checked. This had no effect on results (data not shown).

In order to eliminate the compound as a reason for the negative results BpV(HOpic) was tested. HCT116 and NIH 3T3 cells were treated with bpV(HOpic) in various concentrations. It was earlier demonstrated (Schmid et al. 2004) that treatment of NIH 3T3 with 40 nM bpV(HOpic) induces the phosphorylation of Akt. However, in this project only 500 nM of this compound caused activation. This was the case for both HCT116 and NIH 3T3 cell lines

(Figure 6.17(A,B)). Various concentrations of fresh insulin demonstrated similar levels of Akt phosphorylation as for VO-OHPic (Figure 6.17(C)).

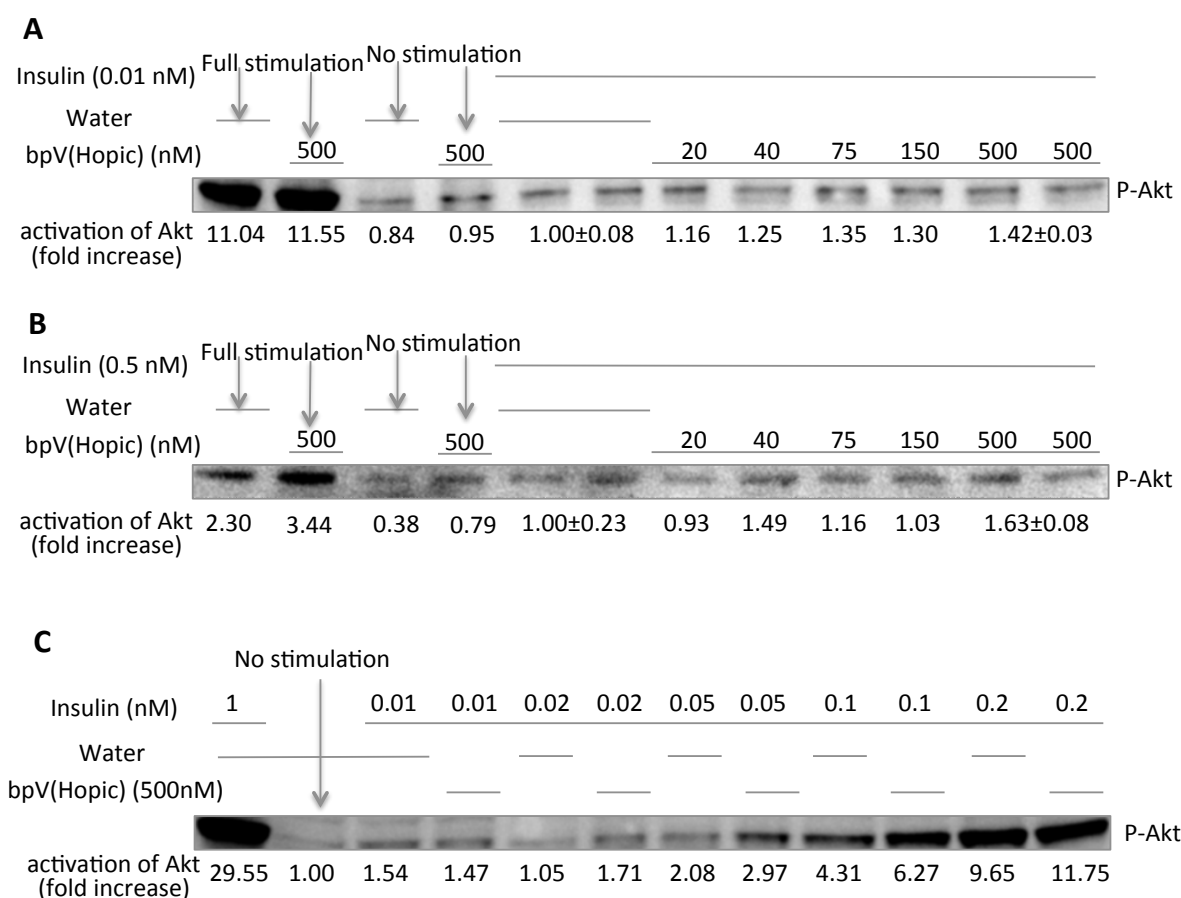


Figure 6.17. Akt activation upon bpV(HOPic) treatment. **(A)** HCT116 cells were treated with 20-500 nM bpV(HOPic) or water (1.3 hours) after 43 hours starvation in FBS-free growing media, and then were stimulated with 0.02 nM fresh insulin. High insulin concentration (1 nM) was used as full stimulation. Only 500 nM bpV(HOPic) treatment provoked Akt phosphorylation in comparison with water (1.42 ± 0.03 fold). **(B)** NIH 3T3 cells were treated with 20-500 nM bpV(HOPic) or water (2 hours) after 43 hours starvation in NCS-free growing media, and then were stimulated with 0.5 nM fresh insulin. High insulin concentration (10 nM) was used as full stimulation. Only 500 nM bpV(HOPic) treatment provoked Akt phosphorylation in comparison with water (1.63 ± 0.08 fold). **(C)** HCT116 cells were treated with 500 nM bpV(HOPic) or water (1.3 hours) after 43 hours starvation in FBS-free growing media, and then were stimulated with 0.01 – 0.2 nM insulin. High insulin concentration (1 nM) was used as full stimulation. Total protein levels were calculated and loaded according to Bio-Rad DC protein assay, then analysed by Western blotting. Tubulin levels were measured in all samples for loading control.

Unfortunately, none of these optimisation experiments gave P-Akt activation in a dose dependent manner with either VO-OHPic, or bpV(HOPic) in HCT116 or NIH 3T3 cells. However, it is possible that this effect is cell dependent and using even the same cell line as published before (NIH 3T3) but different batches could affect the specificity of the assay. Moreover, only higher concentrations of VO-OHPic (0.5-5 μ M) were shown to elevate Akt

phosphorylation in human Hep3B cells with medium PTEN levels and no effect in PLC/PRF/5 cells with high PTEN levels indicating that this assay is indeed cell line-dependent.

In conclusion, both VO-OHpic and BpV(HOpic) demonstrated PTEN inhibition *in vivo* at high nano-molar concentration (500 nM) in tumorigenic HCT116 cells but not in immortalised MRC-5 and PNT1A cell lines.

6.5. Cell viability in PTEN^{+/-} cells upon VO-OHpic treatment

There was no PICS phenotype detected upon PTEN inhibition and only a partial response in the P-Akt assay in cells developed within this project. We next assessed if VO-OHpic (or BpV(HOpic)) caused any change in cellular viability depending on intracellular PTEN status.

The MTS assay was chosen for cell survival evaluation due to its high throughput efficiency, ease of use, rapid indication of toxicity, accuracy and sensitivity in a 96-well format (Berg et al. 1994, Riss et al. 2004). The method is based on combination of intermediate electron acceptor reagent Phenazine Methyl Sulfate (PMS) with tetrazolium reagent (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, MTS) that is reduced by NADH dehydrogenase in metabolically active cells yielding colourful formazan product that is soluble in cell culture media (Figure 6.18).

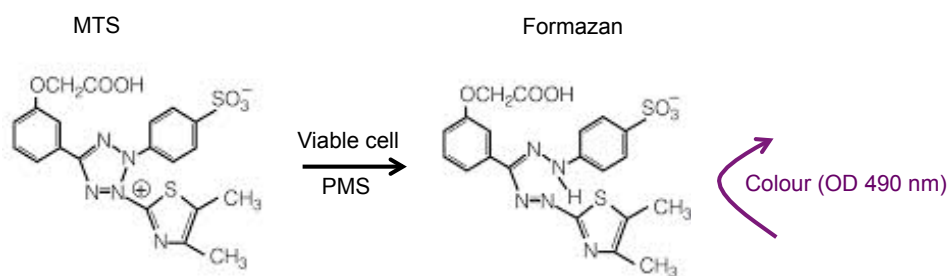


Figure 6.18. MTS cell viability assay scheme. The negatively charged tetrazolium compound (MTS) is soluble in tissue culture media but is not cell permeable. Addition of an intermediate electron acceptor reagent (PMS) that penetrates viable cells and transfers electrons from NADH in the cytoplasm, initiates the reduction of the MTS to the coloured formazan-like product. The signal can be recorded by measuring absorbance at 490 nm.

In order to assess cytotoxicity via the MTS assay, absorbance values from the treated and mock-treated cells were fitted to a normalised sigmoidal curve and in the case of a visual difference between these curves, the normalised growth rate (GR) inhibition with the GR50 parameter was calculated as a quantitative measurement. This metric is superior to conventional cell potency parameters such as IC50 (half-maximum inhibitory concentration) or EC50 (concentration at half-maximal effect) (Hafner et al. 2016). It was recently demonstrated theoretically and experimentally that current metrics vary dramatically from cell-to-cell variability

(Fallahi-Sichani et al. 2013), time-dependent bias (Harris et al. 2016), division rate, cell type, seeding density and medium composition (Hafner et al. 2016). On the contrary, an alternative GR metrics that are calculated by comparing growth rates under treated and untreated conditions, was shown to be robust to variation in cell growth, density, genotype and culture conditions and thus should replace IC50 in studies involving comparison of different cell lines. The GR value is just the ratio between growth rates in the presence or absence of compound and can be estimated from endpoint measurement of cell number (or a surrogate of cell number such as MTS staining) in treated and untreated samples, from a common starting point (initial cell seeding).

Considering, that PTEN modification in the developed lines indeed caused a difference in the growth rate even in the un-treated state⁺⁺⁺⁺, GR50 values were calculated in combination with IC50s for the MTS assays.

MTS cell viability assays for each compound and each cell line were performed at least two times and each time was done in triplicates. For this, cells were incubated with different concentrations of VO-OHpic or BpV(HOpic). Similar to the senescence assays, the effects of the compounds on PTEN^{+/-} cells was compared to the PTEN^{+/+} and PTEN^{-/-} cells.

Initially, cell density and length of assay was optimised for each cell line. A seeding density was determined such that cells would reach near 80% confluent state on the day of detection. The inhibitor concentration range was set in order to test the whole range of the dose response curve. All cell lines exhibited no significant difference in proliferation rate regardless of the PTEN genotype except HCT116 treated with BpV(HOpic) (Figure 6.19(A)). A different view of this graph is shown on Figure 6.19(B) to emphasise significant difference of the values for PTEN^{+/-} cells compared to homozygous PTEN cell lines. This statistically valid result was reproducible with a different time of treatment with BpV(HOpic) (Figure 6.19(C)) and indicates that PTP inhibition leads to reduced cell viability in human colon cancer cells (HCT116) with PTEN^{+/-} genotype but not in immortalised non-tumorigenic cells (MRC-5, PNT1A).

⁺⁺⁺⁺ PTEN^{-/-} cells demonstrated slower proliferation speed compared to PTEN^{+/+} and PTEN^{+/-} cells in all three HCT116, MRC-5 and PNT1A cell lines (Figure 6.22 and data not shown), which was consistent with earlier reports from (Chen et al. 2005, Lin et al. 2010), showing that complete inactivation of PTEN in MEFs decreased cell proliferation speed due to elevated levels of cellular senescence.

(A)

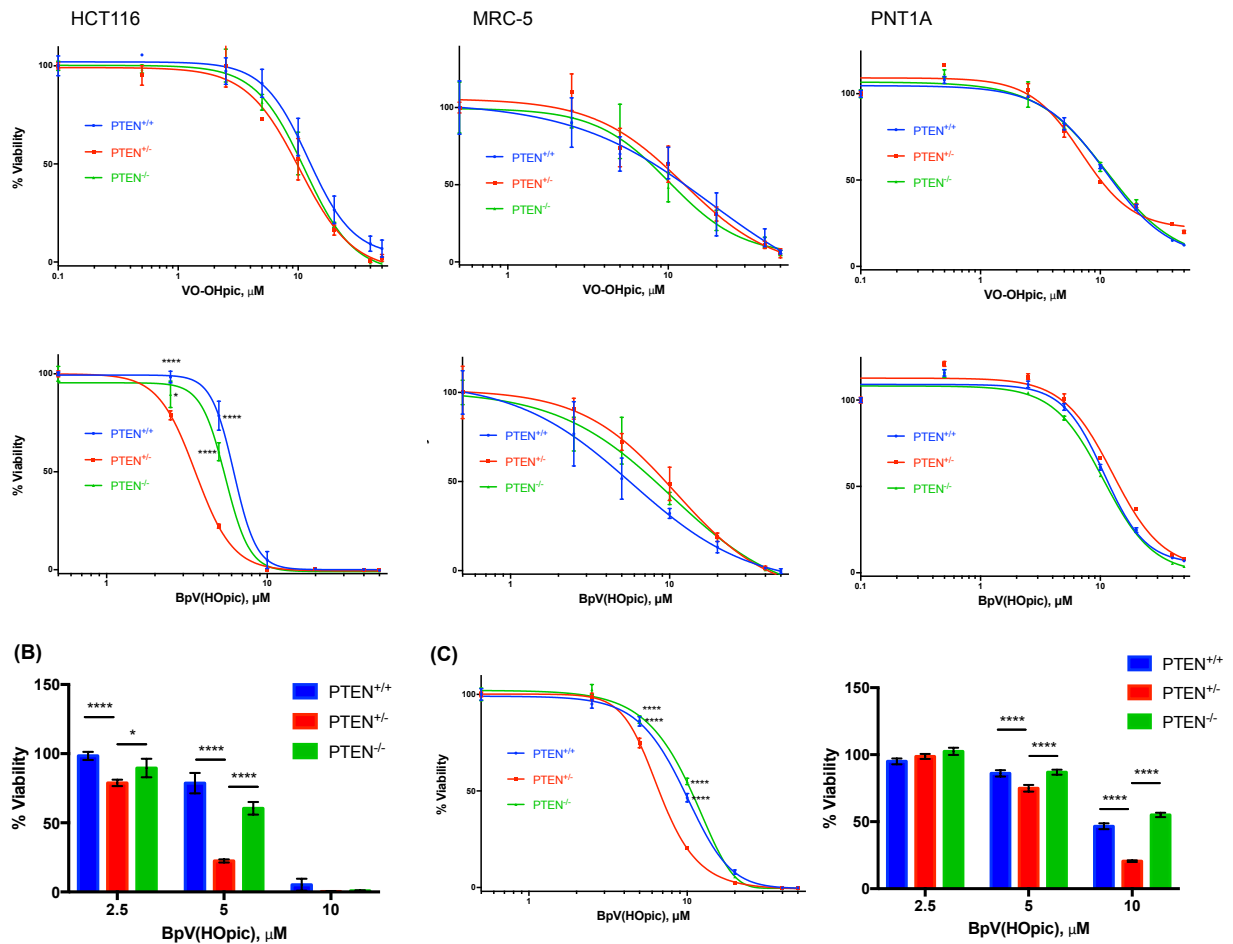


Figure 6.19. VO-OHpic and BpV(HOpic) effect on cell viability. **(A)** HCT116 (0.06×10^4 cells/well), MRC-5 (0.08×10^4 cells/well) and PNT1A (0.3×10^4 cells/well) cells with different PTEN status were plated into 96-well plate and treated with increasing concentrations (0.5-50 μM) of VO-OHpic (top row) or BpV(HOpic) for 6 days (HCT116 and MRC-5) or 3 days (PNT1A). The cell viability was determined by MTS assay. Negative control cells were treated with 0.5% DMSO (for VO-OHpic) or H₂O (for BpV(HOpic)). **(B)** A segment of the cell viability graph representing a significant difference for PTEN^{+/-} HCT116 cells treated with BpV(HOpic) compound. **(C)** HCT116 cells (0.35×10^4 cells/well) treated with BpV(HOpic) for 3 days prior MTS detection demonstrated the same result. Each value represents the mean $\pm$ SD out of three replicates. Significant differences (two-way ANOVA) to the PTEN^{+/-} cell values are indicated by stars (* $p < 0.1$, **** $p < 0.0001$).

All the collected data were used to calculate GR and GR50. These metrics also confirmed the visual results from the sigmoidal survival curves with similar cell viability upon VO-OHpic or BpV(HOpic) treatment in MRC-5 and PNT1A cells regardless of *PTEN* genotype, while PTEN^{+/-} HCT116 cells treated with BpV(HOpic) (but not VO-OHpic) exhibited significant decrease in cell survival. Representative GR values from these calculations are shown on Figure 6.20.

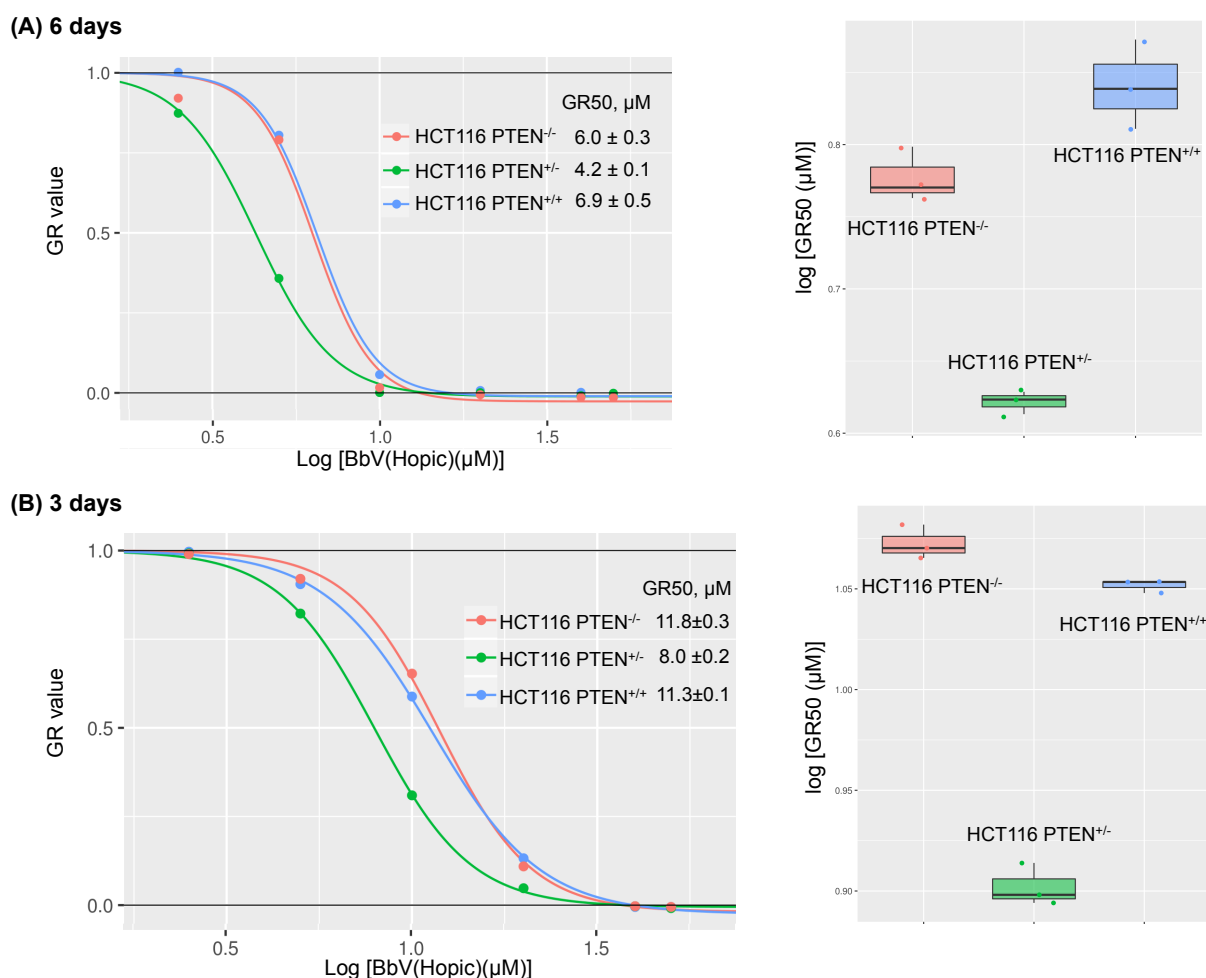


Figure 6.20. Evaluation of cell viability data via GR metrics. Representative calculations of GR and GR50 in PTEN^{+/+}, PTEN^{+/-} and PTEN^{-/-} HCT116 treated with BpV(HOpic) for **(A)** 6 days, or **(B)** three days. In left panels, GR data shown derived from one of three biological replicates. GR50 data are calculated as means from three replicates ± SD. In right panels, the distribution of GR50 is plotted. On the box plot the rectangle represents the first and third quartiles, the bar (vertical line inside) indicates the median value, the vertical line connects minimum and maximum values. GR metrics were calculated via online calculator on <http://www.grcalculator.org>

Firstly, this result suggests that inhibiting just PTEN phosphatase with a more specific VO-OHpic inhibitor does not lead to changes in cellular proliferation and inhibiting a range of PTPs does, because BpV(HOpic) possess broader specificity compared to VO-OHpic that is reported to be exclusively PTEN inhibitor (Rosivatz et al. 2006). This could be due to the activity of other PTPs that may compensate for the decrease in PTEN levels in HCT116 cells. Secondly, the immortalised cells could partially compensate for the PTEN tumour suppression function for instance via p21 and/or p16 activity. Indeed, Alcorta *et al.* reported that SV40-immortalised MRC-5 cells expressed elevated levels of p21 (Alcorta et al. 1996). However, decrease in p21 expression followed by an elevation of p16 protein and mRNA levels caused cellular senescence in the same cells through inhibiting CDK4/CDK6 expression (Hara et al. 1996,

Alcorta et al. 1996). Another study demonstrated that p16 failed to induce senescence in SV40-immortalised IMR90 fibroblasts in the absence of p21 (Stein et al. 1999). Thus, p16 and especially p21 activity could be the major senescent regulators in immortalised MRC-5 (and similarly, PNT1A) cells, which could overwrite PTEN and other PTP inhibition effect.

These data partially explain why the more mild senescence phenotype was not detected in these cells in earlier experiments.

Having determined a reproducible result in cell proliferation rate in PTEN^{+/-} cells compared to PTEN^{+/+} and PTEN^{-/-} upon BpV(HOpic) treatment, it was interesting to assess if the addition of PTEN mutations would affect this result. CRISPR-Cas9 PTEN KI cell lines were the only cells introducing one copy of the gene into PTEN^{-/-} cells. Thus, PTEN^{WT/-} and PTEN^{mut/-} were tested in the same cell viability assay and compared to the cells with various endogenous PTEN levels. Unfortunately, PTEN^{WT/-} Clone 15 (the clone that was characterised as a PTEN^{+/-} phenotype) responded to the BpV(HOpic) treatment the same way as PTEN^{+/+} and PTEN^{-/-} cells rather than PTEN^{+/-} cells (Figure 6.21(A)), suggesting that ectopic expression of PTEN under the CMV promoter in the safe harbour locus on chromosome 19 did not lead to the same PTEN expression or post-translation modification as endogenous protein. Consequently, both PTEN^{K13E&K289E/-} clones demonstrated the same cellular viability response as PTEN^{WT/-} Clone 15. Surprisingly, PTEN^{WT/-} Clone 6 showed a very different behaviour compared to all other cell lines tested, showing an increase in cell survival rate, indicating that perhaps there was indeed an off-target mediated via the CRISPR-Cas9 KI recombination^{####}.

The Sleeping Beauty (SB) KI Clones that contain multiple copies of *PTEN* were also treated with BpV(HOpic) and screened in the MTS assay. Interestingly, cell viability in WT_PTEN, C124S_PTEN and V403A_PTEN KI clones was affected in a similar rate to PTEN^{+/-} cells, while all other mutants behaved more similar to PTEN^{+/+} or PTEN^{-/-} cells (Figure 6.21(B)).

See Chapter 5.3.2 for more about Clone 6 and Clone 15 characterisation upon CRISPR-Cas9 PTEN_WT KI. Briefly, both Clone 6 and Clone 15 were confirmed as carrying one copy of *PTEN* gene via PCR assay but western blot analysis indicated that only Clone 15 expresses functional and active PTEN protein.

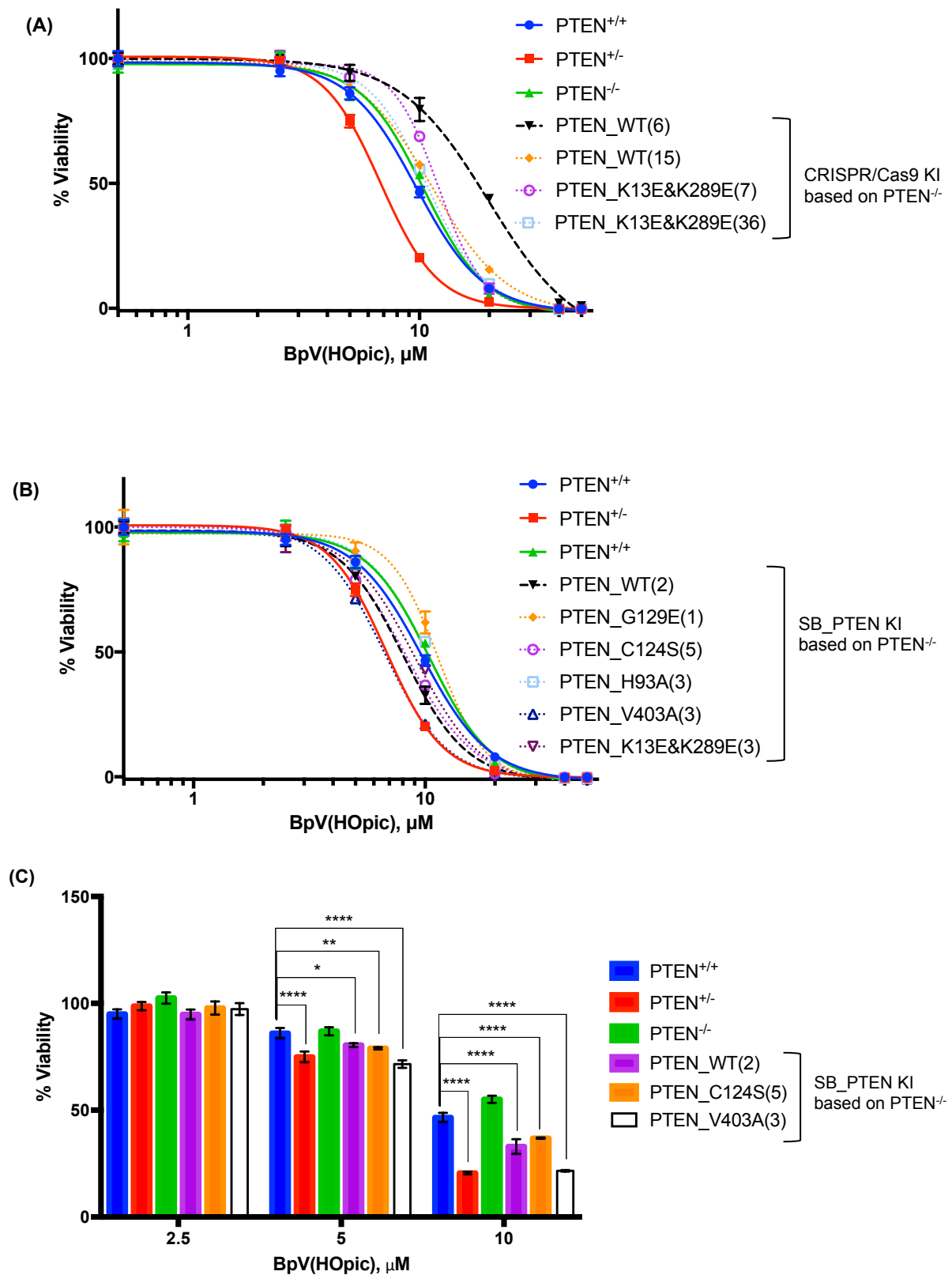


Figure 6.21. BpV(HOpic) effects on cell viability in PTEN KI cell lines. HCT116 KI clones (0.35×10^4 cells/well) were plated in 96-well plates and treated with increasing concentrations (0.5-50 μ M) of BpV(HOpic) for 3 days: **(A)** CRISPR/Cas9 based PTEN KI clones; **(B)** Sleeping Beauty (SB) based PTEN KI clones. The cell viability was determined by MTS assay. HCT116 PTEN^{+/+}, PTEN^{+/-} and PTEN^{-/-} cells were analysed for comparison. Negative control cells were

treated with H₂O. **(C)** A segment of cell viability graph based on SB KI clones representing significant difference for PTEN_WT, PTEN_C124S and PTEN_V403A KI clones treated with BpV(HOpic) compound. Each value represents the mean $\pm$ SD in three independent experiments. Significant differences (two-way ANOVA) to the PTEN^{+/+} cell values (set as control) are indicated by stars (*p < 0.1, ****p < 0.0001).

Even though this result was statistically valid, there is no genetic correlation between these clones, so most likely this result can be explained by individual properties of the clones that might be heterogeneous due to other factors or off-target mutations, which would affect intracellular pathways in different ways.

6.6. Metabolomics in PTEN^{+/-} cells upon PTEN inhibition

It has been shown that elevation of PTEN activity *in vivo* triggers resistance to oncogenic transformation, as well as promotes reduced glucose and glutamine uptake (Garcia-Cao et al. 2012). These latter effects result from altered metabolic pathways. We therefore assessed the changes in levels of these and other cell metabolites upon treatment with VO-OHpics.

HCT116 PTEN^{+/+}, PTEN^{+/-} and PTEN^{-/-} cells in the same seeding density (25x10⁴ cells/well in 6-well dish) were cultured in growth media supplemented with dialysed FBS (Sigma, UK) for 18 hours following by treatment with DMSO (0.05%) or VO-OHpics (500 nM) for 27 or 50 hours. This time was chosen in order to detect the original changes in cellular metabolism, rather than accumulative and secondary effects that could arise from prolonged treatment. Comparative NMR measurements of media were performed by Dr Chung-Ho Lau in Dr Hector Keun's laboratory. Changes in metabolite levels such as amino acids, glucose and vitamins upon PTEN inhibition were compared between HCT116 derivatives and between time points.

The results were consistent with the earlier senescence data: there was no difference in any metabolites checked based on all cell lines treated with VO-OHpics or vehicle. Unfortunately, this implies that this VO-OHpics concentration and time of treatment was not sufficient to cause any detectable changes in cellular behaviour.

Apart of the data for VO-OHpics treatment, HCT116 cell lines with different PTEN levels were compared with each other. Interestingly, PTEN^{-/-} cells produced more lactate and alanine but had a slower growth rate. At the same time, PTEN^{+/+} and PTEN^{+/-} cells had higher glucose uptake, which was in a good correlation with published data (Garcia-Cao et al. 2012).

Surprisingly, PTEN^{+/-} cells treated with VO-OHpics exhibited a higher growth rate when compared to the mock-treated PTEN^{+/-} cells (Figure 6.22).

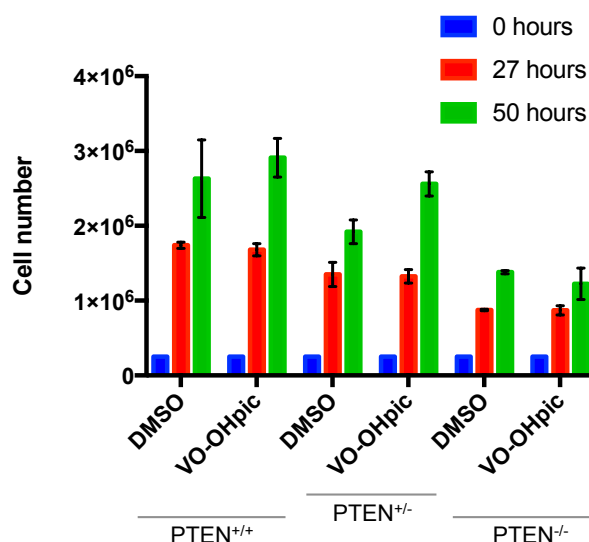


Figure 6.22. Growth rate in of HCT116 cells with different PTEN status treated with DMSO (0.05%) or VO-OHpic (500 nM) for 27 or 50 hours. A slight increase in growth rate in VO-OHpic treated PTEN^{+/-} cells comparing to mock-treated cells was detected. The growth rate correlated with the PTEN endogenous levels: PTEN^{+/+} > PTEN^{+/-} > PTEN^{-/-}.

This is actually the opposite effect to the PICS phenotype in which proliferation would be expected to be reduced. This result indicated that early time points after PTEN inhibition in cells with partial PTEN activity (up to 50 hours) cause pro-proliferative phenotype in HCT116 cells. Possibly higher compound concentrations and longer treatment time would reverse this phenotype but this was not confirmed with the cell lines studied within the timeframe of this study.

6.7. Conclusions

Taking together these results, it was confirmed that VO-OHpic and BpV(HOpic) compounds inhibited PTEN *in vitro*, whereas an *in vivo* effect was detected in HCT116 derived cells but not in MRC-5 or PNT1A derived lines. Moreover, there were indications that VO-OHpic might have an off-target effect within the Akt pathway in HCT116 cells. It is also important to mention that PTEN^{+/+}, PTEN^{+/-} and PTEN^{-/-} HCT116 cells generated within this study and HCT116 cells from Todd Waldman's laboratory (Lee, Kim & Waldman 2004) behaved identically in all experiments.

However, PTEN inhibition with these compounds still did not cause the SA-β-Gal response in PTEN^{+/-} human cells generated within this project. Although, in other studies VO-OHpic was reported to induce senescence in PTEN^{+/-} MEFs (Alimonti et al. 2010) and hepatocellular carcinoma Hep3B cell lines expressing low levels of PTEN (Augello et al. 2016) ; there are no reports with VO-OHpic senescence studies based on human diploid cells upon our knowledge.

Thus, it was hypothesized that VO-OHpic-mediated senescence response could be inhibited in these cells.

MTS data demonstrated a reduction in HCT116 PTEN^{+/-} cell viability upon BpV(HOpic) treatment. This effect was significant in the concentration range 2.5-5 μ M compound, which suggests that using higher concentration than 1 μ M could improve P-Akt assay reproducibility. Indeed, more recent studies analysed 0.25-5 μ M range of VO-OHpic in P-Akt assay and only 2.5 and 5 μ M demonstrated a substantial increase in Akt phosphorylation, while lower concentrations elevated P-Akt levels in a similar rate to HCT116 cells (0.9-1.6). However, such a high concentration was too toxic for the long-term senescence experiments in the cells studied here, which was in correlation with published senescence reports that used 0.25-0.5 μ M of VO-OHpic treatment (Alimonti et al. 2010, Augello et al. 2016).

Similarly to senescence and MTS data, metabolomics results also suggested no significant cellular response upon PTEN inhibition with VO-OHpic up to two days after treatment.

In conclusion, the human diploid cells with partial PTEN expression levels studied within this project did not confirm the PICS phenotype upon further PTEN inhibition possibly due to compensation effect from other Akt regulators, for instance PHLPP1 phosphatase.

Moreover, Augello *et al.* recently reported that VO-OHpic treatment mediated a significant increase in activation of not only the Akt pathway, but also Extracellular signal-Regulated Kinase (ERK)1/2 pathway *in vivo* (Augello et al. 2016). Their experiments also demonstrated that activation of both pathways upon VO-OHpic treatment reduced levels of cell proliferation and strongly suppressed tumour growth in Hep3B mouse xenograft model. These findings were in good correlation with earlier studies, in which PTEN was characterised as a negative regulator of the ERK1/2 pathway (Gu, Tamura & Yamada 1998, Chetram, Hinton 2012) alongside the studies showing that over-activation of the ERK pathway induces growth arrest and cellular senescence (Cagnol, Chambard 2010, Steelman et al. 2011, Deschenes-Simard et al. 2014). Thus, activation of both the Akt and ERK pathways via VO-OHpic can induce cellular senescence at least in HCC human cells. Within this project only the P-Akt pathway was analysed, so it would be important to study MEK-ERK1/2 pathway upon PTEN inhibition in HCT116, MRC-5 and PNT1A cells in the future work.

7. Results and discussion: synthetic lethality in PTEN^{-/-} cells

7.1. Introduction

PTEN inhibition in cancer cells may be useful for sensitising cancer cells to other reagents or inhibition strategies, such as PARP inhibitors (Mendes-Pereira et al. 2009), TTK inhibitors (Brough et al. 2011b), Skp2 inhibition (Lin et al. 2010, Chan et al. 2013), NLK inhibition (Mendes-Pereira, Lord & Ashworth 2012) and curcumin treatment (Chen et al. 2013). In the recent studies it was demonstrated that all these treatments act selectively to cause synthetic lethality in PTEN null cells and thus may be potentially used for treatment of PTEN-deficient cancers. Synthetic lethality (or lethal sensitivity) is an association between two genes where defects in either gene are compatible with cell viability or survival, while simultaneous defects in both genes cause cell death (Dobzhansky 1946). Within this PhD project, it was planned to (i) analyse the lethal sensitivity phenotype in human PTEN KO HCT116, MRC-5 and PNT1A cells upon treatment with these reagents and (ii) assess the effects of introducing PTEN mutants into the PTEN null background. Furthermore, it was interesting to explore the hypothesis that treating the PTEN^{+/-} human cells with VO-OHpic prior to treatment with compounds inducing synthetic lethality in PTEN null cells could lead to the same synthetic lethality in this different genetic background.

7.2. Synthetic lethality via PARP inhibition

7.2.1. Introduction

Shen *et al.* reported that PTEN^{-/-} MEFs exhibited genomic instability due to defects in RAD51-mediated DNA double strand break repair (DSBR) by homologous recombination (HR) (Shen et al. 2007). Later Mendes-Pereira *et al.* demonstrated that PTEN deficient colorectal HCT116 and endometroid adenocarcinoma HEC1A cells (developed in Todd Waldman's laboratory (Lee, Kim & Waldman 2004)) expressed reduced levels of RAD51 causing 5-fold decrease in DSBR by HR when compared to isogenically matched wild type cells (Mendes-Pereira et al. 2009). Similarly, reduced expression of RAD51 was observed in a panel of PTEN deficient human endometroid endometrial carcinoma (EEC) tumour lines (Dedes et al. 2010) and PTEN null human glioblastoma U-87 MG cells (Majuelos-Melguizo et al. 2015) when compared to PTEN proficient cells of the same type. Reduced transcription of RAD51 paralogs (but not RAD51 itself) was also observed in primary PTEN^{-/-} mouse astrocytes (glioma cells)

(McEllin et al. 2010). Consequently, these reports indicated that this HR repair defect in PTEN null cells sensitised those cells to DNA-DSB inducers such as PARP inhibitors. For instance, Mendes-Pereira *et al.* showed that HCT116 PTEN^{-/-} cells were up to 20 times more sensitive to the PARP inhibitor KU0058948 (Farmer et al. 2005) and up to 25 times more sensitive to the PARP inhibitor AZD2281 (also known as KU0059436 or Olaparib (Evers et al. 2008, Fong et al. 2008, Fong et al. 2009, Khan et al. 2011)) than their wild type counterparts. A similar effect was detected when a panel of human tumour lines with wild type PTEN (breast MCF7, prostate DU145) vs PTEN null cells (prostate PC3, glioma A172, bladder UM-UC-3, breast MDAMB468, melanoma RPMI-7951, breast HCC70) was analysed. Interestingly, *PTEN* heterozygosity in HCT116 cells did not cause the same decrease in RAD51 expression as in PTEN^{-/-} cells, nor sensitise cells to PARP inhibition. Moreover, ectopic expression of wild type PTEN or C124S catalytically inactive mutant in PTEN null PC3 cells restored resistance to PARP inhibition, whereas the cytoplasmically constrained K289E mutant expressed in PC3 cells partially but not completely elicited resistance to PARP inhibitors. Based on these findings, the authors concluded that a non-phosphatase activity of PTEN determined sensitivity to PARP inhibition, while disruption of the nuclear localisation of PTEN at least partially modulated sensitivity to PARP inhibitors.

However, later studies demonstrated that PTEN^{+/-} MEFs also showed significant growth inhibition upon exposure to AZD2281 similar to PTEN^{-/-} cells (Gonzalez-Billalabeitia et al. 2014). The authors also showed that this inhibition was accompanied by a significant and dose-dependent senescence response in MEFs of both genotypes. These results were also reproduced in human prostate cancer LnCap cells that lack PTEN. Interestingly, removal of p53 in these cell lines did not change the cell growth inhibition phenotype upon AZD2281 treatment but this time it was accompanied by significant increase in apoptosis rather than cellular senescence (Gonzalez-Billalabeitia et al. 2014). Thus, the authors concluded that PARP inhibition triggers a p53-dependent cellular senescence in PTEN-deficient models. However, Chatterjee *et al.* (Chatterjee et al. 2013) observed an increase in senescence upon treatment with another PARP inhibitor rucaparib (Plummer et al. 2008) in both p53 proficient (LnCap, C4-2) and p53 null (PC3) prostate cancer cell lines suggesting that pathways other than p53-mediated might be involved in the senescence response.

Further, these and other studies suggested additional synthetic lethality partners to PARP inhibition in PTEN deficient cells, for example (i) low-dose ionising radiation in combination with PARP inhibitor rucaparib in PTEN null prostate cancer PC3, LnCap and C4-2 cells enhanced the DNA damage and triggered an even greater increase in the cellular senescence response (Chatterjee et al. 2013) ; however, treatment with PARP inhibitor PJ34 (Abdelkarim et al. 2001) did not cause synergistic effects in glioblastoma U-87 MG cells upon ionising radiation (Majuelos-Melguizo et al. 2015) ; (ii) co-treatment with an EGFR blocker, erlotinib, elicited a

combinatory robust reduction in tumour development in mice inoculated with U-87 MG cells, but contradictory U-87 MG culture cells alone did not exhibit decrease in viability upon this treatment (Majuelos-Melguizo et al. 2015) ; (iii) PI3K inhibition with BKM-120 in combination with PARP inhibitor AZD2281 synergistically increased growth suppression in PTEN null MEFs and prostate cancer LnCap cell lines as well as caused a significant and robust tumour regression in PTEN deficient mice and human xenograft mouse models (Gonzalez-Billalabeitia et al. 2014) ; (iv) hormonal therapy reducing estrogen levels in combination with PARP inhibitor AZD2281 achieved a substantial reduction in tumour size in PTEN deficient endometrial cancer mouse cell lines from high estrogen milieu, where no response was observed when treated with AZD2281 alone (Janzen et al. 2013) ; (v) cisplatin combination treatment with PARP inhibitor AZD2281 also potentiated the synergistic effect on PTEN deficient lung cancer cells and human xenograft mouse model (Minami et al. 2013).

Therefore, there are many pre-clinical studies on PARP inhibition synthetic lethality in PTEN deficient cells. However, the comparison between all these works could be complicated, given all different PARP inhibitors that likely to have distinct off-target effects.

Whereas there are many pre-clinical data on the relationship between PTEN deficiency and sensitivity to PARP inhibition, this correlation has not been widely investigated in clinical trials yet. However, a case report of a patient diagnosed with PTEN-deficient metastatic endometrial cancer who was treated with AZD2281 in a Phase I trial demonstrated a significant clinical response (Forster et al. 2011).

Together with pre-clinical studies these findings suggest that the synthetic lethality from PARP inhibition may be therapeutically beneficial in various types of cancer with PTEN loss (Benafif, Hall 2015), thus it would be interesting to extend the published data with PTEN structural requirements for this synergistic effect based on the cell lines developed within this study.

Within this project initially it was planned to reproduce the findings of Mendes-Pereira *et al.* and optimise cell viability assay using the same PARP inhibitor, AZD2281, and the same isogenic cell lines (PTEN^{+/+}, PTEN^{+/-} and PTEN^{-/-} HCT116 cells kindly provided by Todd Waldman's laboratory (Lee, Kim & Waldman 2004)), and then apply the optimised conditions for the newly developed HCT116, MRC-5 and PNT1A cells with different *PTEN* genotypes.

Lethal sensitivity was evaluated by MTS assays with sequential plotting of survival curves and calculation of GR metrics (see Chapter 6.5 for more details). Even though, the original (Mendes-Pereira et al. 2009) paper utilised sulforhodamine B colourimetric assay (Vichai, Kirtikara 2006), MTS assay was chosen for this work as it required less steps, which potentially should reduce error rates and improve sensitivity ^{§§§§§}.

^{§§§§§} This assay relies on the ability of sulphorhodamine B to bind to cellular protein content after fixation by trichloroacetic acid. Sulphorhodamine B is a pink dye that binds to amino-acid residues on proteins under mild acidic conditions, and dissociates under basic conditions (Vichai, Kirtikara 2006). The method has one drawback in that there

All MTS assays for each compound and each cell line were performed at least two times and each time was done in triplicates.

7.2.2. Results

Firstly, PTEN^{+/+} and PTEN^{-/-} HCT116 cells provided by Todd Waldman (Lee, Kim & Waldman 2004) (TW HCT116) were incubated with AZD2281 (Figure 7.1(A), Stratech, UK) in increasing concentrations (0-50 μ M) for six days with media and compound replaced after three days. Percentages of viable cells were determined after staining with MTS/PMS reagents according to the manufacturer's protocol (CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay, Promega). Time of treatment, inhibitor concentration and cell number were determined so that (i) mock-treated cells would reach 70-90% confluence on the day of analysis; (ii) selected concentrations would fall within the linear portion of the curve; and (iii) mock-treated cells would give an absorbance value between 0.75 and 1.25.

As a result TW HCT116 cells demonstrated no difference in cell viability in response to AZD2281 exposure regardless of PTEN status (Figure 7.1(B)). Interestingly, the sensitivity of PTEN^{+/+} TW HCT116 cells to AZD2281 was comparable to that published for the same cells in (Lee, Kim & Waldman 2004) (IC₅₀ of PTEN^{+/+} (Mendes-Pereira et al. 2009) was 5 μ M and IC₅₀ of PTEN^{+/+} (this study) was 2.2 μ M). Whereas, PTEN^{-/-} TW HCT116 cells exhibited significantly different sensitivity within this study compared to (Mendes-Pereira et al. 2009) (IC₅₀ of PTEN^{-/-} (Mendes-Pereira et al. 2009) was 0.2 μ M *versus* 2 μ M found here). Nevertheless, this comparison could be considered only as an indirect indication of AZD2281 sensitivity, given that different incubation time, sample size and read-out were used within these two studies (6 days incubation in 96-well plates using MTS staining - within this project, and 15 days incubation in 6-well plates using sulphorhodamine B staining – in (Mendes-Pereira et al. 2009)).

In order to determine if this result was due to the TW cell lines, sensitivity to PARP inhibition in HCT116, MRC-5 and PNT1A cells with different PTEN statuses developed within this project were also assessed. All three cell lines confirmed the data from TW HCT116 cells. No correlation with the *PTEN* genotype was reproduced in at least two experiments, representative examples of which are shown in Figure 7.1(C).

Alternative models were then sought to test the previously published data and verify that the AZD2281 was functional. For this, PTEN null human prostate adenocarcinoma PC3 and brain glioblastoma U-87 MG as well as normal prostate PNT1A cells expressing wild type PTEN were incubated with AZD2281 for four or six days. For both incubation time points PTEN deficient PC3 cells demonstrated higher sensitivity towards PARP inhibition than PNT1A cells

are many steps from extracting the signal after staining: cells have to be fixed, washed to remove excess dye and finally basified prior measuring absorbance.

(Figure 7.1(D)), verifying the published data and the functionality of the PARP inhibitors used here.

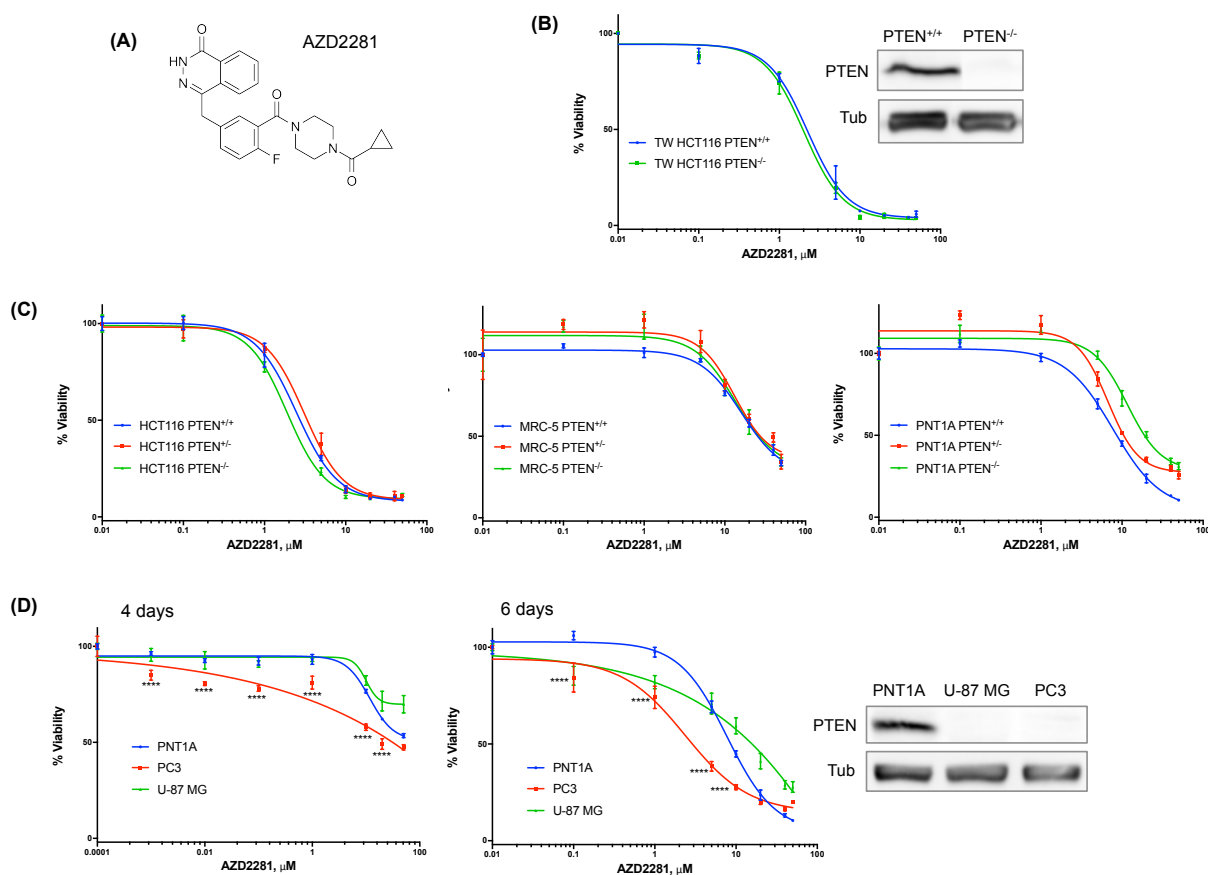
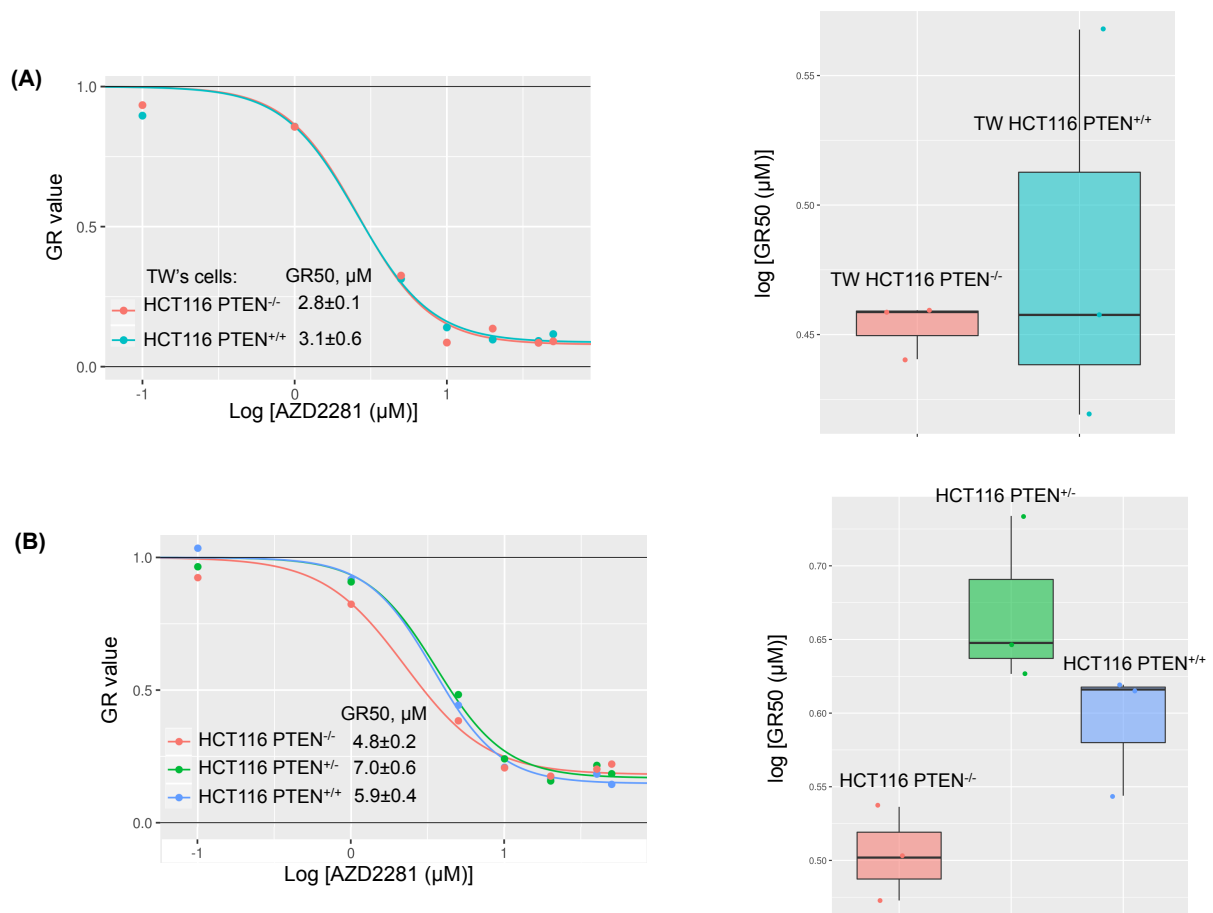


Figure 7.1. Sensitivity of human PTEN deficient cells to PARP inhibition with AZD2281. **(A)** Chemical structure of PARP inhibitor AZD2281 (Olaparib, KU0059436). **(B)-(D)** Cells with various PTEN statuses were plated into 96-well plates and treated with increasing concentrations of compound (0.1-50 μM) for six days: **(B)** PTEN^{+/+} and PTEN^{-/-} Todd Waldman's (TW) HCT116 cells (0.03x10⁴ cells/well) **(C)** PTEN^{+/+}, PTEN^{-/-} and PTEN^{-/-} cells developed within this study: HCT116 (0.05x10⁴ cells/well), MRC-5 (0.08x10⁴ cells/well) and PNT1A (0.25x10⁴ cells/well). **(D)** PTEN WT PNT1A cells (0.6x10⁴ or 0.3x10⁴ cells/well), PTEN deficient PC3 (0.4x10⁴ or 0.22x10⁴ cells/well) and U-87 MG (0.16x10⁴ or 0.05x10⁴ cells/well) cells were incubated with AZD2281 for four (left panel) or six days (right panel) respectively. The compound and media were renewed on day three. The cell viability was determined by MTS assay. Each experiment was performed at least two times in triplicates. Negative control cells were treated with 0.5% DMSO and on the day of the assay reached 70-90% confluence. Each value represents the mean $\pm$ SD out of three replicates. Significant difference in cell viability was calculated and compared to the PTEN WT lines on survival curves (****p < 0.0001 vs PTEN^{+/+} cells, two-way ANOVA). Western blot inserts in **(B)** and **(D)** show cellular PTEN expression levels, tubulin was used as a loading control.

Interestingly, U-87 MG cells displayed limited sensitivity to AZD2281. While PC3 cells are widely used as a PTEN^{-/-} model cell line for PARP inhibition (Mendes-Pereira et al. 2009, Fraser et al. 2012, Chatterjee et al. 2013), there are few studies on U-87 MG cells. One published study indicated that U-87 MG cells displayed a very weak increase (if any) in sensitivity towards AZD2281 PARP inhibitor via cell viability assay while significantly higher

cytotoxic effect was induced by the less specific PJ34 PARP inhibitor in these cells when compared to PTEN proficient glioblastoma LN229 cells (Majuelos-Melguizo et al. 2015) partially agreeing with the data presented here.

All the collected data were used to calculate GR and GR50. Statistics also confirmed the visual results from the sigmoidal survival curves with no sensitivity to PARP inhibition in PTEN null TW HCT116, MRC-5, PNT1A, and U-87 MG cells, while PC3 sensitisation was significant. Representative calculations from these lines are shown on Figure 7.2. The only difference from proliferation curves was that GR50 from PTEN KO HCT116 cells developed within this study, showed a marginal sign of sensitisation to AZD2281 (Figure 7.2(B)). However, similar to survival curves, only PTEN deficient PC3 cells produced a statistically significant sensitisation to the treatment with PARP inhibitors.



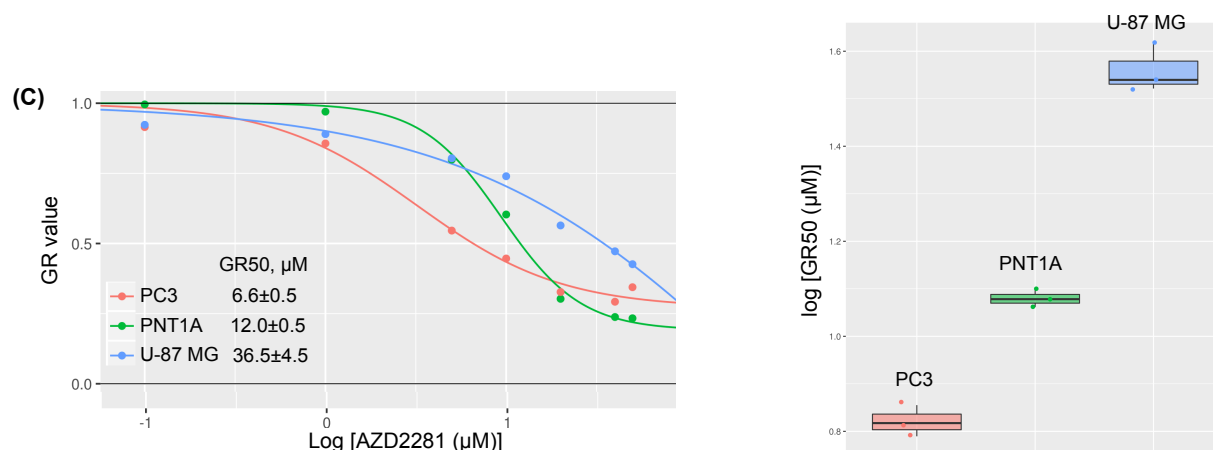


Figure 7.2. Evaluation of sensitivity to PARP inhibition via GR metrics. Representative calculations of GR and GR50 in **(A)** PTEN^{+/+} and PTEN^{-/-} Todd Waldman's (TW) HCT116; **(B)** PTEN^{+/+}, PTEN^{+/-} and PTEN^{-/-} HCT116 from this study; and **(C)** PNT1A, PC3 and U-87 MG cells treated with AZD2281 for six days. In left panels, GR data shown derived from one of three biological replicates. GR50 data are calculated as means from three replicates ± SD. In right panels, the distribution of GR50 is plotted. On the box plot the rectangle represents the first and third quartiles, the bar (vertical line inside) indicates the median value, the vertical line connects minimum and maximum values. GR metrics were calculated via online calculator on <http://www.grcalculator.org>.

7.3. Synthetic lethality to TTK inhibition

7.3.1. Introduction

Brough *et al.* (Brough et al. 2011b) exploited synthetic lethality pairs in breast tumour cell models using high-throughput RNAi screening and discovered PTEN-null cancer cells dependence upon mitotic spindle assembly checkpoint (SAC) kinases. The most significant dependence was attributed to the TTK protein kinase (also known as MPS1). Although the synthetic lethality dependence upon PTEN status was not found in all breast cancer cells, further the authors showed that TTK inhibition was also selective for PTEN deficient cancer cells using isogenic TW HCT116 and HEC1A cells with TTK silencing via siRNA or chemical inhibition (with two distinct TTK small molecule inhibitors CCT132774 or AZ3146 (Hewitt et al. 2010)). While, the synthetic lethality in these cells was not very high ($p < 0.05$), Brough *et al.* concluded that targeting TTK potentially could be therapeutically beneficial in PTEN deficient cancers.

This synthetic lethality combination could be explained by the fact that TTK is critical for mitotic checkpoint complex formation and recruitment of SAC cascade proteins to unattached kinetochores (Dou et al. 2015, Abrieu et al. 2001). SAC prevents chromosome missegregation by arresting the mitotic progression until all chromosomes are correctly attached to the mitotic spindle (Foley, Kapoor 2013, Dominguez-Brauer et al. 2015). Many cancers have disrupted

SAC responses that weaken checkpoint integrity and cause aneuploidy. However, complete inactivation of SAC leads to cell death. Consistent with this, it was demonstrated that TTK inhibition causes chromosome missegregation, aneuploidy and premature exit from mitosis (Martinez et al. 2015). In cancer cells with an aneuploidy phenotype, further TTK disruption leads to accumulation of even more genomic instability, aneuploidy and consequently cell death (Kwiatkowski et al. 2010, Colombo et al. 2010, Dominguez-Brauer et al. 2015, Jemaa et al. 2016). Moreover, genomic instability, aneuploidy and DSB are often found in cells with PTEN loss (Puc, Parsons 2005, Puc et al. 2005, Shen et al. 2007, Saal et al. 2008) and the frequency of abnormal metaphases is substantially higher in PTEN-deficient cells (Brough et al. 2011b). Brough *et al.* showed that subsequent TTK inhibition in such cells increased rates of abnormal metaphases even further, indicating a potential reason for the synthetic lethality between TTK and PTEN.

Earlier findings had indicated that TTK inhibition was potentially of therapeutic benefit in targeting cancers characterised with high levels of chromosome instability or disruption in mitotic progression and spindle assembly checkpoint (de Cárcer, Perez de Castro & Malumbres 2007, Colombo et al. 2010, Dominguez-Brauer et al. 2015, Martinez et al. 2015, Jemaa et al. 2016). The findings of Brough *et al.* indicated that TTK inhibition could also be a novel therapeutic strategy for treating PTEN deficient cancers (Aarts, Linardopoulos & Turner 2013). Thus, the implications of TTK inhibition was tested on isogenic PTEN^{-/-} cell lines developed during this study.

7.3.2. Results

Initially, the same reagents as in (Brough et al. 2011b) were tested. For this, PTEN^{+/+} and PTEN^{-/-} TW HCT116 cells were incubated with increasing concentration of TTK inhibitor AZ3146 (Figure 7.3(A)) for seven days. Unfortunately, no sensitivity was detected in PTEN deficient TW cells in two independent experiments. Representative survival curves are shown on Figure 7.3(B). However, AZ3146 IC50 values for PTEN proficient TW HCT116 cells were comparable between this study and (Brough et al. 2011b) : IC50 of PTEN^{+/+} (Brough et al. 2011b) was ~1 µM and IC50 of PTEN^{+/+} (this study) was 0.63 µM. Whereas, IC50 of PTEN^{-/-} was ~0.3 µM *versus* IC50 of PTEN^{-/-} (this study) still was 0.61 µM. The difference between these two experiments was only in the incubation step involving changing the media and the compound on day three (this study) or addition of the same amount of compound to the same media on day three (Brough et al. 2011b).

Further, isogenic HCT116, MRC-5 and PNT1A cells with various PTEN statuses developed within this study (Figure 7.3(C)) alongside cells with intrinsic *PTEN*-WT or *PTEN*-deficient genotypes (Figure 7.3(D)) were treated with AZ3146 for six days. The assays for each cell line were performed at least two times and each time was done in triplicates. No differences in

sensitivity to TTK inhibition was detected in isogenic lines regardless of PTEN status except that PTEN null U-87 MG cells were significantly more vulnerable upon TTK inhibition compared to PTEN proficient PNT1A cells. These results were also statistically verified via GR values (Figure 7.3(D)).

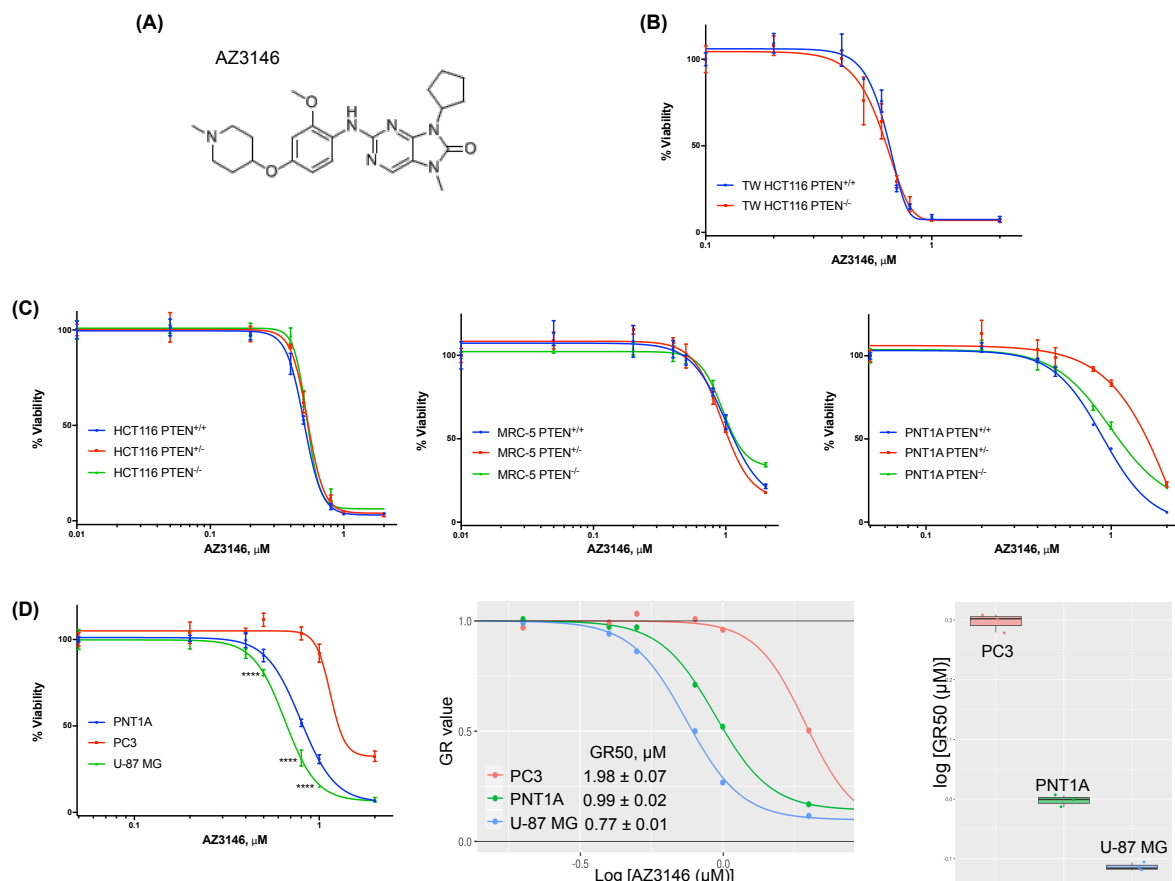


Figure 7.3. Sensitivity to TTK inhibition in human cells. **(A)** Chemical structure of TTK inhibitor AZ3146. **(B)-(D)** Cell viability assay upon AZ3146 treatment. Cells with different *PTEN* genotype were plated into 96-well plates and treated with increasing concentrations of compound (0.01-2 μM) for 5-7 days: **(B)** PTEN^{+/+} and PTEN^{-/-} Todd Waldman's (TW) HCT116 cells (0.02x10⁴ cells/well) **(C)** PTEN^{+/+}, PTEN^{+/-} and PTEN^{-/-} cells developed within this study: HCT116 (0.03x10⁴ cells/well), MRC-5 (0.2x10⁴ cells/well) and PNT1A (0.25x10⁴ cells/well); **(D)** PTEN WT PNT1A cells (0.2x10⁴ cells/well), PTEN deficient PC3 (0.08x10⁴ cells/well) and U-87 MG (0.05x10⁴ cells/well) cells. AZ3146 and media were renewed every 2-3 days. The cell viability was determined by MTS assay. Each experiment was performed at least two times in triplicates. Negative control cells were treated with 0.5% DMSO. Each value represents the mean ± SD out of three replicates. Significant difference in cell viability was estimated and compared to the PTEN proficient lines on survival curves (****p < 0.0001 vs PTEN^{+/+} cells, two-way ANOVA). **(D)** In the middle panel, GR data shown derived from one of three biological replicates. GR50 data are calculated as the mean from three experiments ± SD. In the right panel, distribution of GR50 is plotted. GR metrics were calculated on <http://www.grcalculator.org>.

However, PTEN null PC3 cells appeared to be the most resistant to AZ3146 exposure, emphasising the cell type specificity of this phenotype. It is of note that PC3 cells lack p53 (Komarova et al. 1998, Ben Sahra et al. 2010, Creighton et al. 2010, Lin et al. 2010) and as

p53 is involved in the post-mitotic checkpoint to prevent aneuploidy (Maire et al. 2013). TTK inhibition sensitivity may be p53-dependent. Indeed according to Huang *et al.*, TTK directly phosphorylates p53 at Thr18 disrupting its interaction with MDM2 and subsequently stabilising p53 by abrogating MDM2-mediated p53 ubiquitination (Huang, Chang & Shieh 2009). The authors also demonstrated that p53 phosphorylation at Thr18 enhanced p53-dependent activation of p21, which mediated the post-mitotic checkpoint. This indicates that TTK allows p53 to participate in the post-mitotic spindle assembly checkpoint, whereas p53 deficient PC3 cells can not activate this p53-dependent checkpoint, which may explain why TTK inhibition in these cells did not cause lethal sensitivity regardless of PTEN status.

7.4. Synthetic lethality to Curcumin treatment

7.4.1. Introduction

According to the Chen *et al.* report (Chen et al. 2013), PTEN deficient cancer cells could be targeted with a natural anti-inflammatory agent, curcumin. Those authors generated isogenic set of HCT116 cell lines with different *PTEN* genotype (similar to Todd Waldman's HCT116 cells (Lee, Kim & Waldman 2004)), and the cytotoxic effects from a set of compounds were determined on these cells. It was shown that lack of PTEN increased sensitivity to curcumin. Additionally, different expression patterns of the proteins related to the Akt/p21 pathway in PTEN null cells upon curcumin treatment was demonstrated, while no additional increase in apoptotic markers was observed. Thus, the authors concluded that such sensitivity arose from curcumin-mediated G0/G1 phase arrest rather than curcumin-induced apoptosis.

Earlier, curcumin-induced inhibition of cell proliferation was observed in PTEN null PC3 (Yu et al. 2008, Hilchie et al. 2010, Guo, Xu & Fu 2015), LnCap (Hilchie et al. 2010) and U-87 MG (Campos et al. 2013) cells. Similar, Narayanan *et al.* (Narayanan et al. 2009) demonstrated that curcumin treatment decreased tumour growth from prostatic adenocarcinoma *in vivo* and suppressed cell growth and induced apoptosis *in vitro* in PTEN^{-/-} prostate cancer models and Yang *et al.* showed curcumin-induced apoptosis and cytotoxic effects in a PC3-injected nude mouse model (Yang et al. 2015). Du *et al.* reported comparable findings in PTEN inactivated multiple glioma cell lines (U-87 MG and T98G^{*****}) and a U-87 MG-implanted nude mouse model, in which curcumin suppressed tumour cell proliferation, migration, colony formation and induced apoptosis *in vitro*, and reduced tumour volume and prolonged the survival period *in vivo* (Du et al. 2013). Earlier Aoki *et al.* revealed that curcumin induced non-apoptotic autophagic cell death and G2/M cell cycle arrest in PTEN null U-87 MG and U-373 MG cell lines and in a mouse xenograft model of U-87 MG cells (Aoki et al. 2007).

***** Multiple glioma T98G cell line expresses a catalytically inactive PTEN (Maccario et al. 2007)

These data indicated that curcumin treatment could be beneficial for cancers lacking PTEN. Therefore, this compound was also tested on cells developed within this study.

This compound has been extensively studied for its broad range of biological activities, such as anti-inflammatory, anti-oxidative, anti-bacterial, anti-infection, rheumatoid arthritis, Alzheimer's, psoriasis, diabetes and especially anti-cancer (reviewed in (Ramsewak, DeWitt & Nair 2000, Terlikowska et al. 2014, Mock, Jordan & Selvam 2015)). Curcumin's anti-cancer properties have been investigated both *in vitro* and *in vivo* for cancers including breast, ovarian, lung, colorectal and pancreatic (Shehzad, Wahid & Lee 2010, Rahmani et al. 2014, Mock, Jordan & Selvam 2015, Ramasamy et al. 2015). Curcumin has entered clinical trials for a range of cancers such as endometrial, pancreatic, prostate, breast and colorectal among others (Shehzad, Wahid & Lee 2010, Kanai 2014, Terlikowska et al. 2014). Curcumin and its analogues increased cytotoxicity in tumour cells on their own or in combination with other therapies, such as chemotherapy or radiotherapy (Li et al. 2007). Among others synergistic effects were detected upon curcumin co-treatment with (i) non-steroid anti-inflammatory drug, diclofenac, in colon tumour models (Rana et al. 2015) ; (ii) natural plant antioxidant agent, resveratrol, in prostate cancer cells and mice model (Narayanan et al. 2009) ; (iii) PI3K inhibitor, wortmannin, or (iv) Skp2 silencing in breast cancer cell lines (Fujimoto et al. 2014). Moreover, curcumin was attributed as a successful chemosensitiser agent that opposes drug resistance targeting multiple signalling pathways (Saha et al. 2012, Ramasamy et al. 2015). More than 25 curcumin-sensitive signalling pathways have been identified (Terlikowska et al. 2014). Many of them are directly involved in anti-cancer activity. For instance, curcumin was shown to decrease cell proliferation and viability, down-regulate PI3K and Akt activity in colon (Fujimoto et al. 2014, Rana et al. 2015), breast (Mock, Jordan & Selvam 2015), prostate (Narayanan et al. 2009) and ovarian (Terlikowska et al. 2014) cancer models. In addition, in these reports and others (Du et al. 2013, Yang et al. 2015, Shankar, Srivastava 2007) curcumin was shown to promote apoptosis via elevating the expression of pro-apoptotic Bcl-2 members (Bax and Bad) as well as Caspase-3, -7, -8 and -9, and increasing reactive oxygen species (ROS) generation, while decreasing the anti-apoptotic Bcl-2 protein levels (via direct interaction with the Bcl-2 protein-binding interface by multiple hydrogen bonding (Rana et al. 2015)). Other reports showed curcumin-induced (i) inhibition of pro-proliferative and anti-apoptotic Nuclear Transcription Factor-Kappa B (NF- κ B) (Guo et al. 2013, Kanai 2014) ; (ii) inhibition of mTOR signalling (Johnson et al. 2009) ; and (iii) activation of cell-cycle suppressor, p21 (Guo, Xu & Fu 2015). Moreover, in some cancer cells curcumin mediated up-regulation of anti-proliferative proteins p16, p21, p27 or p53 and initiated G2/M cell cycle arrest or p16-dependent senescence (reviewed in (Terlikowska et al. 2014, Mock, Jordan & Selvam 2015)).

Moreover, curcumin has a very important advantage over other therapeutic approaches such as low toxicity because it is widely used in food industry as a common spice of a natural origin. All these studies indicate that curcumin could be a safe therapeutic agent against a range of cancers and within this study its effect on isogenic cells with various PTEN levels was assessed.

7.4.2. Results

Similar to previous lethal sensitivity experiments, isogenic cell lines were treated with increasing concentrations of curcumin (Figure 7.4(A)) for three days and cell survival was estimated via MTS assay. Each cell line was analysed at least two times and each time was done in triplicates. Both HCT116 cell lines developed within this project and donated Todd Waldman, as well as PNT1A cells, demonstrated similar cell viability distribution regardless of *PTEN* genotype (Figure 7.4(B)).

Interestingly, PTEN^{-/-} MRC-5 cells were more sensitive to curcumin compared to WT PTEN cells. The sensitivity was statistically significant at $p < 0.0001$.

PTEN deficient PC3 and U-87 MG cells were also analysed after curcumin exposure. Similar to TTK inhibition, curcumin was considerably more cytotoxic only in U-87 MG cells but not PC3 when compared to WT PTEN PNT1A cells (Figure 7.4(C)). Interestingly, curcumin GR50 values for U-87 MG cells (GR50 10.2 μM) were lower than IC50 values reported (Aoki et al. 2007) in a similar cell viability experiment (IC50 35 μM). However, comparable values were observed in PC3 cells: GR50 23.2 μM within this study *versus* IC50 ~25 μM in (Hilchie et al. 2010).

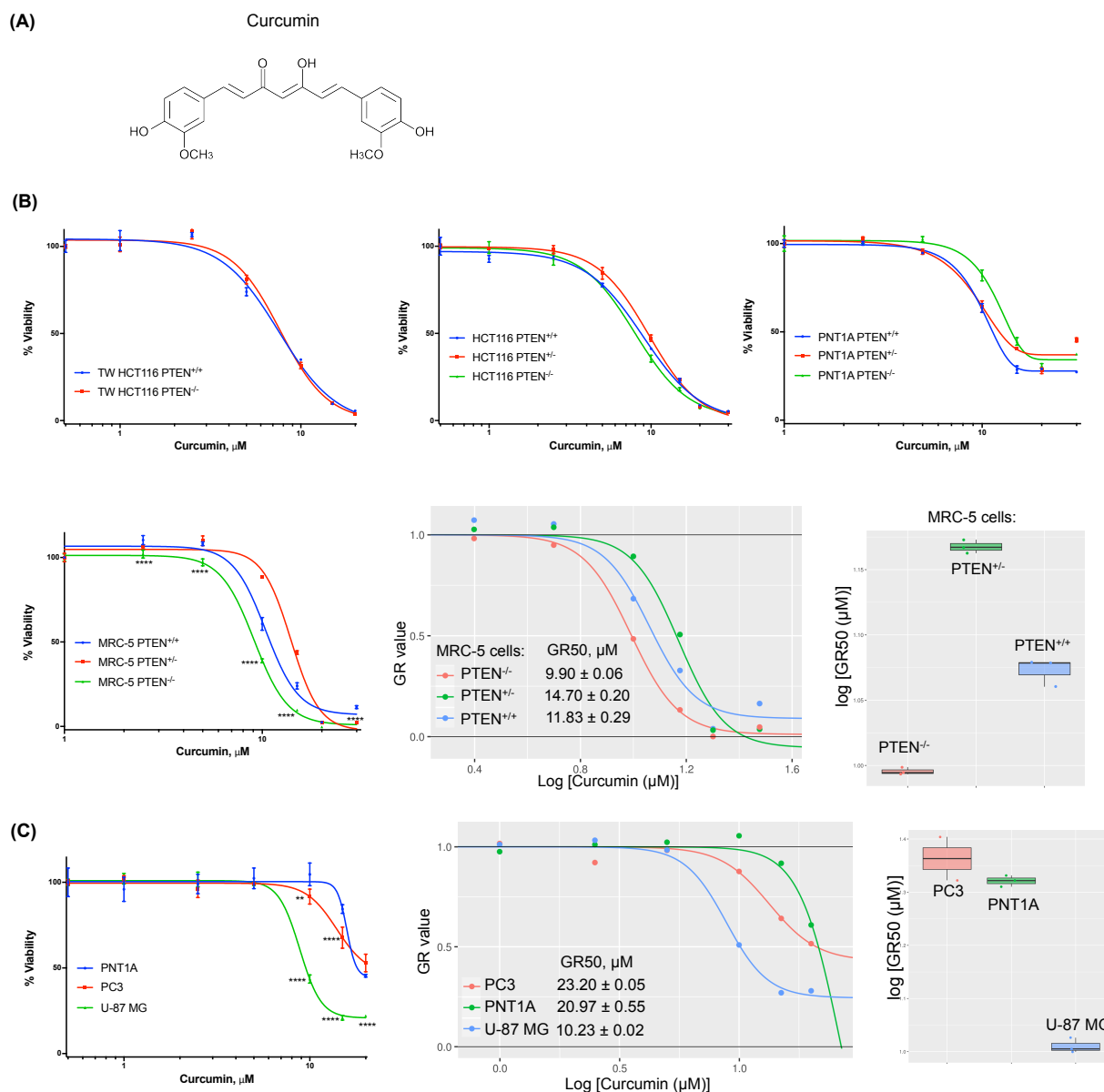


Figure 7.4. Sensitivity of PTEN deficient human cells to curcumin. **(A)** Chemical structure of curcumin. **(B)-(C)** Cell viability assay upon curcumin treatment. Cells with different PTEN status were plated into 96-well plates and treated with increasing concentrations of compound (1-30 μM) for three days: **(B)** PTEN^{+/+} and PTEN^{-/-} Todd Waldman's (TW) HCT116 cells (0.2×10^4 cells/well); and PTEN^{+/+}, PTEN^{+/-} and PTEN^{-/-} cells developed within this study: HCT116 (0.2×10^4 cells/well), MRC-5 (0.7×10^4 cells/well) and PNT1A (0.7×10^4 cells/well); **(C)** PTEN WT PNT1A cells (0.8×10^4 cells/well), PTEN deficient PC3 (0.25×10^4 cells/well) and U-87 MG (0.4×10^4 cells/well) cells. The cell viability was determined by MTS assay. Each experiment was performed at least two times in triplicates. Negative control cells were treated with 0.5% DMSO. Each value represents the mean $\pm$ SD of three repeats. A significant difference in cell survival was calculated and compared to the PTEN WT lines on survival curves (**** $p < 0.0001$, ** $p < 0.01$ vs PTEN^{+/+} cells, two-way ANOVA). GR data shown derived from a single representative data set of one of the three biological replicates. GR50 data are calculated and plotted as the mean from the three repeat experiments $\pm$ SD. GR metrics were calculated on <http://www.grcalculator.org>

Following the cell viability results, levels of several intracellular cell proliferation regulators were assessed in HCT116 cells to compare with the data in (Chen *et al.* 2013). According to their report, curcumin exposure reduced Akt phosphorylation and induced p21 expression in HCT116 cells with WT PTEN. Whereas, the opposite results were detected in HCT116 PTEN^{-/-} cells, which showed increased levels of P-Akt and reduced levels of p21. Additionally a decrease in Cyclin D1 expression was observed in PTEN^{-/-} cells with no change in WT PTEN cells upon curcumin exposure.

A similar analysis was performed with TW HCT116 cells that were incubated with curcumin (10 and 20 μ M) for 24 hours. After lysis and SDS-PAGE separation, the above proteins, as well as PTEN and PCNA (proliferating-cell marker accumulating in cell nuclei during S phase (Landberg, Roos 1991, Schönenberger *et al.* 2015)) were analysed via western blotting. Supporting the cell viability data, there was no difference in expression patterns between the two cell lines. However, the cell cycle regulators demonstrated different behaviours than that observed by Chen *et al.* Thus, P-Akt levels on both Ser and Thr sites as well as p21 expression were elevated in a dose-dependent manner regardless of PTEN status. On the contrary, Cyclin D1 expression was suppressed upon curcumin treatment in PTEN^{-/-} cells and potentially in WT PTEN cells (although different patterns were seen for 10 and 20 μ M treatments) (Figure 7.5).

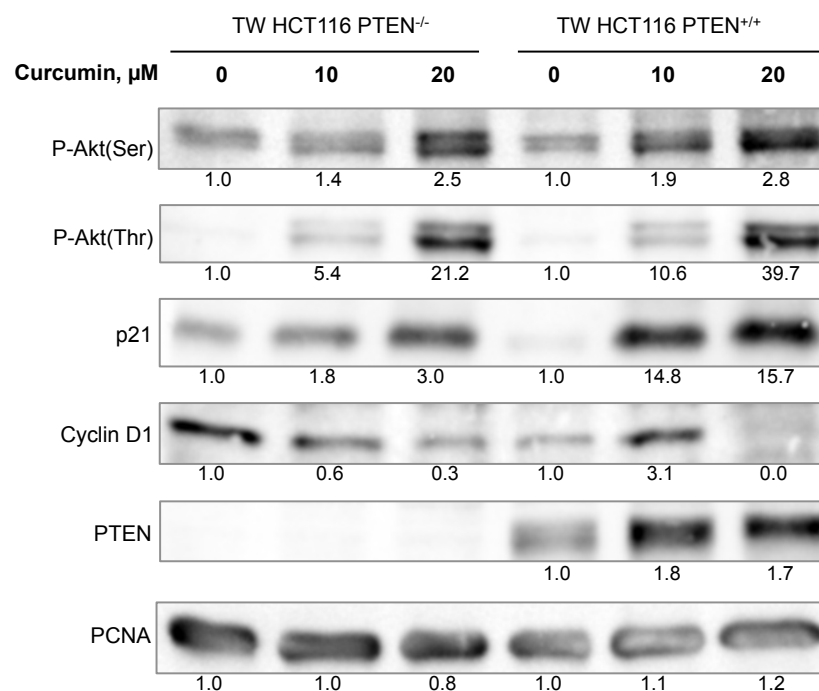


Figure 7.5. Curcumin effect on intracellular proliferation regulators. Todd Waldman's HCT116 cells (PTEN^{-/-} and PTEN^{+/+}) were treated with 10-20 μ M curcumin or 0.5% DMSO (control) for 24 hours then lysed with RIPA buffer. Total protein levels were calculated and loaded according to Bio-Rad DC protein assay, then analysed by western blotting for the indicated proteins. The numbers under the bands represent the ratio of protein expression with control

(DMSO-treated) samples arbitrary set as 1.0 for each cell line and protein determined by ImageStudioLite (LI-COR Biosciences).

Even though current P-Akt data do not correlate with the published results of Chen *et al.*, according to which Akt phosphorylation was down-regulated in WT HCT116 cells, they were in a good agreement with the data of Johnson *et al.* demonstrating dose-dependent accumulation of Akt(Ser) phosphorylation in WT HCT116 cells upon curcumin treatment (Johnson *et al.* 2009). These authors attributed this effect to a decrease in the levels of another phosphatase, PHLPP1, that is known to dephosphorylate Akt directly (Gao, Furnari & Newton 2005, Chen *et al.* 2011).

On the other hand, curcumin-induced increase in P-Akt levels in PTEN^{-/-} cells observed in current study and in Chen *et al.* work is contradictory to a wide range of reports, where curcumin caused inhibition of Akt phosphorylation in PTEN deficient cells, such as prostate cancer PC3 (Chaudhary, Hruska 2003, Yu *et al.* 2008) LnCap (Chaudhary, Hruska 2003) and PTEN-Cap8 (Narayanan *et al.* 2009), glioblastoma U-87 MG (Aoki *et al.* 2007), and breast cancer MDA-MB-468 (Yadav *et al.* 2012) cells.

Taken together, these results indicate that again, PTEN synthetic lethality with curcumin appears not to be reproducible and likely to be very cell line specific.

Interestingly, curcumin treatment led to elevation of PTEN levels in WT PTEN cells. This result correlated well with previous reports showing that curcumin and his analogues enhanced PTEN expression in colon cancer (Rana *et al.* 2015, Roy *et al.* 2013), neuroblastoma (Picone *et al.* 2014), pancreatic cancer (Bao *et al.* 2011) and chronic myelogenous leukemia cells (Taverna *et al.* 2015) possibly through regulation of microRNA-21 (miR-21), the upstream PTEN suppressor, as curcumin can decrease miR-21 levels via both inhibiting the transcription of the *miR-21* gene by binding to its promoter, and increasing miR-21 disposal through exosome exclusion from the cells (Chen, Xu & Chen 2015). Thus, curcumin treatment led to accumulation of both PTEN and P-Akt levels in WT PTEN HCT116 cells suggesting that curcumin interacted with multiple pathways in these cells.

In conclusion, curcumin-mediated alterations on tested cell-cycle regulators did not correlate with *PTEN* genotype in HCT116 cells. Curcumin-induced Akt phosphorylation, increased in p21 levels and suppressed Cyclin D1 expression, the latter two changes likely to underlie a G0/G1 cell cycle arrest leading to reduced cell numbers as revealed by the MTS assays. However, PCNA protein that is involved in DNA replication was not decreased after incubation with curcumin, indicating that there was no accumulation of cells in G0/G1 phase. More experiments would be beneficial to determine if curcumin activated either apoptotic or senescence responses in tested cells. However, this was not pursued as curcumin clearly did not cause significant lethal sensitivity in a PTEN-dependent manner.

7.5. Synthetic lethality to Skp2 inhibition

7.5.1. Introduction

A critical component of the Skp2-SCF complex, the Skp2 E3-ubiquitin ligase, has also been shown to exhibit lethal sensitivity with PTEN deficiency (Lin et al. 2010, Chan et al. 2013). The oncoprotein Skp2 is one of the four subunits of the Skp2-SCF ubiquitin E3 ligase complex. Skp2 was shown to be involved in regulation of apoptosis, proliferation and cell cycle progression by promoting the ubiquitination and degradation of some of its anti-proliferative substrates, p21 (Bornstein et al. 2003) and p27 (Tsvetkov et al. 1999, Nakayama et al. 2004). In a different role, the Skp2-SCF complex triggers Akt ubiquitinylation that activates the kinase activity of Akt leading to enhanced aerobic glycolysis as found in many cancer cells (Chan et al. 2012). Skp2 overexpression is often observed in human cancers (Hershko 2008) (such as breast (Signoretti et al. 2002, Wang et al. 2012a, Zhang et al. 2016), lung (Yokoi et al. 2004), prostate (Yang et al. 2002, Wang et al. 2012b), gastric carcinoma (Ma et al. 2005) and oral squamous cell carcinoma (Yamada et al. 2016) among others) and was found to promote cancer metastasis and invasion, while its deficiency inhibited this process (Chan et al. 2010). Moreover, ectopic expression of Skp2 promoted tumour growth in the LnCap prostate cancer xenograft model (Lin et al. 2009) and breast cancer cells (Zhang et al. 2016). Skp2 silencing was shown to suppress survival of human tumour cell lines including those from lung, liver, prostate and in osteocarcinoma and cancer stem cells (Yokoi et al. 2004, Chan et al. 2013). Thus, targeting Skp2 appears a promising strategy for cancer treatment (Wang et al. 2012a, Wang et al. 2012b).

Lin *et al.* reported that Skp2 inactivation restricts tumourigenesis by triggering cellular senescence only in oncogenic conditions and demonstrated that cancers with impaired tumour suppressor responses, for instance disrupted PTEN or p53, could potentially benefit from Skp2 inhibition (Lin et al. 2010). The authors observed a slower growth rate and massive senescence response (measured by SA- β -Gal staining) in PTEN^{+/-}Skp2^{-/-} MEFs but not in individual mutants. Notably, cellular senescence was triggered by a p53-independent pathway and could be compromised by knocking-down of Skp2 substrates, p21 or p27. The senescence response was also observed *in vivo* in prostate-specific PTEN^{pc/-}Skp2^{-/-} mice.⁺⁺⁺⁺⁺ Skp2 deficiency significantly reduced cellular proliferation and enhanced senescence in mouse prostates upon PTEN inactivation. This response was also detectable over time, whereas SA- β -Gal staining was barely visible in late age Skp2^{-/-} or PTEN^{pc/-} mice. Skp2 loss also profoundly reduced tumour masses in PTEN^{pc/-}Skp2^{-/-} mice compared to PTEN^{pc/-} mice. In addition, Skp2

⁺⁺⁺⁺⁺ PTEN^{pc/-} is transgenic mice that has prostate-specific PTEN knock-out via Cre recombinase expressed in the prostate epithelium after puberty. This genotype causes prostate cancer (pc) in mice.

silencing with shRNA caused a senescence response in PTEN null PC3 and p53 null DU145 cells. Supporting this, inhibition of another component of Skp2-SCF complex, Cullin 1, with small molecule MLN4924 (Soucy et al. 2009) triggered cellular senescence in PC3, DU145 cells and suppressed tumour growth in PC3 xenograft nude mice models.

Later, Chan *et al.* confirmed these findings with the newly developed Skp2 inhibitor, Skp2-25 (also known as Compound #25 or SZL-P1-41). In their study, the authors performed structure-based high-throughput *in silico* screening and identified and characterised a specific Skp2 small molecule inhibitor (Chan et al. 2013). Skp2-25 was shown to bind to Skp2 directly and preventing it from interacting with another important subunit of Skp2-SCF complex, Skp1, thus inhibiting Skp2-SCF ligase activity. As such, Skp2 inhibition with Skp2-25 caused accumulation of Skp2 degradation targets, p21 and p27, *in vitro* and *in vivo* and inhibited Skp2-mediated Akt phosphorylation. Further, the authors showed that Skp2-25 triggered elevated levels of cytotoxicity, cellular senescence, apoptosis and G2/M phase cell-cycle arrest in PTEN deficient prostate cancer PC3 and LnCap cells when compared to PTEN WT prostate PNT1A cells. Interestingly, the effect of Skp2-25 on cell viability was diminished upon Skp2 knock-down with two independent shRNAs or upon stable ectopic expression of two Skp2 mutants that were resistant to the binding of the inhibitor, indicating that Skp2-25 action was through Skp2 specific inhibition.

In vivo, Skp2-25 suppressed tumour growth in a dose-dependent fashion in a PC3 xenograft mouse model. Additionally, Skp2 inhibition with Skp2-25 was shown to further attenuate survival and sensitise PC3 cells to other chemotherapeutic agents, such as cyclophosphamide and doxorubicin (Chan et al. 2013). Other authors also demonstrated that the combination of doxorubicin with Skp2 knock-down in PC3 cells induced cellular senescence and growth arrest (Lin et al. 2010).

A potential explanation for the lethal sensitivity of PTEN disrupted cells towards Skp2 inhibition, could be due to the fact that PTEN deficiency may cause a decrease of p27 levels with concomitant increase of Skp2 levels as was demonstrated in mouse embryonic cells (Mamillapalli et al. 2001), human kidney adenocarcinoma cells (Shanmugasundaram et al. 2013), human gastric carcinoma (Ma et al. 2005), prostate and colon cancer samples (Yang et al. 2002, van Duijn, Trapman 2006, Lin et al. 2009). Moreover, silencing of PTEN with siRNA in prostate DU145 cells showed PTEN/Akt-dependent up-regulation of Skp2 and down-regulation of p27; while genetic inhibition of both PTEN and Skp2 elevated p27 levels (van Duijn, Trapman 2006). Conversely, ectopic expression of WT PTEN (but not C124S or G129E mutants) in PTEN null U-87 MG cells was shown to cause p27 accumulation accompanied by Skp2 reduction, while ectopic expression of Skp2 was sufficient to reverse PTEN-mediated p27 accumulation (Mamillapalli et al. 2001). On the other hand, Akt was shown to phosphorylate Skp2 directly and thus trigger Skp2-SCF complex formation (Lin et al. 2009) and control Skp2

stability by disrupting its interaction with the APC-Cdh1 ubiquitin ligase complex that is involved in Skp2 degradation (Gao et al. 2009). These findings indicate that PTEN could act as a negative regulator of Skp2-SCF pathway (Liang, Slingerland 2003, Ma et al. 2005). The direct correlation between PTEN and Skp2/p27 expression was not confirmed in some cell lines (for instance, it was not found in prostate PTEN null LnCap and PC346 cells (van Duijn, Trapman 2006)). Despite this heterogeneity of the effect of PTEN disruption on Skp2/p27, the synthetic lethality displayed may be therapeutically useful, causing cellular senescence through p27/p21 accumulation.

Collectively, these studies suggest that pharmacological inhibition of Skp2 on its own or in cooperation with chemotherapy could be particularly effective as a potential pro-senescence therapy in PTEN deficient cancers (Chan et al. 2014). These observations prompted the testing of the Skp2 inhibitor Skp2-25 on cells with different *PTEN* genotype and mutations developed within this project.

7.5.2. Results

Initially, Skp2 inhibition with Skp2-25 (Figure 7.6(A)) was tested on cancer cells with PTEN loss similar to the experiments of Chan *et al.* (Chan et al. 2013). Non-tumourigenic PNT1A cells with WT PTEN and PTEN null PC3 and U-87 MG cells were incubated with increasing concentrations of Skp2-25 for six days with the compound being refreshed on day three. At least two independent experiments were performed for all cell lines and each time was done in triplicates. As expected, both PTEN deficient cell lines were more sensitive to Skp2 inhibition (Figure 7.6(B)).

Next, the inhibitory effect of Skp2-25 on cell proliferation of isogenic cell lines with various *PTEN* genotypes was assessed. PTEN^{-/-} cells exhibited significantly higher cytotoxicity from the compound than PTEN^{+/+} in all four cell lines tested with PNT1A cells giving the weakest difference (Figure 7.6(C)). Interestingly, the cell viability of HCT116 PTEN^{+/-} cells developed within this study and to a smaller extent in PNT1A PTEN^{+/-} cells was reduced by Skp2 inhibition similar to PTEN^{-/-} cells, whereas in other lines proliferation of PTEN^{+/-} cells was comparable to WT PTEN cells as found by Lin et al in PTEN^{+/-}Skp2^{-/-} MEFs (Lin et al. 2010). Thus, the results found here largely agree with the published data indicating that complete PTEN loss is more favourable for sensitivity to Skp2 inhibition and demonstrate that in some cellular settings PTEN heterozygosity can lead to enhanced sensitivity to Skp2 inhibition.

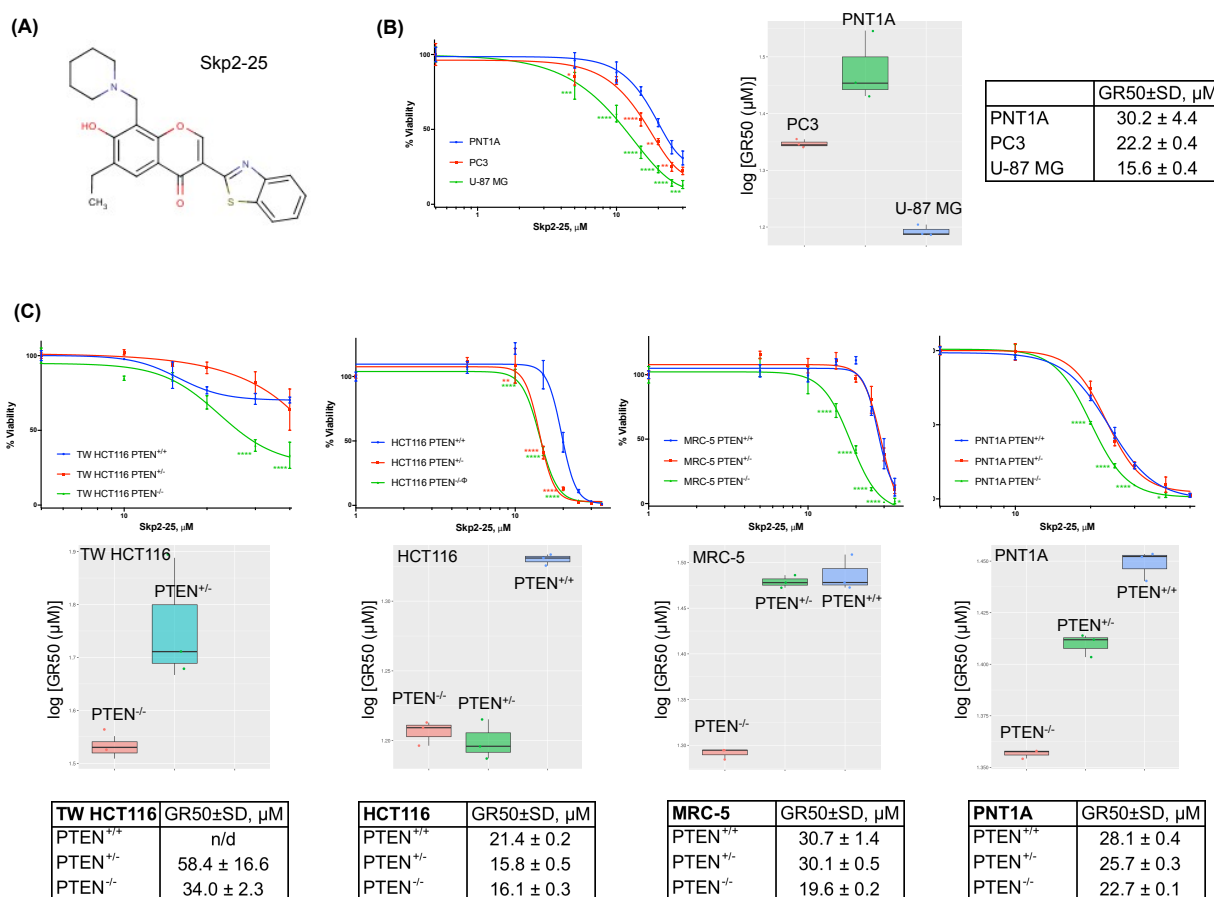


Figure 7.6. Sensitivity of PTEN deficient human cells to Skp2 inhibition with Skp2-25. **(A)** Chemical structure of Skp2-25. **(B)-(D)** Cell viability assay upon treatment with Skp2-25. Cells with different PTEN status were plated into 96-well plates and treated with increasing concentrations of Skp2-25 (0.5-50 μM) for six days. The compound and media were renewed on day three. **(B)** PTEN WT PNT1A cells (0.3×10^4 cells/well); and PTEN deficient PC3 (0.17×10^4 cells/well) and U-87 MG (0.05×10^4 cells/well) cells. **(C)** Isogenic cell lines: PTEN^{+/+}, PTEN^{+/-} and PTEN^{-/-} Todd Waldman's (TW) HCT116 cells (0.015×10^4 cells/well); and cells developed within this study: HCT116 (0.05×10^4 cells/well), MRC-5 (0.08×10^4 cells/well) and PNT1A (0.35×10^4 cells/well). The cell viability was determined by MTS assay. Each experiment was performed at least two times in triplicates. Control cells were treated with 1% DMSO. Each value represents the mean $\pm$ SD out of three independent experiments. Significant difference in cell viability was calculated and compared to the PTEN^{+/+} lines on survival curves (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.1$ vs PTEN^{+/+} cells, two-way ANOVA). GR50 data are calculated for each cell line and plotted as mean from three experiments $\pm$ SD. GR metrics was computed on <http://www.grcalculator.org>. PTEN^{- Φ} cells represent HCT116 PTEN^{-/-} Clone Φ .

Given the differences in sensitivity of PTEN^{+/-} cells to Skp2 inhibition, it was of interest to test if additional inhibition of PTEN in PTEN^{+/-} lines would sensitise these cells to Skp2 inhibition similar to PTEN^{-/-} cells. For this, PTEN^{+/-} HCT116, MRC-5 and PNT1A cells were co-treated with 500 nM of PTEN inhibitor VO-OHpic and increasing concentrations of Skp2-25. However, VO-OHpic inhibition had no effect on viability of Skp2-25 treated PTEN^{+/-} cells (Figure 7.7). Therefore, further inhibition of PTEN with VO-OHpic would not be beneficial for increasing lethal sensitivity of cells with partial PTEN activity towards Skp2 inhibition.

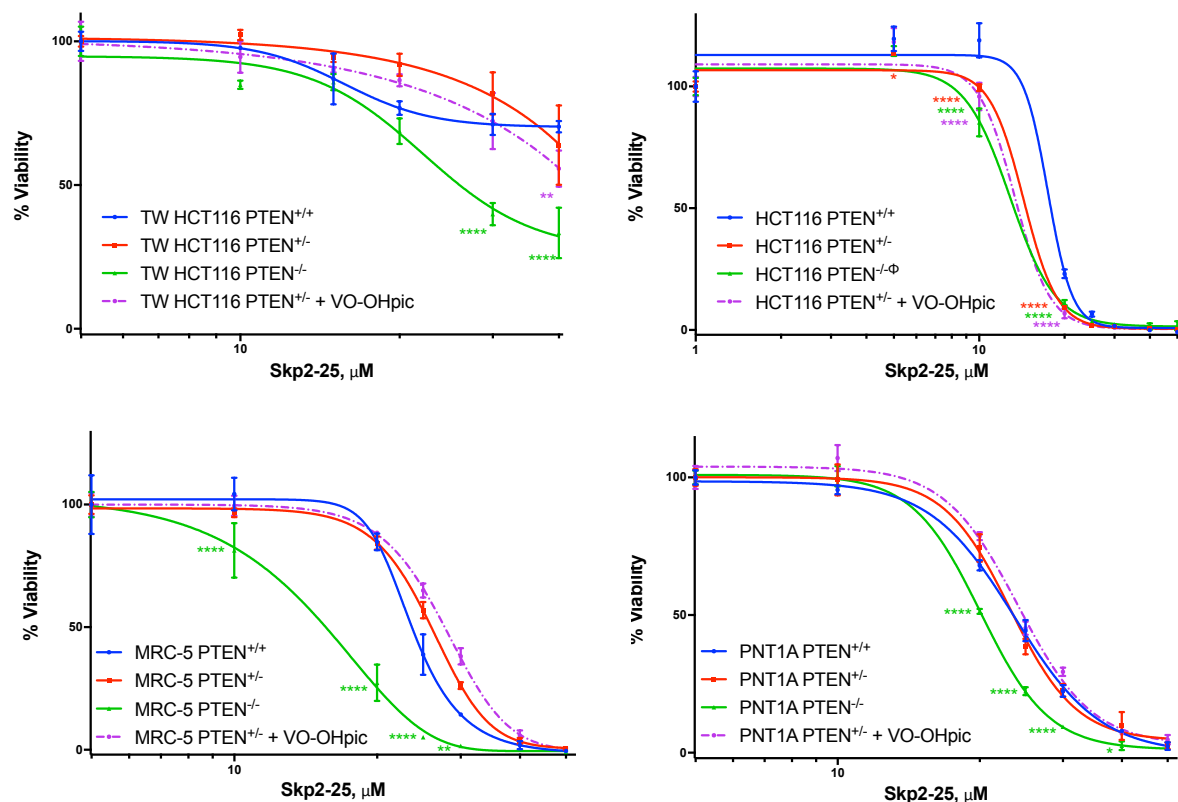


Figure 7.7. Effect of combining PTEN inhibition with Skp2 inhibition in PTEN^{+/-} cells. Isogenic cell lines with different PTEN status were plated onto 96-well plates and incubated with 1-50 μ M Skp2-25 for six days: TW HCT116 cells (0.015×10^4 cells/well); and cells developed within this study: HCT116 (0.055×10^4 cells/well), MRC-5 (0.07×10^4 cells/well) and PNT1A (0.35×10^4 cells/well). PTEN^{+/-} cells were co-treated with 0 or 500 nM of VO-OHpic. Media and compounds were changed after three days. Mock-treated control cells were incubated with 1% DMSO. The cell viability was determined by MTS assay. Each value represents the mean $\pm$ SD out of three independent replicates. Significant difference in cell viability was calculated and compared to the PTEN^{+/+} lines on survival curves (**** $p < 0.0001$, ** $p < 0.01$, * $p < 0.1$ vs PTEN^{+/+} cells, two-way ANOVA). PTEN^{-/- Φ} cells represent HCT116 PTEN^{-/-} Clone Φ .

Similar to previous findings with other lethal sensitivity compounds, HCT116 cells developed within this project and in the Todd Waldman (TW) laboratory, showed distinct behaviour upon Skp2 inhibition (Figure 7.7). Although Skp2-25 cytotoxicity in PTEN^{-/-} cells in both lines was stronger than on PTEN^{+/+} cells, the magnitude of proliferation inhibition differed almost two-fold (see GR50 values on Figure 7.6(C)). One possible explanation for such a difference could be increased resistance of TW isogenic cells to cytotoxic agents because of potential higher rate of pro-survival mutations due to PTEN disruption as discussed earlier. Such mutations could be accumulated over passaging and thus dominate more in older generation of TW cells (the passage number of the TW cells was not known when they were donated to us). In order to evaluate this hypothesis, early (P10-12) and late (P25-26) passage HCT116 cells with different PTEN statuses generated in this study were incubated with Skp2-25 for six days. Surprisingly, the toxic effect of Skp2-25 was compromised in PTEN^{-/-} and PTEN^{+/-} cells of a later passaging

(Figure 7.8). This result potentially supports the above hypothesis regarding increased sensitivity towards anti-proliferation treatment in TW HCT116 cells compared to their younger analogues. Therefore all further experiments were performed only with early passages of cells to avoid accumulation of extra mutations.

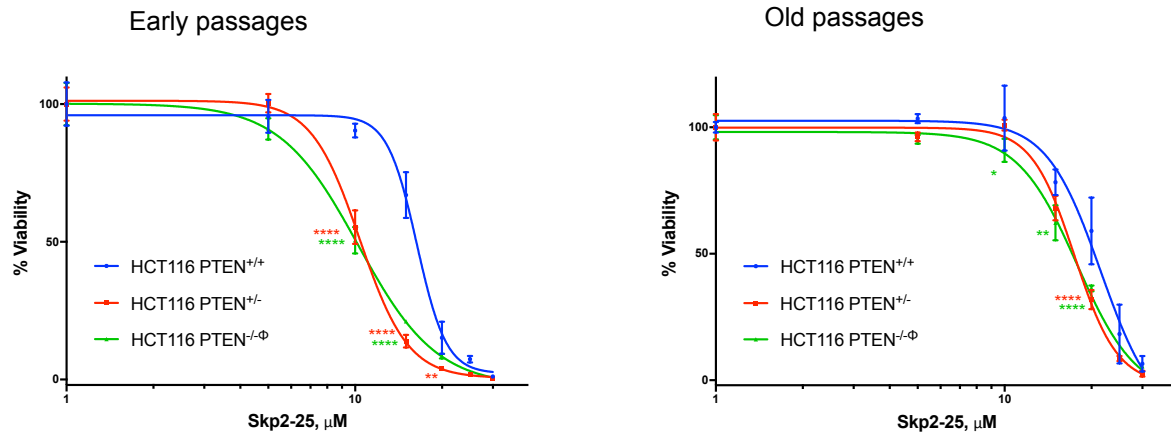


Figure 7.8. Effect of population aging on Skp2 inhibition in HCT116 cells. HCT116 cells from early (P10-12) and late (P25-26) passages (0.055×10^4 cells/well) were incubated with Skp2-25 and analysed via MTS assay. Significant differences in cell survival were compared to the $PTEN^{+/+}$ lines on survival curves (**** $p < 0.0001$, ** $p < 0.01$, * $p < 0.1$ vs $PTEN^{+/+}$ cells, two-way ANOVA). $PTEN^{-/-\Phi}$ cells represent HCT116 $PTEN^{-/-}$ Clone Φ .

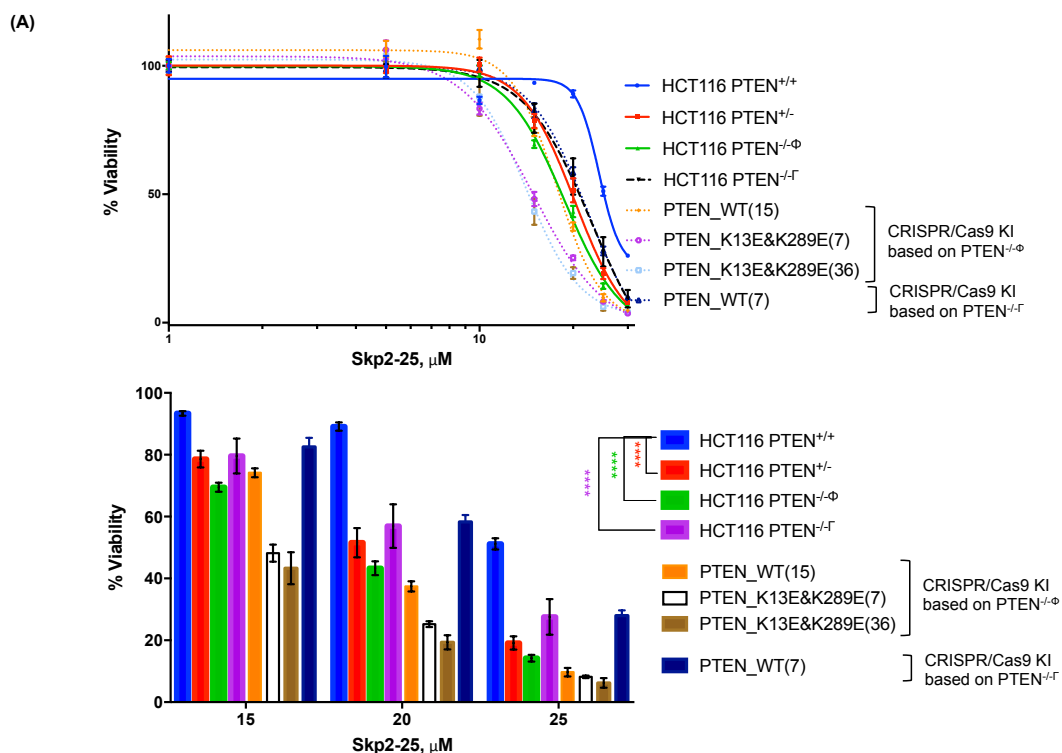
Having established the lethal sensitivity between $PTEN^{-/-}$ cells and Skp2 inhibition, it was of interest to test the PTEN knock-in cell lines and assess the effects of PTEN mutations on this sensitivity. Thus, CRISPR-Cas9 and Sleeping Beauty (SB) knock-ins were subject to Skp2-25 treatment alongside their parental lines. Introduction of a single WT or mutant PTEN copy ($PTEN_{G129E}$, $PTEN_{C124S}$, $PTEN_{H93A}$, $PTEN_{V403A}$ or $PTEN_{K13E\&K289E}$) via CRISPR-Cas9 did not alter sensitivity to Skp2 inhibition in HCT116 $PTEN^{-/-}$ cells (Figure 7.9(A)). This may be due to the fact that the cytotoxicity effect from Skp2 inhibition was similar in the $PTEN^{-/-}$ and $PTEN^{+/-}$ HCT116 cells generated in this study, therefore $PTEN^{WT/-}$ and $PTEN^{mut/-}$ HCT116 cells are expected to proliferate similar upon Skp2-25 treatment. More precisely, $PTEN^{-/-}$ and $PTEN^{+/-}$ HCT116 cells from this work were both sensitive to Skp2 inhibition so putting one copy of PTEN (WT or mutant) back in to null cells essentially recapitulated the $PTEN^{+/-}$ state that was still sensitive to Skp2-25. Also it did not matter whether or not the PTEN was functional. This result indicates that PTEN expression levels in CRISPR/Cas9 knock-in lines were not massively over-expressed as if they were, cells would recapitulate the $PTEN^{+/+}$ state and lose their sensitivity, which they did not.

Interestingly, two PTEN KO based on HCT116 cells exhibited slightly different sensitivity to Skp2 inhibition. As such, cell growth was inhibited in a greater degree in $PTEN^{-/-\Phi}$ cells than in $PTEN^{-/-\Gamma}$. This finding indicates that all individual KO and knock-in clones may have different genotype and phenotype due to either original or acquired heterogeneity in the HCT116 cell

line, which potentially effects all phenotypic studies even in what are believed to be isogenic lines.

Next, SB PTEN knock-in cells that express higher protein levels than PTEN^{+/-} cells (see Chapter 5.4.3) were analysed upon Skp2 inhibition. SB clones with PTEN_WT, PTEN_H93A (lacking near 75% of PTEN lipid phosphatase activity) and PTEN_K13E&K289E (lacking PTEN nuclear localisation) demonstrated significant rescue of Skp2-25 sensitivity at least at one chosen Skp2-25 concentration (Figure 7.9(B)). However, PTEN mutants lacking lipid phosphatase activity (PTEN_G129E) and lipid/protein phosphatase activity (PTEN_C124S), as well as the mutant with the disrupted PDZ domain and potentially decreased PTEN stability (PTEN_V403A) demonstrated the same behaviour as PTEN^{-/-} cells. These results suggest that PTEN lipid activity and stability but not nuclear localisation are at least partially responsible for resistance to Skp2 inhibition in HCT116 cells.

Interestingly, none of SB PTEN knock-in pools rescued sensitivity to Skp2 inhibition (Figure 7.9(C)), indicating that heterogeneity of these cells could affect the PTEN effect on sensitivity to Skp2 inhibition even though total levels of the protein expression were higher than in PTEN^{+/-} cells.



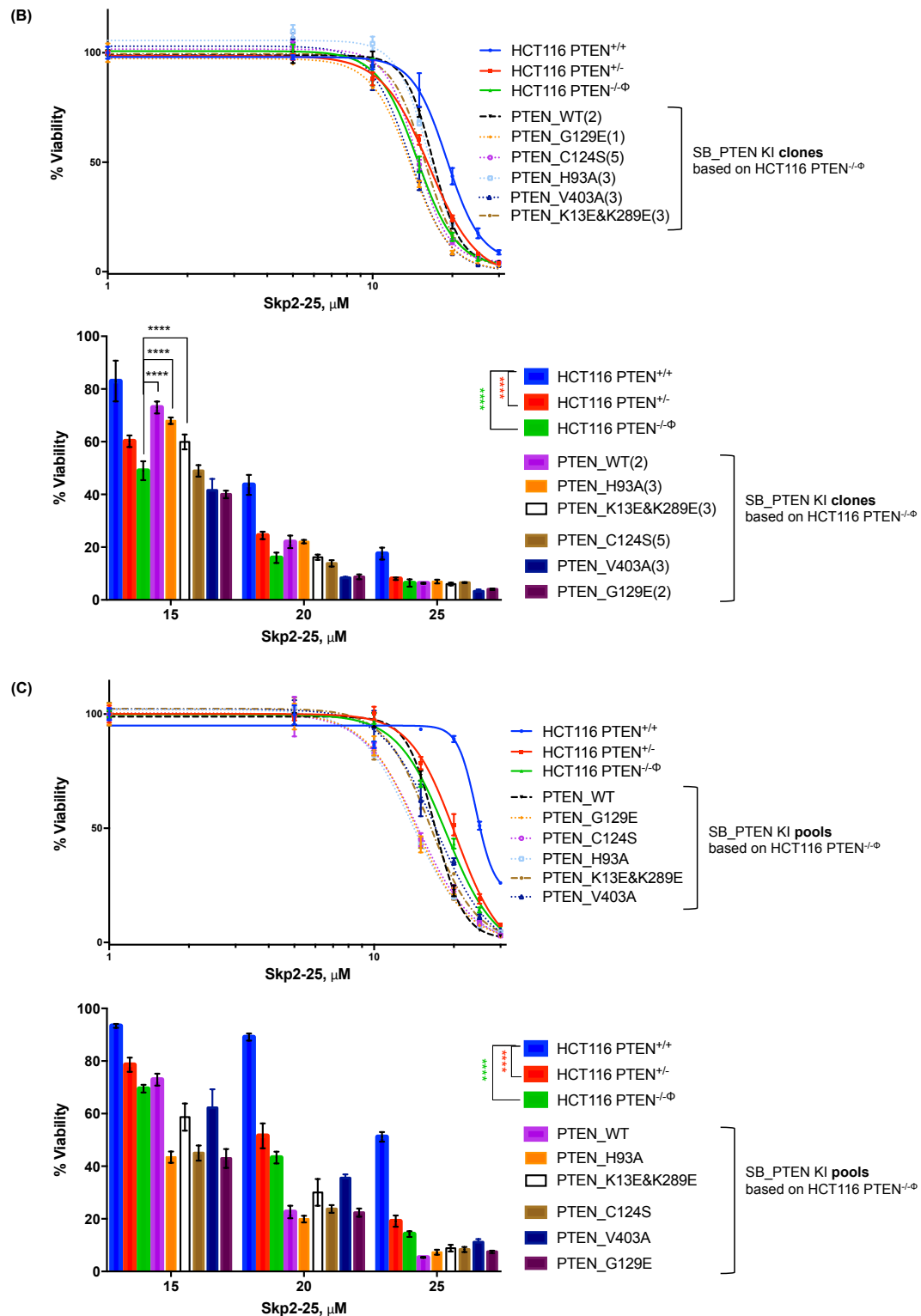


Figure 7.9. PTEN structural requirements for sensitivity to Skp2 inhibition in HCT116 cells. PTEN^{WT} or PTEN^{mutant} knock-in cells derived from HCT116 PTEN^{-/-} cells alongside PTEN^{+/+}, PTEN^{+/-} and PTEN^{-/-} cells (0.08×10^4 cells/well) were treated with increasing concentrations of Skp2-25 and analysed with MTS assay as described above: **(A)** CRISPR/Cas9-based knock-in clones; **(B)** Sleeping Beauty (SB)-based knock-in clones and **(C)** pools. Bar plot (bottom panel) shows representative points from the survival curves (top panel). Each value represents the mean $\pm$ SD out of three independent replicates. Significant differences in cell survival were compared to the PTEN^{+/+} lines (and PTEN^{-/-} lines on **(B)**) (****p

< 0.0001, ***p < 0.001, two-way ANOVA). Clone numbers are indicated in brackets. PTEN^{-/-Φ} and PTEN^{-/-Γ} cells represent HCT116 PTEN^{-/-} Clone Φ and Clone Γ respectively.

Surprisingly, SB knock-in clones derived from PTEN^{-/-} MRC-5 cells demonstrated no differential cytotoxicity towards Skp2-25 compared to the original PTEN^{-/-} cells, suggesting that PTEN levels expressed in this cell line were not sufficient to rescue sensitivity to Skp2-25.

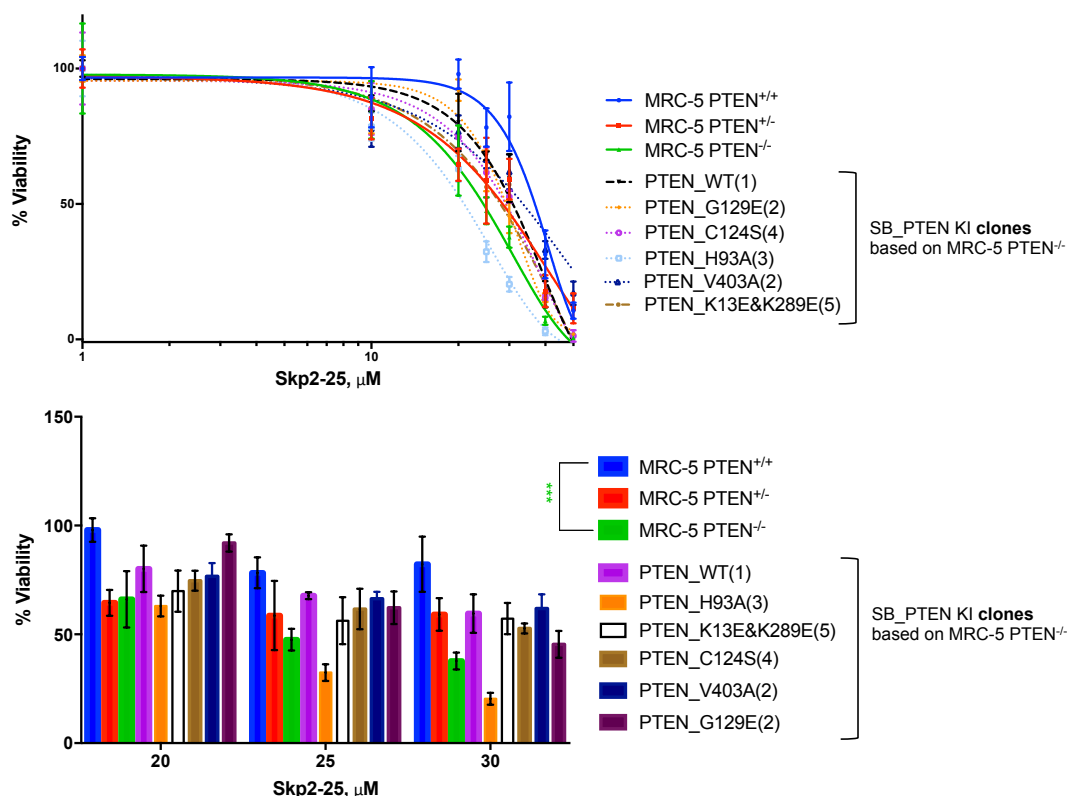
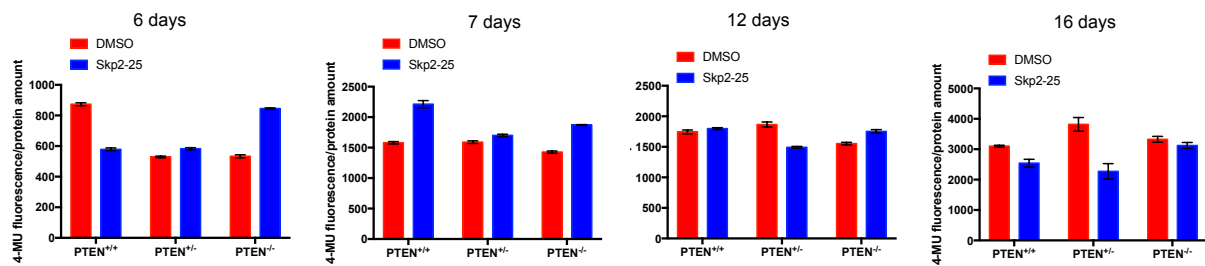


Figure 7.10. PTEN structural requirements for sensitivity to Skp2 inhibition in MRC-5 cells. PTEN_WT or PTEN_mutant Sleeping Beauty (SB) knock-in clones derived from MRC-5 PTEN^{-/-} cells alongside PTEN^{+/+}, PTEN^{+/-} and PTEN^{-/-} cells (0.09x10⁴ cells/well) were treated with increasing concentrations of Skp2-25 and analysed with MTS assay as described above. Bar plot (bottom panel) shows representative points from the survival curves (top panel). Each value represents the mean ± SD out of three independent experiments. Significant difference in cell survival was compared to the PTEN^{+/+} lines (***p < 0.001 vs PTEN^{+/+} cells, two-way ANOVA).

The mechanism of sensitisation to Skp2 inhibition in PTEN deficient and mutant cells was assessed by determining if the reduced viability was actually a reflection of senescence onset. Even though Chan *et al.* assessed senescent cells through measuring SA-β-Gal activity by X-Gal staining, the protocol based on SA-β-Gal activity detection by measuring MUG hydrolysis into the fluorescent product, 4-MU, was used within this study as described in Chapter 6.2. Initially, SA-β-Gal activity was assessed in HCT116 and MRC-5 cells with different PTEN statuses after their incubation with 20 μM of Skp-25 for 6-16 days. Representative examples of resulting plots are shown on Figure 7.11.

(A) HCT116



(B) MRC-5

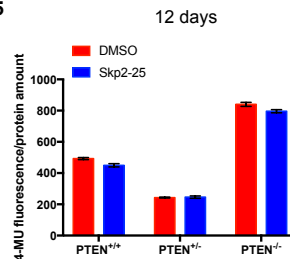


Figure 7.11. SA-β-Gal activity assessment in Skp2-25 treated cells. **(A)** HCT116 and **(B)** MRC-5 cells with different PTEN status were incubated with 1% DMSO or 20 μM Skp2-25 for 6-16 days, then lysed and stained with SA-β-Gal substrate, MUG (0.85 μM). Fluorescence intensity from hydrolysed product, 4-MU, was measured in a 96-well plate and normalised by the total protein levels. MUG solution without cells was used as a background control. Data are reported as mean ± SD of three independent experiments.

After measuring SA-β-Gal activity at different time points, it was concluded that the amount of SA-β-Gal activity was not consistently different in any genetic setting, indicating that senescent cells accumulated over the time of the assay regardless of Skp2-25 treatment or PTEN status. A similar trend was observed in PTEN knock-in clones (Figure 7.12).

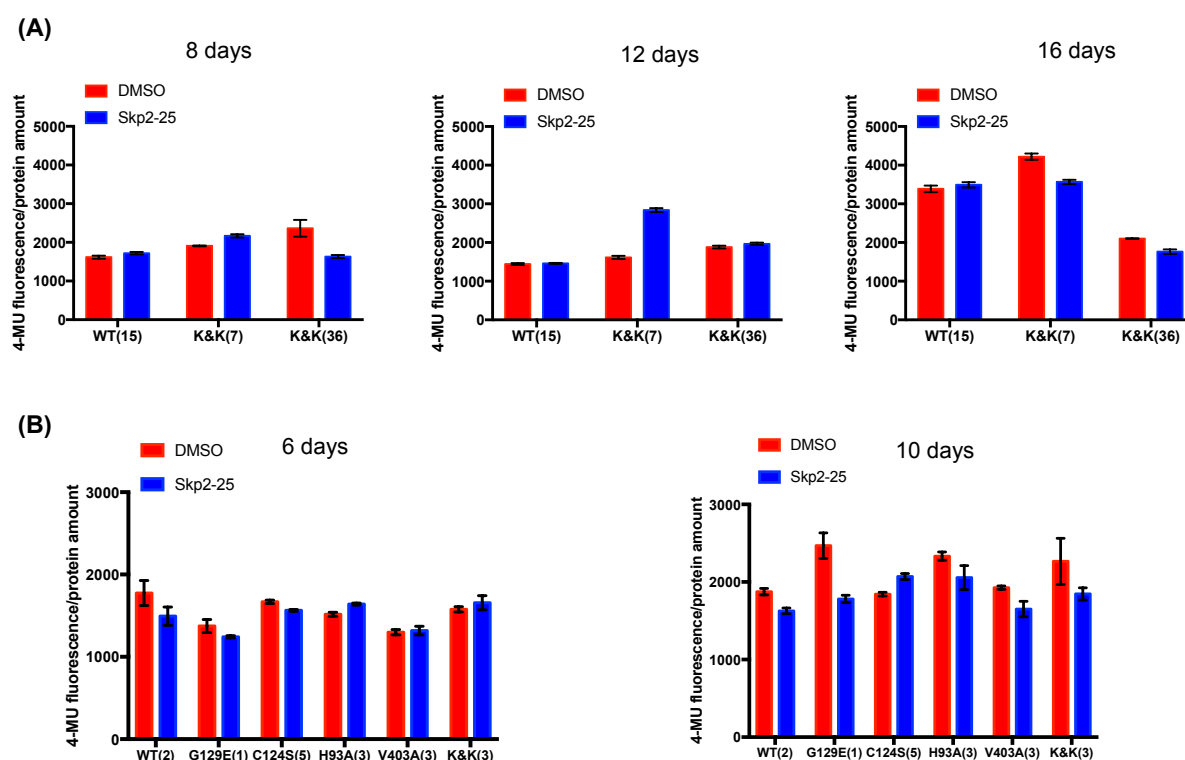


Figure 7.12. Effect of PTEN mutations on SA- β -Gal activity in Skp2-25 treated HCT116 cells. PTEN_WT or PTEN_mut **(A)** CRISPR/Cas9 knock-in clones and **(B)** Sleeping Beauty (SB) knock-in clones based on HCT116 PTEN^{-/-} cells were incubated with 1% DMSO or 20 μ M Skp2-25 for 6-16 days, then lysed and stained with MUG (0.85 μ M). 4-MU fluorescence intensity was measured in a 96-well plate and normalised by the total protein levels. MUG solution without cells was used as a background control. Data represent the mean values $\pm$ SD of three independent experiments. Clone numbers are shown in brackets. K&K – K13E&K289E.

This result did not correlate with published data (Lin et al. 2010, Chan et al. 2013), which showed accumulating X-Gal positive cells in PTEN deficient PC3 and MEFs upon Skp2 inhibition either by Skp2-25 or shRNA. As described earlier in Chapter 6.3 SA- β -Gal activity is not always associated with senescence and alternative markers of senescence were assayed. One such marker is accumulation of hypophosphorylated retinoblastoma protein which suppresses cell growth, DNA replication and induces senescence (Dyson 1998, Chicas et al. 2010). Similar, a decline in expression of the cell proliferation nuclear marker, PCNA (Landberg, Roos 1991, Schönenberger et al. 2015) should be detected in non-proliferating cells. p53 levels could be increased in cellular senescence if it is triggered via p53 pathway (Chen et al. 2005), however Skp2 inhibition was shown to cause senescence through a p27/p21-dependent pathway causing no alterations in p53 expression (Lin et al. 2010, Chan et al. 2013). In contrast, p21 and p27 levels are expected to be elevated in senescent cells and upon Skp2 inhibition in particular as p21 and p27 are downstream substrates of Skp2 (Tsvetkov et al. 1999, Nakayama et al. 2004, Bornstein et al. 2003).

In order to detect changes in these protein patterns, immunoblotting assays were performed with PTEN^{+/+} and PTEN^{-/-} HCT116 or MRC-5 cells treated with 20 μ M of Skp2-25 for 7-16 days. Unfortunately, no trends were detected in any of these markers (Figure 7.13).

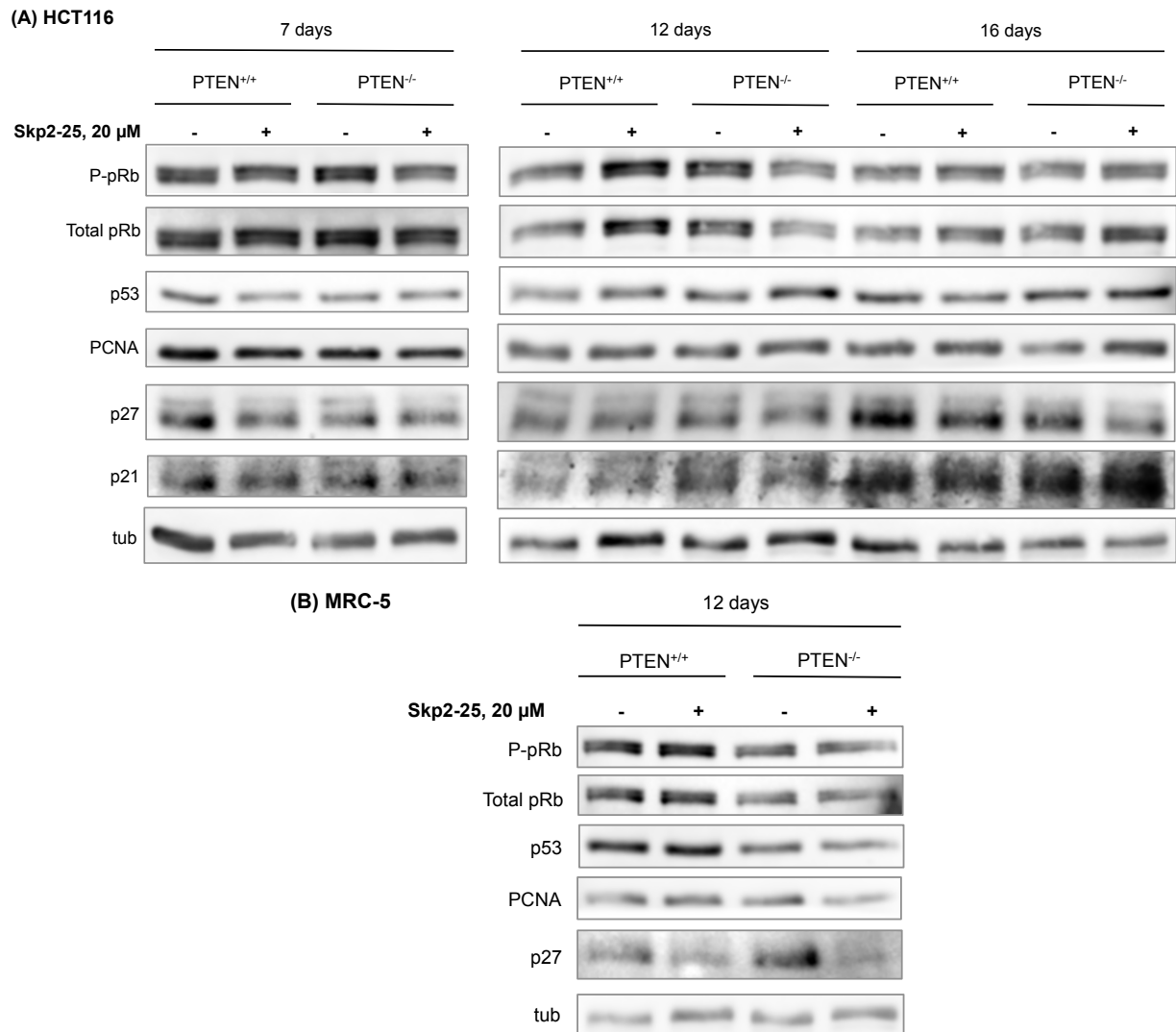


Figure 7.13. Skp2-25 effect on intracellular proliferation regulators. PTEN^{+/+} and PTEN^{-/-} **(A)** HCT116 and **(B)** MRC-5 cells were treated with 20 μ M of Skp2-25 or 1% DMSO (control) for 7-16 days then lysed with RIPA buffer and subject to western blotting for the indicated proteins. Tubulin was blotted as a loading control.

Further, the immunoblotting technique and antibodies were verified on a positive example of senescence described earlier in Chapter 6.2. It was demonstrated there that Pyridostatin treated A549 cells accumulate SA- β -Gal-positive cells over time compared to mock-treated cells. Therefore, these cells were tested for the same intracellular markers as Skp2-25 treated cells. Interestingly, all the markers underwent alterations in expression levels as expected upon entering the senescence state (Figure 7.14).

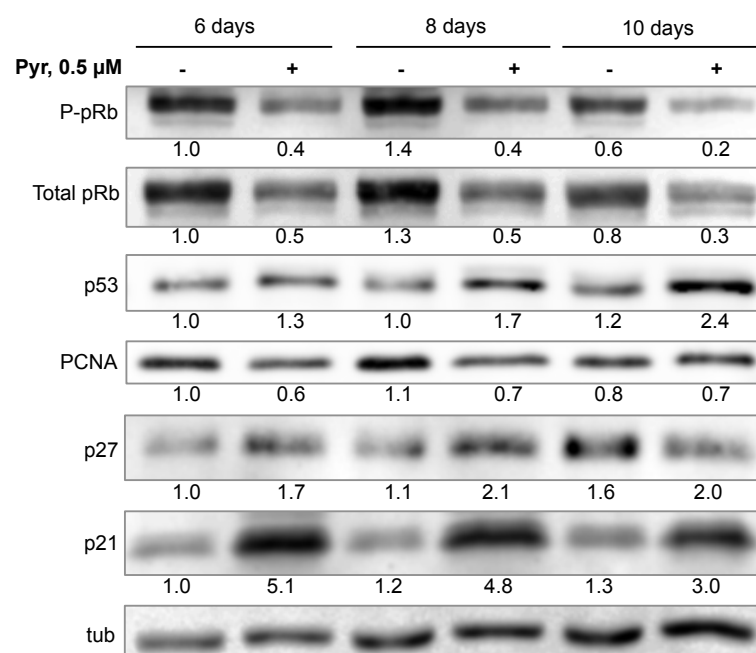


Figure 7.14. Effects of pyridostatin on intracellular proliferation regulators upon senescence induction. A549 cells were treated with 0.5 μ M of pyridostatin (Pyr) or 0.5% DMSO (control) for 6-10 days. Senescence induction in pyridostatin treated cells was confirmed via MUG assay (Figure 6.5(A) in Chapter 6.2.3) then samples were lysed with RIPA buffer and subject to western blotting for the indicated proteins. Tubulin was blotted as a loading control. The numbers under the bands represent the ratio of protein expression with control (DMSO-treated for 6 days) samples arbitrary set as 1.0 for each protein and determined by ImageStudioLite (LI-COR Biosciences).

These results validated the immunoblotting assay. However, the absence of the senescence response was accompanied with a lack of changes in expression of Skp2 substrates, p27 and p21, which would be predicted to increase upon Skp2 inhibition. Thus, it became questionable if Skp2-25 was indeed inhibiting Skp2. Potentially this could be explained by the degradation or wrong composition of the inhibitor. In order to check that Skp2-25 composition was correct, the stock solution of the compound was analysed by ESI⁺-MS. The chromatogram displayed clear and sharp single peak of expected mass (ESI⁺-MS (m/z) for [M+H]⁺ = 421, Figure 7.15).

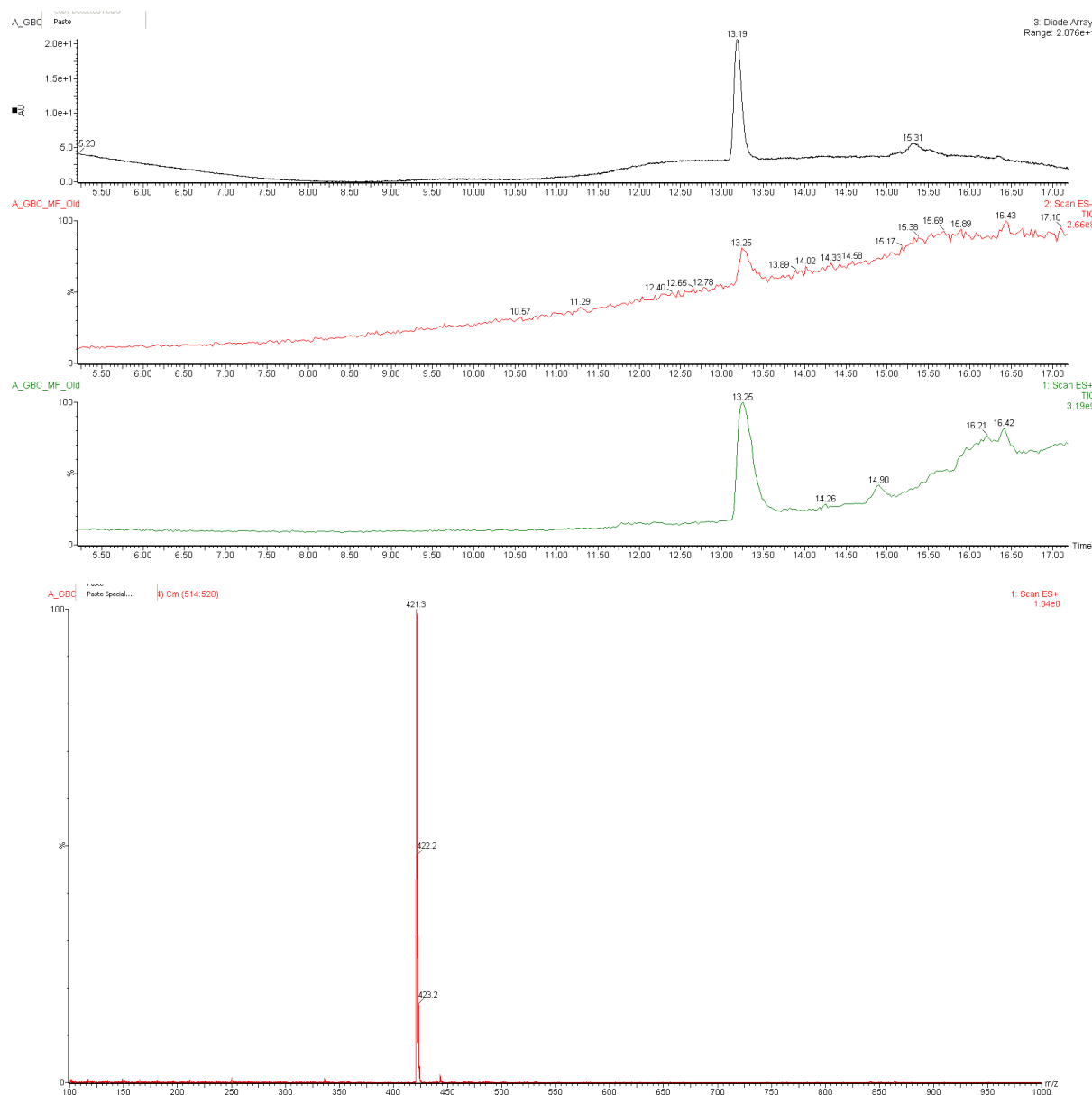


Figure 7.15. LC-MS chromatogram of Skp2-25 stock solution (10 mM in DMSO) (top panel) with ESI⁺-MS spectrum of the representative 13.19 peak (bottom panel). Mass spectrum peak at m/z 421 is due to the Skp2-25 [M+H]⁺.

This chromatogram confirmed that there were no signs of degradation of the compound. However, its activity could potentially be lowered due to low solubility. Indeed, some undissolved residues were present in all batches of Skp2-25 stock solution in DMSO (10 mM) obtained from two different companies (ChemBridge and Xcess Biosciences Inc.) even after sonication and heating steps. Additional precipitation and crystal formation of Skp2-25 was also observed in tissue culture media when cells were treated with the higher concentrations of the compound ($\geq 10 \mu\text{M}$). Thus, the concentration of the inhibitor in media could be enough to trigger differential cytotoxicity in cells but may be insufficient to inhibit p27 or p21 levels in HCT116 or MRC-5 cells. As an alternative reason, other pro-survival pathways, such as

PI3K/Akt, could suppress p27 and p21 expression under the conditions used. Indeed, PI3K/Akt is known to regulate p27 expression and localisation via transcriptional and post-transcriptional mechanisms, moreover Akt directly phosphorylates p27 at multiple residues, which affects p27 localisation and/or stability depending on cell type (Vervoorts, Lüscher 2008, Shanmugasundaram et al. 2013). To support this hypothesis, an inverse correlation between Skp2 and p27 was not observed in some studies, indicating that this association could be cell line- or other pathway- dependent (Drobnjak et al. 2003, van Duijn, Trapman 2006). For instance, inverse patterns of Skp2 and p27 expression was observed only in approximately 53% of prostate cancer tissues and decreased levels of p27 were detected in the absence of Skp2 overexpression in almost one-third of cases. The authors attributed this to the degradation of p27 by a Skp2-independent pathway, which could be potentially a reason for lack of p27 up-regulation upon Skp2 inhibition in current study. Such alternative pathway was earlier suggested by Hara et al., describing polyubiquitylation of p27 in the cytoplasm of Skp2^{-/-} lymphocytes that contributed to the early phase of p27 degradation, thereby promoting cell cycle progression during G0/G1 phases but not during S/G2 phases (Hara et al. 2001).

7.6. Synthetic Lethality in PTEN^{-/-} cells. Conclusions

PARP inhibition

Data within this work demonstrate that PC3 prostate cancer cells with *PTEN*-deficient genotype displayed enhanced sensitivity to the PARP inhibitor, AZD2281, compared to PNT1A cells expressing PTEN, but not in any of the genomically engineered cells tested. These data are in direct contrast to published studies outlined above but there are reports in the literature that indicate that the responses tested here are not always reproducible. For example, Fraser *et al.* confirmed Mendes-Pereira *et al.* data (Mendes-Pereira et al. 2009) on PTEN^{-/-} TW HCT116 cells demonstrating a weak but significant increase in AZD8821 sensitivity compared to PTEN^{+/+} cells but failed to observe a similar correlation between sensitivity to PARP inhibition and PTEN status in the prostate cancer cell lines PC3 (PTEN null), DU145 (reported as PTEN heterozygous) and 22rv1 (PTEN WT) and found that PTEN WT 22rv1 cells were the most sensitive of the tested lines to PARP inhibition.

Dedes et al. (Dedes et al. 2010) concluded that PTEN deficiency in endometroid endometrial cancers (EECs) determines sensitivity to PARP inhibition based on eight EEC cell lines with different PTEN statuses upon KU0058948 treatment. Similar sensitivity was observed in isogenic PTEN^{-/-} endometroid adenocarcinoma HEC1A cells (Mendes-Pereira et al. 2009) and in PTEN-silenced HEC1B using two independent shRNA (Dedes et al. 2010). Consequently, resistance to PARP inhibition was restored upon ectopic PTEN re-expression in two PTEN null EEC lines (Nou-1 and Ishikawa). Contradictorily, Miyasaka *et al.* (Miyasaka et al. 2014)

indicated that PTEN status was not associated with sensitivity to PARP inhibitor AZD2281 in EECs based on 16 endometrial cancer cell lines some of which were the same as in (Dedes et al. 2010) study. More specifically, PTEN null RL95 and HEC-59 EECs that showed similar sensitivity to PARP inhibitor (KU0058948) in (Dedes et al. 2010) behaved as resistant to PARP inhibitor (AZD2281) in (Miyasaka et al. 2014) when compared to PTEN proficient EECs using similar 14 day survival assay protocols. According to Miyasaka *et al.*, only three of the 12 PTEN deficient cells and 1 of 4 PTEN WT cells were classified as sensitive PARP inhibition; while all the 4 resistant cell lines were lacking PTEN. Overall, PTEN WT cells demonstrated slightly higher sensitivity to PARP inhibition than PTEN^{-/-} EEC cells. Moreover, introduction of WT PTEN into two PTEN null EEC cells (HEC-6 and AN3CA) or knocking-down PTEN by siRNA in HEC1B or HEC-50B did not alter sensitivity to PARP inhibition (Miyasaka *et al.* 2014).

Similar to results in EECs, several contradictory reports were published in prostate cancer sensitisation to PARP inhibition. Several studies indicated that prostate cancer cells with PTEN loss were sensitive to PARP inhibition with KU0058948, AZD2281, rucaparib or niraparib (MK4827 (Jones et al. 2009)) (Mendes-Pereira et al. 2009, Roscilli et al. 2010, Chatterjee et al. 2013, Gonzalez-Billalabeitia et al. 2014) ; (Gonzalez-Billalabeitia et al. 2014) also confirmed this sensitivity in human prostate cancer xenograft model. However as mentioned earlier, Fraser *et al.* (Fraser et al. 2012) reported no effect from PTEN expression on sensitivity to PARP inhibition in prostate cancer cells. Likewise, Sandhu *et al.* (Sandhu et al. 2013) did not observe correlations between PTEN loss, PARP inhibition and antitumour activity in sporadic metastatic prostate cancer patients upon treatment with PARP inhibitor niraparib. In this Phase I trial from BRCA mutation carriers and sporadic (BRCA WT) cancers, niraparib showed a good clinical response but no correlation with PTEN status.

Published data are conflicting not only regarding sensitivity to PARP inhibition but also in characterisation of intracellular processes within PTEN deficient cells by themselves and upon treatment. Thus, while reduced RAD51 levels and subsequent decrease in DSBR homology recombination (HR) rate were observed in MEFs (Shen et al. 2007), human EEC and colon cancer cells (Mendes-Pereira et al. 2009) as well as in human glioblastoma (Majuelos-Melguizo et al. 2015) ; only decreased RAD51 levels but not DSBR HR was associated with PTEN deficiency in EEC cell lines (Dedes et al. 2010). McEllin *et al.* (McEllin et al. 2010) reported HR deficiency association with loss of PTEN in mouse glioma cells, but no decrease in RAD51 expression (rather only in RAD51 paralogs). Others failed to observe any correlation between PTEN deficiency and either RAD51 expression levels or HR even when using the same reagents and cell lines: MEFs (Gupta et al. 2009), human prostate cancer cells or xenografts (Fraser et al. 2012) and EECs (Miyasaka et al. 2014), suggesting that association of RAD51 and PTEN might be different among various types of cells, tissue and tumours.

Another example covers the correlation between PTEN deficiency and MRE11 that is recruited by PARP to DSB for subsequent recombination repair and restart of disrupted replication forks (Bryant et al. 2009). MRE11 recruitment to DNA DSBs was reduced in PTEN^{-/-} TW HCT116 cells (Xu et al. 2010, Fraser et al. 2012) but elevated in PTEN null prostate cancer cells (Fraser et al. 2012).

Results within this PhD and earlier published data demonstrated no agreement between the authors using similar or even the same cell lines, inhibitors and assays.

There are number of possible explanations to such a variability of data.

- (i) Accumulation of additional mutations during culture in key tumour suppressors that can directly or indirectly affect RAD51 levels, with prime candidates being p53 or pRb (Zhang 2014). While p53 can suppress RAD51 transcription either directly or indirectly (Arias-Lopez et al. 2006), loss of function pRb could cause an increase in E2F1 activity, and E2F1 was shown to bind RAD51 promoter and enhance its transcription (Shen et al. 2007, Fraser et al. 2012). Thus, overexpression of RAD51 in cancer cells with pRb or p53 mutations could potentially rescue the HR defects caused by loss of PTEN. In correlation with this hypothesis it is known that p53 is a preferred mutation upon PTEN loss (Carnero et al. 2015). Although such studies were not performed within this project, it would be beneficial to confirm the presence of p53 and/or pRb mutations in the studied cell lines. For instance, mutations causing disruption of p53 activity could be detected by measuring induction of p53-dependent transcription activation of p21 and consequent cell cycle arrest in response to DNA damage (Macleod et al. 1995, Hirao et al. 2000, Wulf et al. 2002, Bartek, Lukas 2001), whilst, mutations disturbing pRb activity could be assessed by western blotting analysis against phospho-pRb (Dyson 1998, Mitnacht 1998).

Moreover, the cells developed within this study by CRISPR-Cas9 KO may have additional underlying genetic lesions caused by off target effects of the Cas9 activity. As discussed earlier in Chapter 3, gRNA for PTEN KO was optimised using <http://chopchop.cbu.uib.no/index.php> (Montague et al. 2014, Labun et al. 2016), which predicted no off-target sites for PTEN gRNA within human genome. The prediction was based on (i) Hsu *et al.* (Hsu et al. 2013) stating that mismatches between sgRNA and target DNA in human genome can be tolerated by Cas9 at any position except in the PAM motif; and on (ii) Mali *et al.* (Mali et al. 2013a) showing that up to three target mismatches could potentially retain Cas9 cleavage activity. Even though this and other existing bioinformatic software are very useful and powerful tools for predicting the most likely off-target sites, accumulating data indicate that off-target effects can be difficult to predict, as the following examples illustrate. Cas9-targeting specificity was demonstrated to tolerate up to five mismatches with the frequencies comparable to (or

even higher than) those observed at the designed on-target site (Fu et al. 2013, Kuscu et al. 2014). Moreover, Cas9 could retain binding to the target site with the total number of mismatches as high as thirteen, with nine of them being even in a row in the PAM-distal region (Kuscu et al. 2014, Wang et al. 2015a). Similarly, Lin *et al.* showed 15 off-target sites from 27 different gRNAs, each harbouring a single-base insertion or deletion in human genome DNA sequence (Lin et al. 2014b). The authors hypothesised that existing bioinformatic search tools could unlikely include the potential off-target sites with such mismatches in the output as there would be too many hits within the genome. Furthermore, Cradick *et al.* demonstrated that two or more target or off-target cleavage sites on the same chromosome might cause chromosomal deletions, rearrangements and mutagenesis (Cradick et al. 2013). This implies that Cas9 can mediate a DSB even with imperfectly matched gRNA-target DNA interfaces in human cells, which suggest that the lines should be fully sequenced to determine if there are other introduced mutations that may impact signalling responses.

- (ii) Alternatively, various behaviours of PTEN^{-/-} cells could be associated with random accumulation of phenotype-affecting mutations due to genomic instability, abrogation of critical cell-cycle checkpoints or defective homologous recombination (Hunt et al. 2012). Indeed, PTEN loss-mediated activation of AKT leads to phosphorylation of one of its substrates, cell-cycle checkpoint kinase Chk1. While active Chk1 phosphorylates CDC25A resulting in its degradation and subsequent cell-cycle arrest, AKT phosphorylation of Chk1 triggers its ubiquitilation and subsequent degradation, leading to reduced Chk1 levels which can contribute to increased genomic instability. Likewise, AKT over-activation of DNA-protein kinase suppresses apoptosis and enhances DNA repair by NHEJ, subsequently leading to defective DSBR (Toulany et al. 2008). This hypothesis could be addressed by Next-Generation Sequencing (NGS) technology. For this it would be interesting to perform NGS of PTEN^{+/+} compared to PTEN^{-/-} cell lines in order to confirm if PTEN^{-/-} cells have more accumulated mutations, as such work has not been reported upon our knowledge.
- (iii) Another possible explanation is that cancer cells that were developed with different *PTEN* genotypes might be functionally distinct from cells in which PTEN was removed or introduced experimentally, possibly due to secondary genetic aberrations that could be developed upon or even before PTEN disruption during tumourigenesis. For instance, PTEN deletion was reported to be quite a late event during prostate cancer development (Hunt et al. 2012), suggesting that additional primary genomic alterations may be necessary to result in tumourigenesis upon subsequent loss of PTEN. As such, early p53 or pRb mutations in some cancers including prostate (Downing, Russell & Jackson 2003, Rivlin et al. 2011) may support this hypothesis. Similarly, elevated E2F1

expression due to disrupted pRb can occur in early stages of tumorigenesis before PTEN loss, subsequently elevating RAD51 expression and HR repair rate regardless of PTEN status (Hunt et al. 2012). The fact that parental colon cancer HCT116 cells have a long list of known mutations (<http://cancer.sanger.ac.uk/cosmic>) including those that appear within tumour suppressors or oncogenes such as loss of p16 (Roninson 2002) and mutation in PI3K catalytic domain (Dan et al. 2010), also indirectly supports the above hypothesis. On the other hand, parental MRC-5 and PNT1A cells were immortalised by transformation with SV40. SV40 is known to bind to p53 and pRb and sequentially inhibit them (Ludlow 1993, Ali, DeCaprio 2001, Saretzki 2010), which also potentially supports the former hypothesis. However, as mentioned earlier, only NGS would allow to confirm this.

- (iv) Finally, a technical possible explanation can be that PARP inhibitors are not as specific as reported and can have some off-target effects altering other pathways in different cell lines. Showing the same response with more than one inhibitor of distinct chemotype could combat this issue. Alternatively, differences could be due to small and subtle changes in experimental techniques. Such technical considerations could explain the contradictory results contained within reports studying the same cell lines and reagents such as (Gupta et al. 2009) against (Shen et al. 2007) or (Dedes et al. 2010) *versus* (Miyasaka et al. 2014) (described earlier in this chapter).

Taking all these contradictory results together including data from this PhD study, one can conclude that (i) PTEN loss does not serve as a universal predictive biomarker for sensitivity to PARP inhibition; (ii) PTEN, homologous recombination and sensitivity to PARP inhibition are unlikely to be connected directly and their relationship is cell specific; and (iii) cells with PTEN alterations have a complex and cell specific phenotype.

TTK inhibition

The lethal sensitivity to TTK inhibition generated here are in line with that for sensitivity to PARP inhibition: no direct dependence upon *PTEN* genotype was observed in isogenic cell lines. However, one out of two PTEN-deficient cancer lines demonstrated a decrease in cell viability upon TTK inhibition.

Even though, data in isogenic cells did not correlate with (Brough et al. 2011b), they correlate well with the earlier data on PARP inhibition in the same cell lines, perhaps again indicating that these cells may have altered genotypes due to elevated genomic instability. In agreement with this hypothesis, one study has reported that drug-resistant mutations causing steric hindrance to inhibitor binding can naturally occur in normal and malignant cells (Gurden et al. 2015). Thus, even three days of treatment of WT HCT116 cells with AZ3146 (0.8 μ M) caused a 50-fold increase in TTK drug-resistant mutations. Accumulation of TTK mutants resistant to a

range of TTK inhibitors was also confirmed in HELA, DLD1 and U2OS cells (Koch et al. 2016). This suggests that such mutations could be stimulated within a six-day treatment experiment in this study and could cause increase in drug resistance.

In contrast, TTK inhibition in cancer cells with PTEN loss was in a good agreement with the previous reports. Thus, Brough *et al.* showed that out of three PTEN null breast tumour cell lines tested only two (SUM149 and CAL51) appeared to be sensitive to TTK inhibition, while one (MDA-MB-468) was resistant to such treatment. Similarly, two breast cancer lines (MCF7 and JIMT) with WT PTEN also exhibited sensitivity towards TTK inhibition (Brough et al. 2011b). Interestingly, according to Brough *et al.* data, the resistant MDA-MB-453 cell line expresses the E307K mutant of PTEN at normal levels but with limited nuclear expression (Singh et al. 2011) whilst the most sensitive CAMA1 cells express the D92H mutation that leads to reduced protein levels (Weigelt, Warne & Downward 2011) #####. Other authors also reported sensitivity to TTK inhibition in non-tumorigenic PTEN proficient MCF10A and immortalised RPE1-hTERT cells (Kwiatkowski et al. 2010, Maire et al. 2013). Moreover, PTEN null MDA-MB-468 and HCC-70 cells demonstrated a higher resistance upon TTK silencing with two independent siRNAs when compared to PTEN WT MDA-MB-231 and MCF10A cells (Maire et al. 2013). In another study, PTEN WT Hs578T and T47D breast cancer cells demonstrated similar sensitivity to TTK silencing via siRNA as PTEN deficient BT-549 breast cancer cell line (Daniel et al. 2011). Interestingly, in this report MCF10A cells did not exhibit any sign of cellular death upon TTK siRNA treatment after 9 and 12 days, showing no correlation with (Kwiatkowski et al. 2010, Maire et al. 2013) reports.

As a result, some reports have correlated synthetic lethality from TTK inhibition with TTK overexpression levels rather than PTEN levels (Kwiatkowski et al. 2010, Daniel et al. 2011, Maire et al. 2013, Al-Ejeh et al. 2014). For instance, RNAi-mediated depletion of TTK in various triple-negative breast cancer (TNBC) cell lines compromised their viability, increased aneuploidy and induced apoptosis (Maire et al. 2013). Later, the same results were achieved with TTK inhibitor AZ3146 against a panel of breast tumour lines with TNBC cell lines being more sensitive to the treatment (Al-Ejeh et al. 2014). In addition, TTK was shown to be specifically overexpressed in TNBC, compared to healthy tissues and other breast cancer subgroups (Maire et al. 2013, Al-Ejeh et al. 2014), in comparison with only 35% of TNBCs that show mutation or loss of PTEN (Kalimutho et al. 2015). Thus, Maire *et al.* suggested that TTK inhibition could be therapeutically beneficial in tumours with TTK up-regulation, which is a common event not only in TBNCs, but also lung, bladder, prostate, hepatocellular carcinoma

MDA-MB-453 cell line expresses PTEN E307K mutant that displays similar lipid phosphatase and tumour suppressor activities comparing to WT PTEN. However, this mutation disrupts PTEN subcellular localisation with partial nuclear exclusion. This mutation is commonly found in metastatic breast cancer and might predispose breast cells to malignant transformation (Singh et al. 2011). CAMA1 cells carry the D92H PTEN mutation that decreases protein expression levels (Weigelt, Warne & Downward 2011).

and anaplastic thyroid tumours, and higher TTK levels correlate with worse clinical prognosis (Carter et al. 2006, Maire et al. 2013, Al-Ejeh et al. 2014, Dominguez-Brauer et al. 2015, Martinez et al. 2015, Liu et al. 2015b). Moreover, TTK belongs to a signature defined by the 25 genes most overexpressed in tumours with chromosomal instability (CIN25) (Carter et al. 2006).

However, great care should be taken in development of therapies based on TTK inhibition as some healthy non-tumourigenic cells that grow fast and have higher number of mitosis (such as MCF10A cell line or bone marrow/stomach proliferative tissues) could suffer from severe side effects. Also, it is important to emphasise that this sensitivity does not work the same way in all TNBC lines overexpressing TTK. For instance, MDA-MB-468 cells were quite resistant to TTK silencing (Brough et al. 2011b, Maire et al. 2013), while showing the highest TTK protein levels (Maire et al. 2013). Thus, there is a possibility of a TTK expression range that could be optimal for sensitivity to TTK inhibition without causing cell death in healthy tissues. Alternatively, some reports indicated that TTK inhibition could sensitise cells to other treatments. For example chemotherapeutic microtubule-targeting agent paclitaxel (taxol) at very low doses was shown a synergistic effect with TTK inhibition exclusively in cells with overexpressed TTK (Janssen, Kops & Medema 2009, Al-Ejeh et al. 2014) and in a human TNBC mouse xenograft model (Maia et al. 2015).

In conclusion, synthetic lethality from TTK inhibition in PTEN deficient cells demonstrated earlier by Brough *et al.* (Brough et al. 2011b), was not reproduced within this study suggesting that the cellular response is not robust to this treatment. Probably therefore the PTEN-TTK synthetic lethality pair is of limited potential for further study with a view to clinical application, although it is possible that it may be relevant in specific cell types or particular genotypes (such as TTK over-expression in PTEN null tumours, perhaps).

Curcumin treatment

The results in this section demonstrate that curcumin-mediated cytotoxicity did not correlate with PTEN status in the majority of tested isogenic lines. However, PTEN null U-87 MG cells were considerably more sensitive to the curcumin treatment (GR50 was near 2 times lower) than WT PNT1A cells. PC3 cells, however, did not exhibit higher cytotoxicity upon exposure to curcumin than wild type PNT1A cells. These findings correlated well with results from Hilchie *et al.* Accordingly, current curcumin GR50 value for PC3 cells (23.2 μ M, Figure 7.4(C)) was comparable with around 50% cell death rate in PC3 cells after three days treatment with 25 μ M curcumin in (Hilchie et al. 2010). However, these data do not correlate with (Guo, Xu & Fu 2015), where low-dose curcumin (0.2-0.8 μ M) inhibited proliferation of PC3 cells via non-apoptotic mechanism detectable even after 48 hours.

Similar to synthetic lethality reports regarding PARP and TTK inhibition, the correlation between PTEN deficiency and curcumin-induced cytotoxic effects suggested in (Chen et al. 2013), did not find an agreement among other studies. For instance, human prostate cancer PTEN proficient DU145 cells exhibited higher sensitivity to curcumin than PTEN^{-/-} LnCap and PC3 cells (Deeb et al. 2007) or equal sensitivity (Shankar, Srivastava 2007) ; similarly brain tumour WT PTEN DAOY cells were more sensitive than PTEN null U-87 MG (Lim et al. 2011). Comparable sensitivity to curcumin and its derivatives was observed in PTEN^{-/-} PC3 and breast cancer MCF7 cells (PTEN^{+/+}) (Rejinold et al. 2011) ; glioblastoma PTEN null human U-87 MG, U-373 MG, U-138 MG and WT PTEN rat C6^{§§§§§} cell lines (Zanotto-Filho et al. 2012) ; breast cancer PTEN null MDA-MB-468 and PTEN proficient MDA-MB-231 (Palange et al. 2012, Thulasiraman, McAndrews & Mohiudddin 2014), SK-BR-3 (Palange et al. 2012) and SKBr3 cells (Yadav et al. 2012). In addition, Shankar *et al.* demonstrated that wild type PTEN actually enhanced curcumin-induced apoptosis when transiently transfected in LnCap cells, while the PTEN-G129E mutant (lipid phosphatase-dead) had no effect (Shankar, Srivastava 2007). Thus, PTEN was shown to play no role or opposite roles upon curcumin treatment to that reported in Chen *et al.* Data in this study demonstrate no direct correlation with PTEN status, but rather cell line specific responses to curcumin.

Interestingly, Jia *et al.* suggested an alternative sensitivity target for curcumin treatment instead of PTEN (Jia et al. 2014). In their study, breast cancer cells MCF-7 and MDA-MB-231 (both PTEN^{+/+}) responded differently to curcumin (MDA-MB-231 exhibited higher sensitivity than MCF7). In the contrary to (Chen et al. 2013), curcumin treatment caused a significant increase in Akt(Ser) phosphorylation of less sensitive MCF7 cell line, while it resulted in decreased P-Akt levels in more sensitive MDA-MB-231 cells. The authors attributed this differential susceptibility to Skp2 activity. In correlation with the fact that Skp2 regulates the ubiquitin-dependent degradation of some cell-cycle regulators, including anti-proliferative proteins p21 and p27 (Tsvetkov et al. 1999, Nakayama et al. 2004), Skp2 was substantially inhibited in MDA-MB-231 cells upon curcumin exposure, whereas only higher curcumin concentrations and extended incubation resulted in Skp2 suppression in MCF7 cells. Subsequently, silencing Skp2 with siRNA overcame curcumin resistance in MCF7 cells. Based on their data, Jia *et al.* proposed that Skp2 inhibition in combination with curcumin treatment might be a better therapeutic approach for breast cancer subtypes. Similar results were reported later based on glioma cells, in which Skp2 down-regulation by siRNA promoted curcumin-induced apoptosis and inhibition of cell-growth, migration and invasion (Wang et al. 2015c). These two studies suggest that curcumin could also inhibit Skp2 in cells studied within this project, thus

§§§§§ A range of malignant glioma cells was checked for PTEN status and U-87 MG and U-373 MG cells showed no PTEN expression (Inaba et al. 2011). Rat C6 cells were included as a wild type PTEN cell line (Malchinkhuu et al. 2009, Zanotto-Filho et al. 2012, Qian et al. 2015).

accounting for the increase in its target, p21, which was observed in TW HCT116 cells regardless of *PTEN* genotype (Figure 7.5).

Taking together these data and results within this study, one can conclude that curcumin appeared to act irrespective of PTEN status, possibly due the fact that this compound alters a very wide range of anti-proliferative intracellular pathways, through which PTEN deficiency could be compensated. Moreover, these data suggest that curcumin effects are quite selective, dose- and time-dependent and also cell-specific.

Skp2 inhibition

The results in this study demonstrate for the first time that Skp2 inhibition by small molecule inhibitor Skp2-25 triggered a lethal sensitivity response in PTEN deficient isogenic human cell lines. This response was cell type specific with the strongest effect in HCT116 and MCR-5 cells compared to PNT1A cells. In addition elevated cytotoxicity was observed in PTEN^{+/-} HCT116 cells. These findings correlated well with previously reported studies in different cell models (Lin et al. 2010, Chan et al. 2013).

Novel to the previous reports, it was demonstrated that lack of PTEN phosphatase activity or disruption in PTEN PDZ domain were sufficient factors for mediating Skp inhibitor sensitivity in HCT116 cells suggesting that Skp2-25 synthetic lethality was dependent on PTEN activity and intact PDZ domain, but not really nuclear localisation. No structural requirements of PTEN were determined in MRC-5 cells.

While Skp2 inhibition with Skp2-25 displayed inhibitory effect on PTEN deficient cell lines in this study, its biological activity did not trigger a cellular senescence response (as described by Chan *et al.* (2013) in the lines tested, indicating that synthetic lethality was not due to senescence response. More work is required to determine the mechanism of cytotoxicity and whether or not it would be useful in a clinical setting.

8. Results and discussion: Dissecting the synthetic lethality between PTEN and NLK

8.1.1. Introduction

Serine/threonine kinase, NLK, contributes to apoptosis, cell differentiation, proliferation, migration and morphological changes by regulating several intracellular pathways, including down-regulation of FOXO transcription factors and the Notch1 signalling pathway and cell-dependent negative or positive regulation of the Wnt/ β -catenin pathway and consequent survival signalling (Ishitani, Ishitani 2013).

Several studies reported that NLK was overexpressed and played tumourigenic functions in liver (Jung et al. 2010, Chen et al. 2015) and gallbladder (Li et al. 2013a) cancers and that NLK expression was significantly up-regulated in liver hepatocellular carcinoma and gallbladder cancer tissue samples compared with normal tissues and was associated with poor survival prognosis for patients with these cancers. Jung *et al.* showed that NLK disruption via siRNA suppressed cell growth and cell cycle progression by inhibition of Cyclin D1 and CDK2 expression with concomitant loss of pRb hyperphosphorylation. Jung *et al.* suggested that NLK regulation affected phosphorylation of pRb protein family through transcription activation of Cyclin D1 and CDK2 in hepatocellular carcinoma cancer. The authors hypothesised that simultaneous regulation of Cyclin D1 and CDK2 by NLK would cause mitogenic stimulation and uncontrolled cell growth during liver tumourigenesis, thus NLK may be a potential therapeutic target for patients with liver cancer (Jung et al. 2010). Similarly, NLK appeared to function as an oncogene in gallbladder cancer cell lines. NLK knock-down with siRNA suppressed cell proliferation and migration of gallbladder cancer cells as well as decreased *in vitro* tumourigenesis in a colony formation assay (Tan et al. 2012).

However, other studies demonstrated an opposite, tumour suppressor, function for NLK in other cancer cells. For instance, NLK over-expression in human colorectal carcinoma DLD1 cells decreased cell growth, caused loss of anchorage independence and induced apoptosis in a kinase-dependent p53-independent manner via suppressing the Wnt/ β -catenin survival signalling (Yasuda et al. 2003). Similarly, NLK negatively regulated brain cancer tumourigenesis in patient-derived glioblastoma xenograft models (Sa et al. 2015). In this study, the authors observed that NLK expression was significantly lower in glioblastoma samples. Restoration of NLK mediated inhibition of Wnt/ β -catenin pro-survival signalling and reduced colonogenic growth, survival *in vitro* as well as decreased tumour growth *in vivo*.

Mendes-Pereira *et al.* performed high-throughput genetic screening on Todd Waldman's isogenic PTEN^{-/-} and PTEN^{+/+} HCT116 cells using a RNAi library with 779 siRNAs targeting a

specific kinase or kinase-related gene (Mendes-Pereira, Lord & Ashworth 2012). The sensitivity of PTEN null cells was determined by cell viability assay. Three genes with clear PTEN selective effects were highlighted: NLK, TTK and Polo Like Kinase 4 (PLK4). Considering that this group had already studied synthetic lethality from TTK inhibition in HCT116 cells and breast cancer lines (Brough et al. 2011b), further studies were performed with NLK and PLK4 siRNAs. Initially, the siRNA result was reproduced in other isogenic cell lines with different PTEN status: endometrial HEC1A and colorectal DLD1 tumour lines. NLK knock-down was successfully re-validated in both cell lines, while PLK4 knock-down failed to exhibit a lethal sensitivity effect in PTEN deficient DLD1 cells. Further cell viability experiments were performed on a panel of 24 human PTEN proficient or deficient tumour cell lines. Overall, NLK siRNA knock-down demonstrated better sensitivity towards PTEN null cells than PLK4 siRNA. Seven out of 13 PTEN deficient lines were sensitive to NLK down-regulation, while nine out of eleven PTEN proficient lines behaved as NLK siRNA-resistant. For PLK4 siRNA, lethal sensitivity numbers correlated less with PTEN status: five out of 13 PTEN null cells and two of eleven PTEN proficient cells, were sensitive to PLK4 knock-down. NLK siRNA follow-up experiments revealed that in PTEN deficient cells, the NLK substrate FOXO1 could trigger tumour suppressive effects by inducing the transcription of cell cycle inhibitors p21 and p27 that promote apoptosis, quiescence or senescence. However, this tumour suppressive effect of FOXO1 can be reversed by Akt phosphorylation of FOXO1 and thus its inactivation in cells with PTEN deficiency and subsequently higher P-Akt levels. FOXO1 phosphorylation by P-Akt causes FOXO1 to be removed from the nucleus and subsequently degraded in the cytoplasm (Trotman et al. 2006). One of the NLK functions is the AKT-independent phosphorylation of FOXO1 leading to FOXO1 exclusion from nucleus and subsequent inactivation (Kim et al. 2010). Mendes-Pereira *et al.* demonstrated that NLK inactivation by siRNA caused reactivation of FOXO1 and its translocation to the nucleus in PTEN deficient HCT116 cells. This process also caused cellular senescence in PTEN null HCT116 cells but not in PTEN proficient cells. The authors also demonstrated that sensitivity of PTEN^{-/-} cells to NLK down-regulation was FOXO1 dependent and FOXO silencing with siRNA reversed the synthetic lethality effect (Mendes-Pereira, Lord & Ashworth 2012).

Thus, NLK knock-down was shown to inhibit PTEN null tumour cells, which potentially could be used in anti-cancer therapy to treat PTEN deficient tumours upon development of selective NLK inhibitors. In the light of our previous observations that did not reproduce published synthetic lethality data, it was interesting to test the data of Mendes-Pereira *et al.* with NLK inhibitors as siRNA knock-down often triggers the up-regulation of secondary kinases to play compensatory roles (Elphick et al. 2009). Indeed chemical inhibition with small molecules disrupts protein activity but not its expression levels, thus there is less chance for the compensatory effects from other proteins and pathways as the protein is still present in the cell.

Within this part of the project it was planned to inhibit NLK in HCT116 cells with different PTEN status and make an additional validation of Mendes-Pereira *et al.* siRNA data using chemical genetic approach.

There are no reported inhibitors available for NLK to our knowledge. However, this is a common situation for kinases as there are over 500 protein kinases in human genome that share a conserved ATP binding site, which makes it extremely difficult to develop mono-specific inhibitors (Elphick et al. 2009, Miller et al. 2013).

Shah et al. offered a solution to these difficulties by employing a chemical genetic approach to engineer an enlarged ATP binding site in the oncogene tyrosine kinase v-Src that would allow the kinase to exclusively accept an ATP analogue with a bulky group at the N⁶ position as a phosphate donor (Shah et al. 1997). The enlarged ATP binding site not only enables the kinase to use bulky ATP analogues, but also suggests a mechanism through which a single kinase can be uniquely inhibited (Figure 8.1). The amino-acid residue suitable for mutation in the kinase ATP binding site, known as a gate keeper residue, usually has a large hydrophobic side chain (Cravatt 2005). The gate keeper mutation to an amino-acid with a smaller side chain such as glycine or alanine expands the ATP binding site of the kinase allowing bulky ATP analogues to act as phosphate donors and bulky ATP-like inhibitors are specific for the mutated kinase (Shah et al. 1997). Such a gate keeper mutant was termed an analogue sensitive (AS) mutant kinase. This chemical genetic method (also known as analogue-sensitive kinase technology) allowed a unique way of studying and inhibiting kinases individually. However, more recent reports have demonstrated that the introduction of a gatekeeper mutation can affect both the activity and structure of the parental kinase by disruption of essential hydrophobic interactions, thus the activity of the gate keeper mutant has to be confirmed and compared to the wild type kinase prior using it for further inhibition (Kornev, Taylor & Ten Eyck 2008, Elphick et al. 2009). The gate keeper residue was reported to be conserved amongst many human kinases and subsequently this chemical genetic approach can be applied widely for various applications: kinase inhibition and studying their biological activity and intracellular substrates (Cravatt 2005).

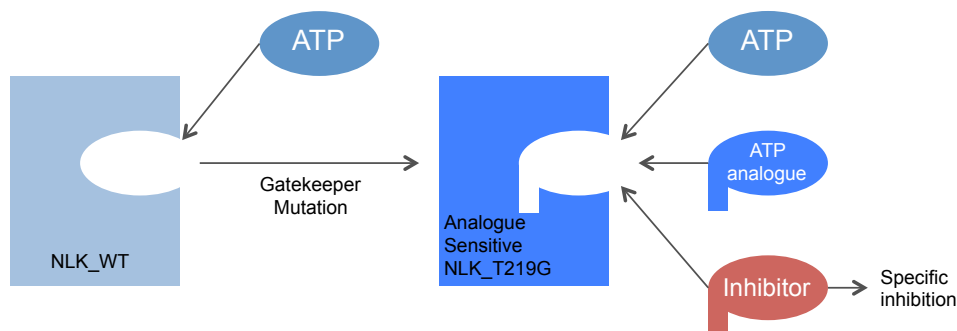


Figure 8.1. Schematic view of chemical genetic approach and analogue sensitive mutant kinase generation. The gate keeper mutation makes an enlarged ATP binding site that can

bind wild type ATP, bulky ATP analogues and specific inhibitors. The wild-type parental kinase can only utilise wild type endogenous ATP.

Given that NLK is a serine-threonine kinase, it was logical to compare its ATP binding domain to another serine/threonine kinase, Cdk2, that was previously well characterised for the chemical genetic approach with the F80G gate keeper mutation (Elphick et al. 2009). It was hypothesised that the mutation of a structurally aligned residue within the NLK ATP binding site would enlarge the pocket and allow binding to bulky kinase inhibitors. Thus F80 gatekeeper residue within Cdk2 was aligned to the T219 residue of NLK (Figure 8.2(A)) on the online Constraint-based Multiple Protein Alignment Tool (COBALT, <http://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi>). This software performs modelling alignment of protein sequences based on collection of pairwise constraints developed from conserved protein domain database and local sequence similarities (Papadopoulos, Agarwala 2007). Given that threonine does not have a large side chain, it was questionable whether replacement with a glycine would create sufficient enough space to allow discrimination between wild type and analogue sensitive mutant, however it could be determined only experimentally.

Having identified the gatekeeper residues of NLK, the PyMol models for ATP binding pockets with gatekeeper mutations were built based on SWISS-MODEL software (<https://swissmodel.expasy.org/>, (Arnold et al. 2006, Biasini et al. 2014)). There is no crystal structure for NLK available, therefore the closest available structural analogue was determined computationally as Mitogen-Activated Protein Kinase 7 (MAPK7) harbouring 46.57% of sequence identity (Figure 8.2(B)). Cdk2 SWISS-MODEL was also built in PyMol for comparison of analogue sensitive mutations in these two kinases (Figure 8.2(C)). Both analogue sensitive (AS) mutants of Cdk2 and NLK were modelled by replacing the bulky residues (F80 for Cdk2 and T219 for NLK) within the ATP binding pocket to a smaller glycine. According to the Pymol models, these gate keeper mutations enabled creation of an enlarged ATP binding site in these kinases.

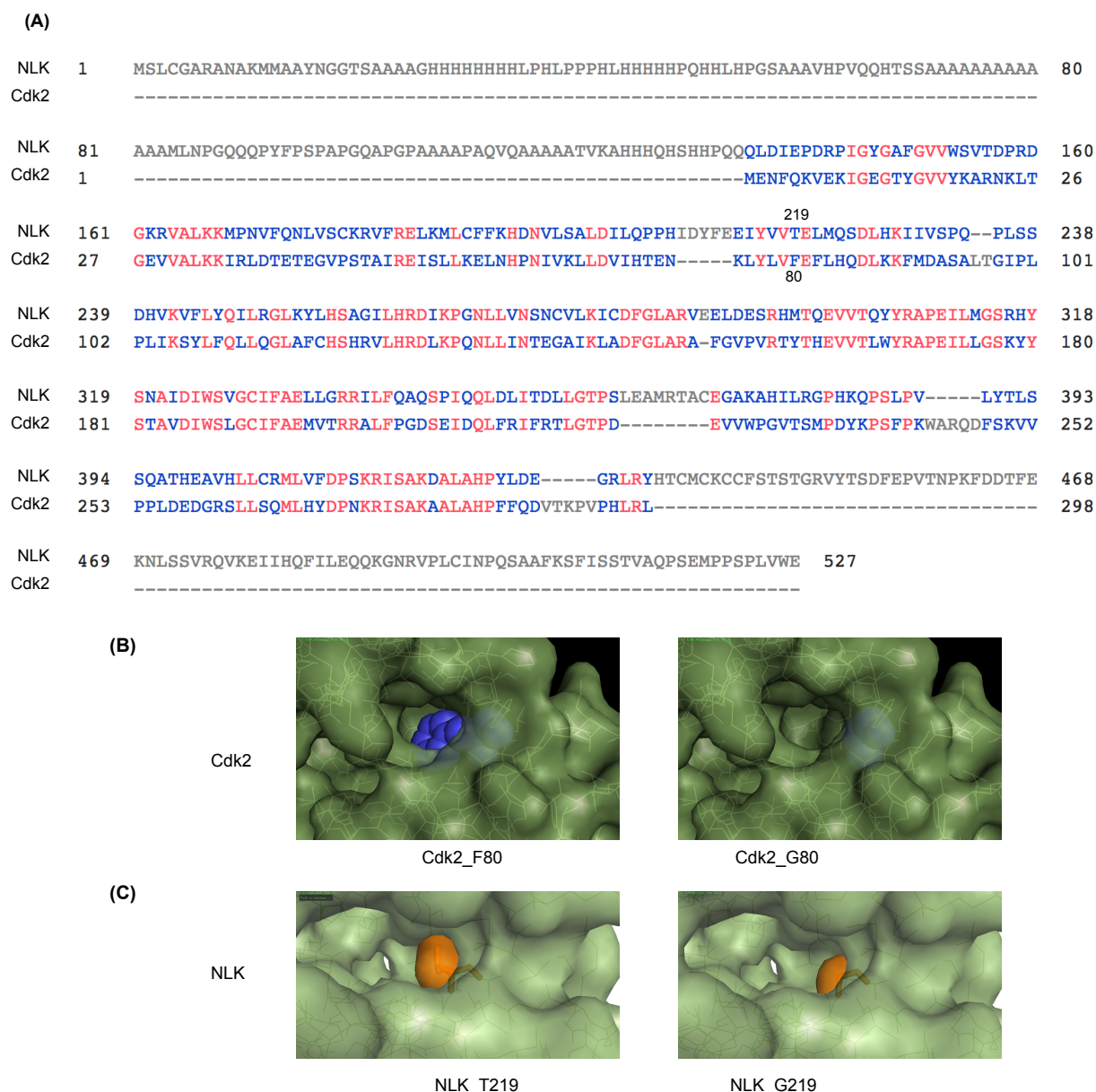
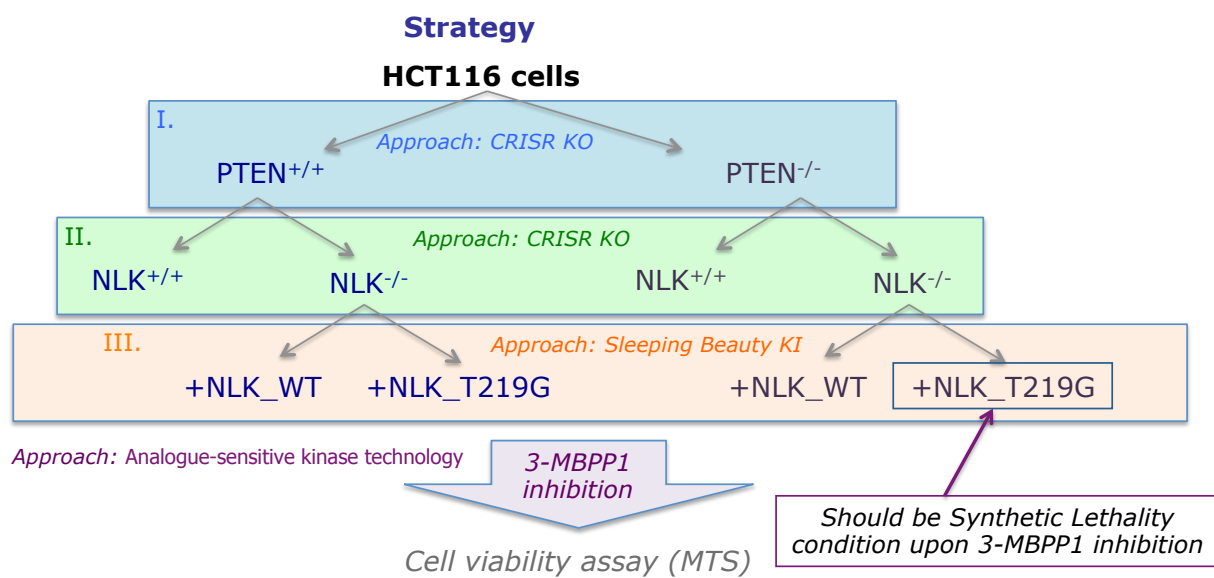


Figure 8.2. PyMol images and alignment of gatekeeper residues in Cdk2 and NLK. **(A)** Gate keeper mutation of F80 residue in Cdk2 and T219 residue in NLK was confirmed by modelling alignment on the online software COBALT (Papadopoulos, Agarwala 2007). Alignment pairs with are coloured in red (identities) or blue (other residues). **(B), (C)** PyMol images of ATP binding pocket and gatekeeper residues before and after introduction of analogue sensitive mutation on glycine amino-acid: **(B)** Cdk2 gate keeper residues (F80 in Cdk2^{WT} and G80 in Cdk2^{AS}) are highlighted in blue. PyMol images were generated on the basis of Cdk2 crystal structure SWISS-MODEL (PDB ID code is 3pxr (Betzi et al. 2011)). **(C)** NLK gate keeper residues (T219 in NLK^{WT} and G219 in NLK^{AS}) are highlighted in orange. PyMol images were build by structural analogy of NLK SWISS-MODEL to MAPK7 crystal structure template (PDB ID code is 4ic8 (Glatz et al. 2013), sequence identity 46.57%). An enlargement of the ATP binding pockets is shown for both kinases.

This analogue-sensitive approach would allow mono-specific inhibition of NLK^{AS} mutant in the presence of all other kinases *in vivo*. However, in order to perform such inhibition, it was necessary to eliminate endogenous wild type NLK in cells. Therefore, the following strategy

was suggested for this part of the project: (i) knock-out of endogenous NLK in $PTEN^{-/-}$ and $PTEN^{+/+}$ HCT116 cells developed by Todd Waldman's group and within this study^{*****}; (ii) generate constructs encoding NLK^{WT} and NLK^{AS} proteins; (iii) verify kinase activity and analogue sensitive inhibition with a bulky kinase inhibitor (3-MBPP1) of NLK^{WT} and NLK^{AS} kinases *in vitro*; (iv) knock-in of NLK^{WT} and NLK^{AS} into NLK KO cells with different *PTEN* genotype; (v) perform cell viability assay upon analogue sensitive inhibition of NLK^{WT} and NLK^{AS} knock-in cells *in vivo* (Figure 8.3).



Result: NLK was characterised as a potential therapeutic agent in *PTEN* deficient cancers

Figure 8.3. Schematic strategy for NLK inhibition via chemical genetic approach in cells with different *PTEN* status.

8.1.2. NLK KO via CRISPR/Cas9

CRISPR/Cas9 KO of *NLK* gene was performed similar to *PTEN* KO described in Chapter 3.2. It involved co-transfection of plasmids encoding Cas9 endonuclease and target-directed gRNA into cells of interest.

8.1.2.1. gRNA design

The DNA construct used for gRNA expression was designed according to the recommendations in (Mali et al. 2013b) (Figure 3.1(B) in Chapter 3.2). It consisted of U6 RNA III polymerase promoter, target DNA sequence, gRNA scaffold and a transcription terminator sequence. Suitable 5'→3' GN₂₀GG gRNA target sequences were chosen within the *NLK* gene

^{*****} It is important to take into consideration that NLK silencing in $PTEN^{-/-}$ cells may cause synthetic lethality during generation of this cell line based on the data of (Mendes-Pereira, Lord & Ashworth 2012). If this is the case, than first two steps of suggested experimental strategy should be swapped (making the knock-in step prior to the NLK KO). However, as discussed earlier there is a possibility, that NLK activity could be compensated by other intracellular kinases upon complete NLK KO. In this case $PTEN^{-/-}NLK^{-/-}$ cells would be still viable and only chemical inhibition of exogenous NLK would show synthetic lethality.

based on following criteria: i) would contain PAM Cas9 binding motif (NGG) at the end of 20 nucleotides starting with the transcription initiating first G residue for U6 promoter; (ii) the position within the gene should be disruptive, thus should be within exon sites and ideally in a close proximity to residues essential for the kinase activity to eliminate the possibility of rescue via downstream initiation (NLK has protein kinase domain in the position 138-427); (iii) the target chosen sequences would have the least potential off-target binding sites within human genome. These criteria (and others that were listed in Chapter 3.2) were incorporated within the online software <http://chopchop.cbu.uib.no/index.php> (Montague et al. 2014, Labun et al. 2016). However, this bioinformatics tool was not developed at the time when NLK gRNAs were designed. Instead, E-CRISP-TEST online software (an older and discontinued version of E-CRISP software, <http://www.e-crisp.org/E-CRISP/> (Heigwer, Kerr & Boutros 2014)) was used to generate the list of possible GN₂₀GG gRNA target sequences within *NLK* (Gene ID used was Ensembl ENST00000407008, *NLK*-001 transcript), and Cas-OFFinder online software (<http://www.rgenome.net/cas-offinder/> (Bae, Park & Kim 2014)) was utilised to find potential off-target sites outside of *NLK* gene.

All sequences close to the NLK active site and with the least off-targets did not contain the transcription initiating first G nucleotide at the 5' end, therefore this residue was incorporated in the vector construct instead. Two top hits (with the least off-target score and closest to the NLK active site) were chosen within exons 5 and 6 (PAM sequences in bold text):

- 1) 5'- TTACATCGAGACATTAAGCC**AGG**-3' (exon 5, TS1^{NLK})
- 2) 5'-TGAATCCCGTCATATGACTC**AGG**-3' (exon 6, TS2^{NLK})

In order to analyse these two targets in detail a later version of online software (<http://chopchop.cbu.uib.no/index.php> (Montague et al. 2014, Labun et al. 2016)) was used. The TS1^{NLK} target sequence was located within exon 5, contained 43% of GC-content (as mentioned earlier gRNAs with a GC-content between 40 and 70% worked more effective (Wang et al. 2014, Tsai et al. 2015)) and no self-complimentary regions longer than 3 nucleotides (self-complementarity within the target sequence or between the target sequence and gRNA backbone could inhibit gRNA efficiency (Labun et al. 2016)). TS1^{NLK} targeted the sequence encoding amino acids 261-268 of NLK protein, located within the protein kinase domain (138-427 aa) including the essential DFG motif, indicating that disruption within this region should cause complete loss of kinase activity. The off-target analysis showed that TS1^{NLK} was unique in the human genome but had one more target with three mismatches: CCTGGCTgAATGTCTtGAgGTAA (mismatches are shown in low cases). This off-target sequence located on the chromosome 7 and was more than 170000 nucleotides away from the

closest known coding sequence, suggesting that even in case of Cas9 off-target cleavage at this site, the effect on cellular genotype and phenotype was unlikely to be significant.

The TS2^{NLK} target sequence was located within exon 6, consisted of 48% of GC-content and 2 self-complementary regions longer than 3 nucleotides. It targeted the region of NLK encoding amino acids 293-299 within the protein kinase domain including the T298 residue involved in enzyme regulation (<http://www.uniprot.org/uniprot>). This residue is part of the TQE activation loop motif in which intermolecular autophosphorylation on T298 residue activated by the non-canonical Wnt signalling pathway is sufficient to activate NLK kinase activity (Ishitani et al. 2003). Similar to the TS1^{NLK} sequence, TS2^{NLK} also was characterised as a unique sequence within human genome and had one off-target with three mismatches: CCTGAGTCATATtAaGtGATTCA. It located on the chromosome 8 and also was more than 1000000 nucleotides away from the closest known coding sequence.

Considering this characterisation and the lowest potential for off-target disruption within the genome out of all potential options at the time of design, TS1^{NLK} and TS2^{NLK} target sequences were incorporated into gRNA expressing constructs. These constructs were ordered as a part of gBlocks harbouring the complete transcriptional unit as described above. Synthesised gBlocks were further A-tailed and incorporated into an empty pGEM-T backbone.

8.1.2.2. CRISPR/Cas9 transfection

PTEN^{-/-} and PTEN^{+/+} HCT116 cells were chosen for NLK KO experiments as these cells demonstrated faster growth rate, higher transfection efficiency and more distinguishable colony formation upon selection than MRC-5 or PNT1A cells, based on previously described experiments. PTEN^{-/-} and PTEN^{+/+} lines based on both HCT116 cells developed by Todd Waldman (TW) and within this study, were co-transfected with Cas9, gRNA encoding plasmids using Lipofectamine 2000 transfection methodology. This time TS1^{NLK} and TS2^{NLK} gRNAs were transfected separately based on the difficulties with the analysing KO clones generated from simultaneous use of two gRNAs outlined in the previous PTEN KO experiments (see Chapter 3.3). Selectable marker plasmids pBabePuro or pBabeHygro were co-transfected into TW HCT116 cells or HCT116 cells developed within this study respectively, to enable selection of transfected cells by the addition of puromycin or hygromycin B. Puromycin selection marker was used as it had shown the fastest killing efficiency on HCT116 cells in previous experiments. However, the HCT116 cells developed within this work have already been selected with puromycin to create PTEN KOs, thus hygromycin B, an alternative selection marker, was required for these lines. The DNA:Lipofectamine 2000 ratio was used the same as for previous PTEN KO experiments (1:3). The composition of transfection mixtures is shown in Table 8.1.

Table 8.1. Composition of Lipofectamine 2000 based transfection mixtures for CRISPR/Cas9 NLK KO. Amounts are shown for cells cultured in 10 cm dishes.

	TW HCT116	HCT116
DNA:Lipofectamine 2000 ratio	1:3	1:3
Total DNA, µg	24	24
DNA mix		
hCas9	5 parts	5 parts
gRNA (TS1 ^{NLK} or TS2 ^{NLK})	5 parts	5 parts
pBabePuro	1 part	
pBabeHygro		1 part

On the day following transfection, cells were replated at a range of different densities and puromycin (0.4 µg/ml) or Hygromycin B (600 µg/ml) were added to select for resistant clones. The concentration of the selection drugs was pre-determined in earlier PTEN knock-out and knock-in experiments (See Chapter 3.2.2). Media and the drugs were renewed every three days and after all non-transfected cells on a control plate were dead (8-9 days), the selection was removed. Cells were cultured further until individual colonies could be identified and collected (on the 20-25th days following transfection). Subsequent targeted genomic changes were assessed via western blotting, enzyme mismatch cleavage (EMC) and PCR assays.

8.1.2.3. Western blot based analysis of NLK KO clones

For each cell line with different PTEN status, 20-30 individual transfectant clones that were resistant to puromycin (for TW HCT116 cells) and hygromycin B (for cells developed in this study) were isolated, and five-six of each were assessed for NLK expression by western blotting. The clones were lysed by RIPA buffer from near-confluent 24-well plate and loaded onto SDS-PAGE according to the protein amount in each sample (measured with Bio-Rad DC Protein Assay). Separated proteins were subject to immunoblotting and probed with anti-NLK antibodies. Initially, a goat polyclonal anti-NLK antibody from Santa Cruz Biotechnology was used as it was previously reported in (Mendes-Pereira, Lord & Ashworth 2012). However, this antibody demonstrated an indistinguishable pattern for non-transfected cells and isolated colonies and therefore a second antibody, a rabbit monoclonal antibody from Abcam, was tested. Unfortunately, the band of approximately 50-60 kDa was clearly seen in all samples again. Attempts to optimise the immunostaining protocol such as antibody concentration, blocking reagent (5% BSA, 3% FBS, 5% milk and their combination), time or temperature of the blocking step (1-24 hours at RT or +4°C), did not change the result. A representative blot with samples derived from individual resistant clones derived from TW HCT116 cells is shown in Figure 8.4.

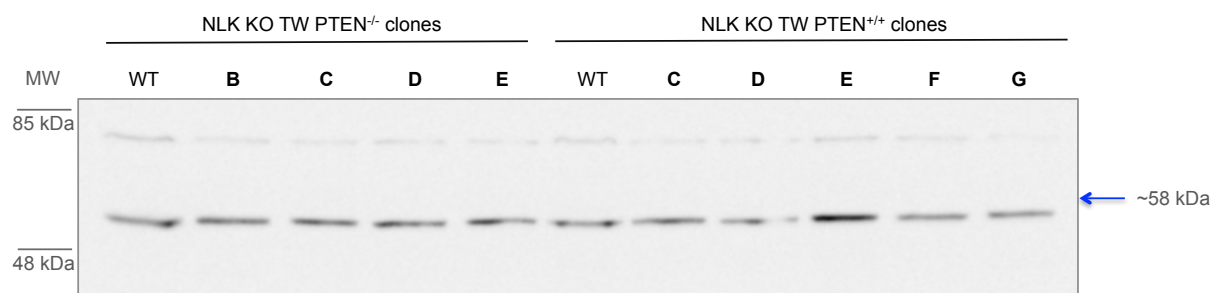


Figure 8.4. Representative Immunoblot to determine the NLK status of cells. Samples from individual colonies isolated after transfection with CRISPR-Cas9 components and TS2^{NLK} gRNA were subject to protein quantification via Bio-Rad DC Protein Assay. Equal amounts of protein were loaded for each sample onto an SDS-PAGE and per lane immunoblotted with mouse anti-NLK antibody (Abcam) and developed using ECL. The predicted NLK MW is 58 kDa (blue arrow).

Such a result could indicate that the CRISPR-Cas9 mediated KO of the NLK gene had failed or that NLK antibodies demonstrated no specific binding. To distinguish between these possibilities the KO clones had to be characterised with an alternative method. The sequencing of the targeted region would be one of the options but this method is quite expensive and time-consuming for a large numbers of samples. Thus, another strategy to identify unknown genetic alterations was chosen, the enzyme mismatch cleavage method.

8.1.2.4. NLK KO clones analysis with enzyme mismatch cleavage assay

The enzyme mismatch cleavage (EMC) assay utilises mismatch-sensitive nucleases that detect heteroduplex DNA formed by hybridisation of wild type DNA strand to DNA strands with CRISPR-Cas9-mediated indels, or of two different mutant DNA strands (Vouillot, Thelie & Pollet 2015, Koo, Lee & Kim 2015). This method can be used to get first estimates of whether there were genetic alterations in the targeted region. Two endonucleases are widely used for this method: CEL1 or T7E1. Vouillot *et al.* demonstrated that CEL1 enzyme was better for detecting single nucleotide changes, while T7E1 outperformed CEL1 in terms of sensitivity with deletion indels (Vouillot, Thelie & Pollet 2015). Thus, both these endonucleases were tested in the EMC assay for the NLK KO clones. Initially, CEL1 was chosen as it did not have any requirements for the minimal number of mismatch nucleotides.

A single-strand specific endonuclease CEL1 (also known as Surveyor nuclease) derived from celery juice extract, cuts heteroduplex DNA only one strand at a time on the 3' end of a mismatch that created a single-strand bulge (Till et al. 2004, Till et al. 2006). CEL1-based protocol (also known as Surveyor assay or Targeting Induced Local Lesions IN Genomes, TILLING) for CRISPR-Cas9 clones consists of following steps: (i) isolating gDNA from WT cells and selected KO clones; (ii) amplifying of the targeted region by PCR; (iii) denaturing and re-annealing the PCR products to allow heteroduplex formation between WT DNA and CRISPR-

Cas9 mutated DNA or between DNA strands with different mutations; (iv) incubating with CEL1; and (v) visualising cleaved and full-length PCR amplicons on agarose gels.

gDNA was purified from 10-15 clones from each cell line and WT original lines, following by PCR amplification of the gDNA region containing target sites in each sample. PCR primers for the TS1^{NLK} and TS2^{NLK} target sites were designed using the Primer3: WWW primer tool (Rozen, Skaletsky 1998) according to the location showed on Figure 8.5.

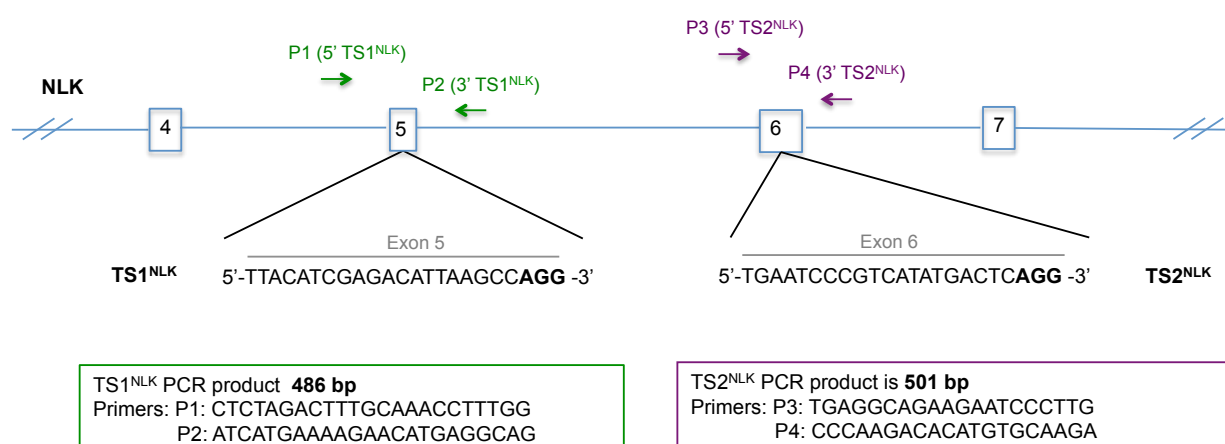


Figure 8.5. The location of two primer sets (TS1^{NLK} and TS2^{NLK}) designed for PCR screening of NLK KO lines.

TS1^{NLK} and TS2^{NLK} PCR primers for the TS1^{NLK} and TS2^{NLK} PCR target sequence were initially tested on gDNA samples isolated from the wild type cells using the express gDNA protocol. PCR reactions were performed with GoTaq G2 polymerase according to the conditions in Table 8.2.

Table 8.2. PCR conditions and two primer sets (TS1^{NLK} and TS2^{NLK}) for PCR screening of NLK KO cell lines.

	Specific primers	T melting, °C	PCR product
TS1 ^{NLK} primers	P1: CTCTAGACTTTGCAAACCTTTGG P2: ATCATGAAAAGAACATGAGGCAG	69.6 69.4	486 bp
TS2 ^{NLK} primers	P3: TGAGGCAGAAGAATCCCTTG P4: CCCAAGACACATGTGCAAGA	70.2 70.6	501 bp

Polymerase	GoTaq G2		
	T, °C	Duration	No of cycles
Initial denaturation	95	2 min	1
Denaturation	95	30 s	25
Annealing	68	1 min	
Extension	72	1 min	
Final extension	72	5 min	1
Hold	4	Indefinite	-

PCR composition (50 µl)
5 µl gDNA (from ~12500 cells) 1x Reaction Buffer 200 ng each primer 500 µM each dNTP 1 M betaine 0.25 µl polymerase

The PCR annealing temperature was first optimised for both primer sets by performing individual PCR reactions at different annealing temperatures (Figure 8.6(A) and data not shown). The temperature that provided with the cleanest and brightest single band for expected amplicons was chosen as 68° C.

Next, the re-annealing procedure was optimised by hybridising either two samples from WT cells with the expectation that the nuclease should not cleave the annealed products which would be identical or a WT and a pool of potential KO samples with the expectation that some small MW products should be generated due to the presence of KOs. Three hybridising protocols were tested on PCR products isolated from WT or KO lines. For this, PCR mixtures were either (i) incubated for 10 min in 1 L of boiling water and allowed to cool until water reached room temperature; (ii) and (iii) incubated at 99° C for 10 min in an Eppendorf Mastercycler machine, following by controlled cooling down at the rate (ii) 1° C per one minute or (iii) 1° C per three minutes. A representative gel with two PCR samples that were denatured and re-annealed under these three conditions and then incubated with CEL1, is shown on Figure 8.6(B). PCR re-annealing and controlled cooling in the Mastercycler machine provided with the cleaner DNA bands. For further experiments the cooling rate 1° C per minute was chosen as additional slowing down to 1° C per three minutes did not improve the result.

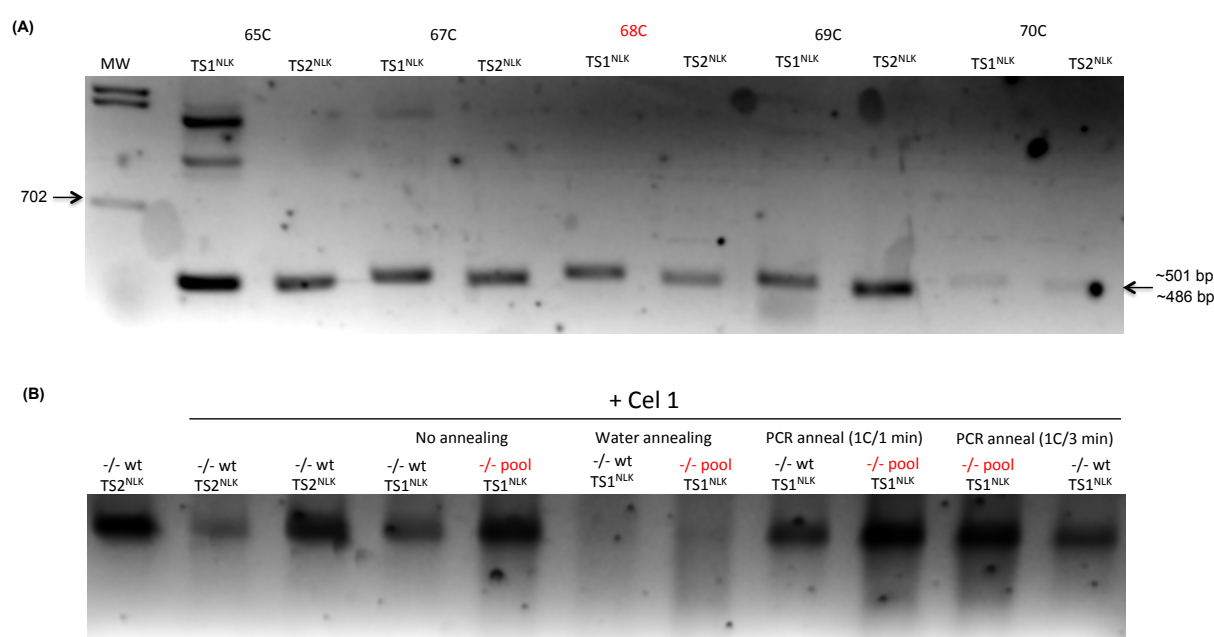


Figure 8.6. Optimising the Enzyme Mismatch Cleavage Assay. Agarose gels with PCR products amplified with TS1^{NLK} and TS2^{NLK} primers from Todd Waldman's (TW) HCT116 cells. **(A)** Optimal annealing temperature (i.e. the temperature at which the expected amplicon was the only one and maximised) was determined. MW-DNA ladder. **(B)** PCR samples amplified from WT cells and KO pools based on PTEN^{-/-} TW HCT116 cells were denatured and re-annealed in (i) boiling water; or (ii), (iii) Eppendorf Mastercycler machine with the cooling rate (ii) 1° C per one minute or (iii) 1° C per three minutes.

Following these optimisation steps, gDNA was isolated from ~20 clones for each guide RNA from both PTEN^{-/-} and PTEN^{+/-} lines (from both Todd Waldman's and this study's HCT116 cells). Individual PCR reactions with TS1^{NLK} or TS2^{NLK} primer sets were performed on each of these isolated gDNA samples, following by denaturation and re-annealing either to themselves or in a mix with TS1^{NLK} or TS2^{NLK} PCR product from the wild type cells respectively. Re-annealed samples were incubated with CEL1 and subject to separation on agarose gels. As expected very bright and clean bands were visible for the unprocessed PCR products, but the bands resulting from CEL1 digestion were often quite weak and indistinct. A representative gel with some of the tested samples is shown on Figure 8.7.

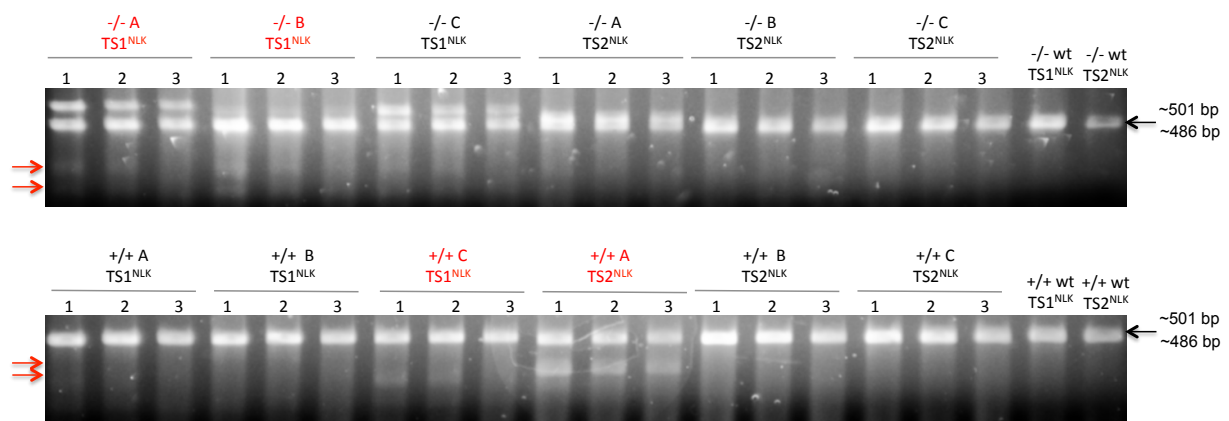


Figure 8.7. EMC assay by CEL1 endonuclease. Agarose gels with PCR products amplified with TS1^{NLK} and TS2^{NLK} primers on the basis of Todd Waldman's (TW) HCT116 cells; PCR products were denatured, re-annealed and incubated with CEL1 endonuclease. In order to detect mutations arising from NLK KO and mismatches in sequences following annealing pairs were analysed: **1.** KO clone + KO clone (amplicons from KO clones annealed to themselves); **2.** KO clone + WT (amplicons from KO clones annealed to the parental cell line); **3.** KO clone + WT (amplicons from KO clones mixed with parental cell line without denaturation and annealing steps). Non-annealed samples and samples from parental lines were included as controls of CEL1 specificity to mismatches. Red arrows indicate digested bands triggered by CEL1 digestion of mismatches in annealed PCR samples from KO clones with mismatches (red font).

Having such a weak signal from the samples that potentially contain CRISPR-Cas9-mediated mutations could lead to underestimated efficacy of the CRISPR targeting. Thus, an alternative mismatch-sensitive enzyme, T7E1 (Endonuclease I of Bacteriophage T7) that cleaves both strands of DNA at the mismatch site (Mean et al. 2004, Kim et al. 2009) was compared to CEL1 in the EMC assay. Incubation with T7E1 was performed according to the manufacture recommendations (M0302, NEB). Similar to previously reported data (Vouillot, Thelie & Pollet 2015), T7E1 demonstrated higher sensitivity than CEL1 for the detection of mismatches in re-annealed samples derived from TW HCT116 clones (Figure 8.8(A)). T7E1-based assay was therefore performed for all isolated clones and some representative gels are shown on Figure 8.8(B).

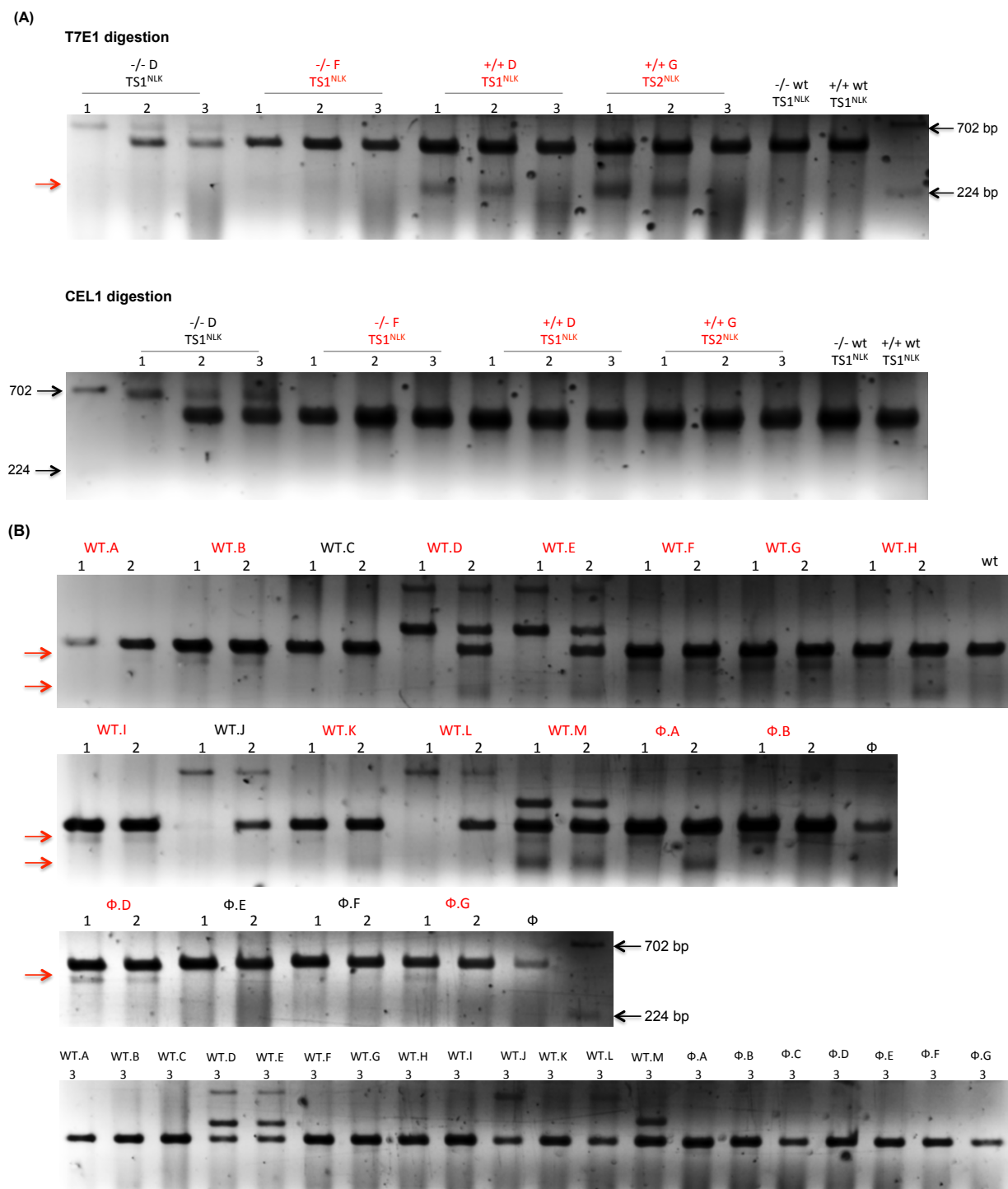


Figure 8.8. EMC assay by T7E1 endonuclease. Agarose gels with PCR products amplified with TS1^{NLK} and TS2^{NLK} primers from **(A)** Todd Waldman's (TW) HCT116 cells; **(B)** this study's HCT116 cells (only with TS1^{NLK} primers). PCR products were denatured, re-annealed and incubated with CEL1 or T7E1 endonuclease. In order to detect mutations aroused from NLK KO and mismatches in sequences following annealing pairs were analysed: **1.** KO clone + KO clone (amplicons from KO clones annealed to itself); **2.** KO clone + WT (amplicons from KO clones annealed to parental cell line); **3.** KO clone + WT (amplicons from KO clones mixed with parental cell line without denaturation and annealing steps). Not annealed samples and samples from parental lines were included as controls of T7E1 specificity to mismatches. Red

arrows indicate digested bands triggered by T7E1 digestion of mismatches in annealed PCR samples from KO clones with mismatches (red font). WT - PTEN^{+/+} HCT116 cells, Φ - PTEN^{-/-} HCT116 cells.

The EMC method allowed discovery of several positive clones in each cell line that were either digested by mismatch-sensitive T7E1 endonuclease or produced a PCR amplicon of a different size from the wild type amplicons that could be directly visualised on agarose gels. A summary of these results is given in Table 8.3.

Table 8.3. Numbers of NLK KO clones determined by EMC assay (T7E1 endonuclease). The rows in the table represent the number of positive clones (with PCR products of a distinct size from the WT amplicons upon cleavage with CEL1 or T7E1) out of total number (in brackets).

	Positive clones according to EMC assay			
	TW HCT116		HCT116 (this study)	
	PTEN ^{-/-}	PTEN ^{+/+}	PTEN ^{-/-} Φ	PTEN ^{+/+}
TS1 ^{NLK}	5 (9)	1 (9)	n/d	n/d
TS2 ^{NLK}	6 (13)	5 (15)	7 (14)	7 (16)

8.1.2.5. Analysis of *NLK* gene in the putative KO clones

All clones that were characterised as potential positive KOs via the EMC assay were further analysed at the genome level in order to get precise validation of their *NLK* status. Initially, all TS1^{NLK} and TS2^{NLK} PCR products from EMC-positive clones were purified, concentrated and submitted for sequencing. Similar to PTEN KO data, due to disrupted NLK target sites all these clones were either (i) distinguishable from non-mutated WT sequences or (ii) mixed sequencing data. Such a result indicated that the EMC assay gave a very precise indication of CRISPR-Cas9-disrupted clones. Further analysis was performed with TS2^{NLK} clones only as they demonstrated higher efficiency by the EMC method.

All potential mutant TS2^{NLK} PCR amplicons were cloned into empty pGEM-T vector and transformed into *E.coli* DH5 α , in order to discriminate between non-identical alleles (see Chapter 3.2.2.2). Plasmid integrity was confirmed by digestion with restriction enzymes (SphI/SacI). Approximately 10 mini-preps for each TS2^{NLK} KO clone were purified and sequenced, and TS2^{NLK} PCR inserts were compared to the WT PCR sequence. This assay allowed characterisation of the precise type of mutation that occurred in each NLK KO clone due to NHEJ repair after CRISPR-Cas9-induced DSB at the TS2^{NLK} target site. This data for some NLK KO clones is shown in Table 8.4.

Table 8.4. Summarised results from the sequencing analysis of NLK KO clones established in PTEN^{+/+} or PTEN^{-/-} HCT116 cells derived from Todd Waldman (TW) clones or created here. The TS1^{NLK} and TS2^{NLK} PCR products were cloned into pGEM-T and DNA alleles were sequenced individually. The clones with NLK^{-/-} phenotype are highlighted in green. PTEN^{-/-} Φ – HCT116 PTEN^{-/-} Clone Φ .

TW HCT116	PTEN ^{-/-}		Genotype
	Allele 1	Allele 2	
Clone A	202 bp insert	WT	NLK ^{+/-}
Clone B	1 bp gap	137 bp insert	NLK ^{-/-}
Clone C	5 bp gap	202 bp insert	NLK ^{-/-}
Clone D	161 bp insert	161 bp insert	NLK ^{-/-}
Clone F	1 bp insert	WT	NLK ^{+/-}
Clone K	WT	WT	NLK ^{+/+}
TW HCT116	PTEN ^{+/+}		Genotype
	Allele 1	Allele 2	
Clone C	12 bp gap	1 bp gap	NLK ^{-/-}
Clone G	1 bp insert	13 bp gap	NLK ^{-/-}

HCT116 (this study)	PTEN ^{-/-Φ}		Genotype
	Allele 1	Allele 2	
Clone ΦA	9 bp gap*	9 bp gap*	NLK ^{-/-}
Clone ΦD	WT	WT	NLK ^{+/+}
Clone ΦG	7 bp gap**	7 bp gap**	NLK ^{-/-}
HCT116 (this study)	PTEN ^{+/+}		Genotype
	Allele 1	Allele 2	
Clone A	1 bp insert**	1 bp insert**	NLK ^{-/-}
Clone E	188 bp insert**	188 bp insert**	NLK ^{-/-}
Clone F	WT	WT	NLK ^{+/+}
Clone H	2 bp gap**	2 bp gap**	NLK ^{-/-}
Clone I	WT	WT	NLK ^{+/+}

* these indels are not identical: they have different location

** identical indels in both alleles

Similar to the PTEN KO results, biallelic mutation was also preferential for NLK gene targeting. This suggests that CRISPR-Cas9 mediates targeted DSB on both chromosomes with a higher frequency even when only one gRNA is used. This data supports similar conclusions in a variety of eukaryotic systems (Canver et al. 2014, Li et al. 2013b), (Wang et al. 2013), (Jao, Wente & Chen 2013, Li et al. 2015, Ma et al. 2015).

For the subsequent experiments, NLK^{-/-} clones were chosen in order to use them as a platform for introduction of NLK^{WT} and NLK^{AS} variants:

- 1) TW HCT116 (PTEN^{+/+}, NLK^{-/-}) Clone G; TW HCT116 (PTEN^{-/-}, NLK^{-/-}) Clone D (derived from Todd Waldman's cells)
- 2) HCT116 (PTEN^{+/+}, NLK^{-/-}) Clone A and Clone E; HCT116 (PTEN^{-/-Φ}, NLK^{-/-}) Clone ΦA and Clone ΦG (derived in this study)

It needs to be emphasised that these clones that were fully characterised as NLK KOs, demonstrated exactly the same expression bands in the Western Blot analysis as NLK WT lines upon staining with both batches of anti-NLK antibodies (Figure 8.4). These data confirm that this antibodies bind non-specifically to another protein of a similar size to NLK in the tested cell lines. Given such results, an alternative method of detecting NLK expression was required in order to screen clones in the following NLK knock-in experiments. For this, a small epitope tag, FLAG⁺⁺⁺⁺⁺, was fused in-frame with NLK. Thus, the cells expressing NLK would also express the FLAG marker, which can be blotted against well-characterised and widely used monoclonal anti-FLAG antibodies.

⁺⁺⁺⁺⁺ The Flag tag consists of the eight amino-acid peptide (DYKDDDDK, 1012 Da). When the FLAG coding sequence is fused to the coding sequence of the gene of interest, the resulting recombinant fusion protein can be detected with monoclonal anti-FLAG antibody (Chubet, Brizzard 1996, Einhauer, Jungbauer 2001).

8.1.3. NLK plasmid generation

To allow ectopic expression, constructs coding for NLK were generated in order to use them in kinase and cellular assays. Initially, the analogue sensitive NLK_T219G mutant was synthesised by Ms Sara Bik using pDONR233-NLK plasmid as a template (Johannessen et al. 2010).

NLK_WT and NLK_T219G coding sequences were then cloned into vectors that were used for further experiments such as (i) NLK protein expression using baculovirus expression system for performing kinase assays; and (ii) NLK knock-in into human cells via sleeping beauty transposons. Two vectors were chosen for these experiments: (i) pFAST-BAC1-GST vector and (ii) pSBbi-GB transposon vector. To enable the introduction of the epitope tag, a pcDNA3-FLAG vector was used as an intermediate vector prior to cloning the fused gene into the final pSBbi-GB transposon vector.

Initially, NLK_WT and NLK_T219G coding sequences were amplified via PCR using primers that were flanked with EcoRI and HindIII restriction sites (Table 8.5). A stop codon (TAA) was incorporated at the 3' primer, and additional 2 nucleotides (CG) were incorporated at the 5' primer prior NLK start codon (ATG) in order to align the NLK frame with the upstream GST and FLAG frames within the target vectors (pFAST-BAC1-GST and pcDNA3-FLAG respectively). Besides, these two nucleotides would contribute to encode an additional proline amino-acid (encoded by the most commonly used codon for this amino acid in human genome, CCG) that is known to be a good linker for fusion proteins (Chen, Zaro & Shen 2013). This is because proline lacks amide hydrogen, which can prevent the formation of hydrogen bonds between other amino-acids within two protein domains, and therefore reduce the potential intramolecular interactions between them. As a result, the addition of proline might increase the structural independence of the domains (Chen, Zaro & Shen 2013).

Table 8.5. Thermocycling conditions and primers designed for NLK_WT and NLK_T219G PCR amplification. 5' primers initiates PCR amplification in 5' -> 3' direction, whereas 3' primer was designed to initiate NLK amplification in 3' -> 5' direction. 5' and 3' primers are flanked with EcoR1 and HindIII restriction sites respectively (shown in low cases and highlighted with bold font). Additional two nucleotides CG in front of the starting codon are highlighted in red. The stop codon is shown in green. The primers read from 5' to 3'.

	Specific primers			T melting, °C	PCR product
NLK primers	5': gaattcCG ATGGCGGCTTACAATGG 3': aagcttA TCACTCCCACACCAGAGG			69.9 69.2	1563 bp

Polymerase	Q5 High-Fidelity			
	T, °C	Duration	No of cycles	
Initial denaturation	98	30 s	1	
Denaturation	98	8 s	30	
Annealing	72	50 s		
Extension				
Final extension	72	2 min	1	
Hold	4	Indefinite	-	

PCR composition (50 µl)
0.5 ng DNA template 1x Reaction Buffer 300 ng each primer 400 µM each dNTP 0.5 µl polymerase

The amplicons were A-tailed and ligated into the pGEM-T vector. The inserts and junctions with the vector backbone were verified by sequencing (Appendix 12.2).

The FLAG-NLK_WT or FLAG-NLK_T219G insets were then cloned into pFAST-BAC1-GST vector via EcoRI and HindIII sites and into pcDNA3-FLAG via EcoRI and NotI sites⁺⁺⁺⁺⁺. Then, the fusion genes from the pcDNA3-FLAG-NLK constructs were cloned into pSBbi-GB sleeping beauty vector via HindIII restriction site. For this, pSBbi-GB was linearised with HindIII and dephosphorylated, followed by ligation of the insert sequences flanked with HindIII sites. The resulting constructs were characterised by restriction digestion and sequencing of backbone vector junctions with inserts (Appendix 12.2). The schematic illustration of construct maps is shown on Figure 8.9.

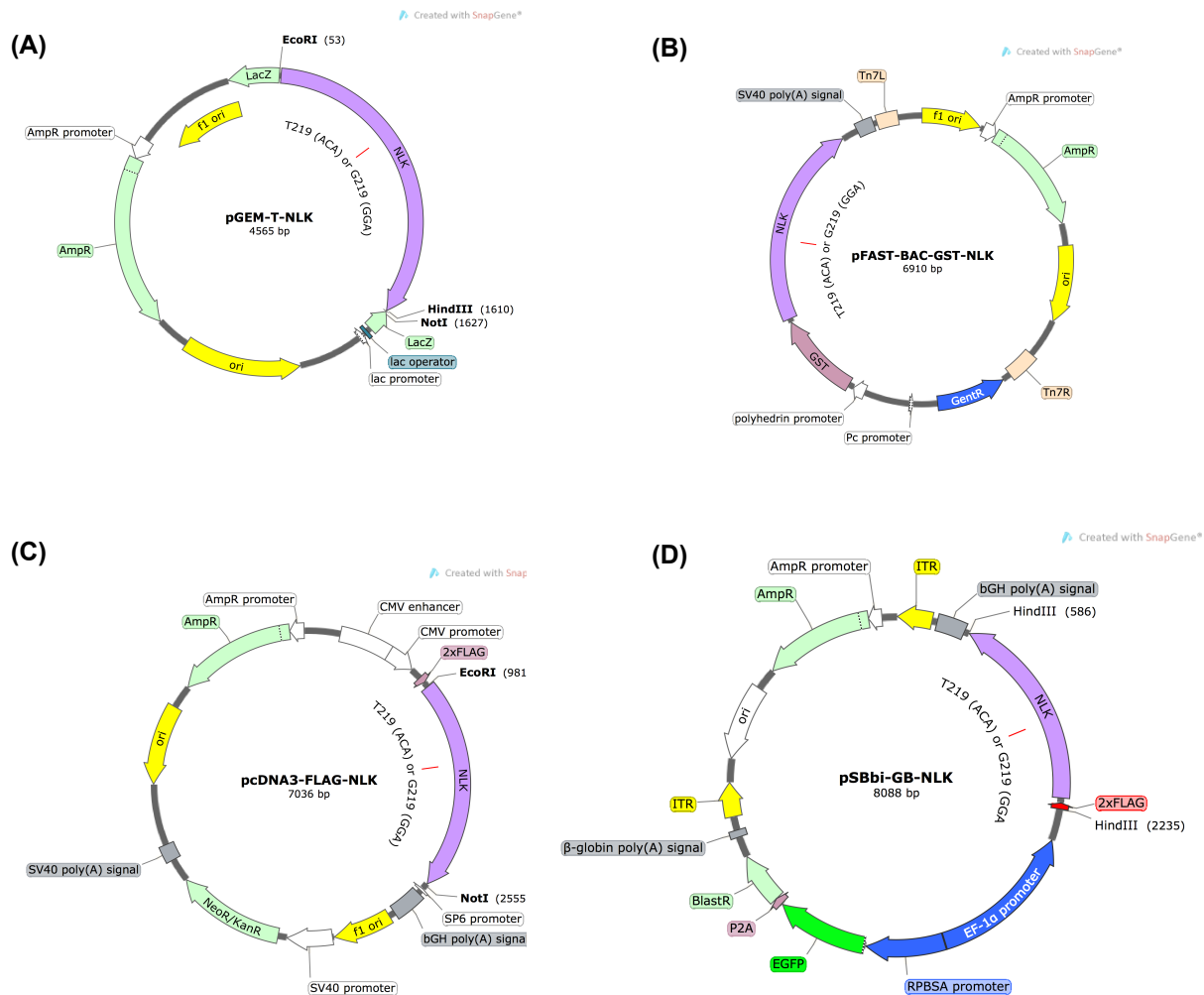


Figure 8.9. Plasmid maps of target vectors encoding NLK_WT or NLK_T219G. **(A)** pGEM-T-NLK. The NLK sequence is flanked with EcoRI and HindIII sites. The NotI site within the pGEM-T vector backbone was used for further cloning into pcDNA3-FLAG. **(B)** pFAST-BAC1-GST-NLK. The GST protein is located in-frame with NLK sequences. Two flanking sites Tn7L and Tn7R are called mini-Tn7 region that attaches to the mini-att-Tn7 site on the bacmid DNA with the help of transposase, which initiates site-specific recombination between these sites

⁺⁺⁺⁺⁺ Prior cloning into the pcDNA3-FLAG vector, the orientation of NLK inserts in pGEM-T backbone was assessed in order to use NotI restriction enzyme at the 3' end of NLK sequences. Such orientation is shown on Figure 8.9(A).

(see next Chapter 8.1.4 for more details). **(C)** pcDNA3-FLAG-NLK. Two tandem FLAG epitope tags are located in frame with NLK sequence. **(D)** pSBbi-GB-NLK. EF1 α /RPBSA bi-directional promoter regulates (i) NLK-FLAG expression in the 3' to 5' direction; and (ii) GFP and blasticidin (BlastR) selectable markers separated by 2A peptide in the 5' to 3' direction. The two inverted ITR segments indicate sites where the transposase can bind and cut. Red lines inside maps show the position of T219 (NLK^{WT}) or G219 (NLK^{AS}).

8.1.4. Bac-to-Bac baculovirus system for NLK expression

8.1.4.1. Introduction

Baculovirus expression system utilises eukaryotic insect cells to produce recombinant proteins. Such a system has several advantages over the bacterial protein expression. Thus, eukaryotic proteins expressed in bacterial cells often can be non-functional because these cells are not equipped to implement the post-translational modifications and/or molecular folding that could be required for protein activity, function, structure, solubility and stability (Yin et al. 2007). For instance, eukaryotic proteins expressed in prokaryotic systems often become insoluble.

The viral vector used in the system, originally isolated from *Autographa californica* nuclear polyhedrosis virus (AcNPV), has a large (130 kb) circular DNA genome. As a result, the method is based on site-specific transposition of a gene of interest from a donor plasmid to a cloned baculoviral DNA (known as bacmid) such that gene of interest is controlled by the strong baculovirus-specific polyhedron promoter (Kost, Condreay & Jarvis 2005). The bacmid contains the bacterial transposon region, mini-att-Tn7, that does not disturb the LacZ reading frame. The donor plasmid encodes a gene of interest flanked with complimentary mini-Tn7 sites (Figure 8.10). Upon transposase-mediated site-specific transposition, the *LacZ* gene is disrupted, which prevents LacZ expression to facilitate generation of recombinant bacterial colonies (Airenne et al. 2003, Laitinen et al. 2005). After successful transposition, recombinant bacmids are used to transfect insect cells for viral amplification, following by infecting fresh insect cells for subsequent protein expression. Such a system is also biosafe as baculovirus is incapable of replicating in mammalian cells and does not naturally infect humans (Kost, Condreay 2002, Hu 2005).

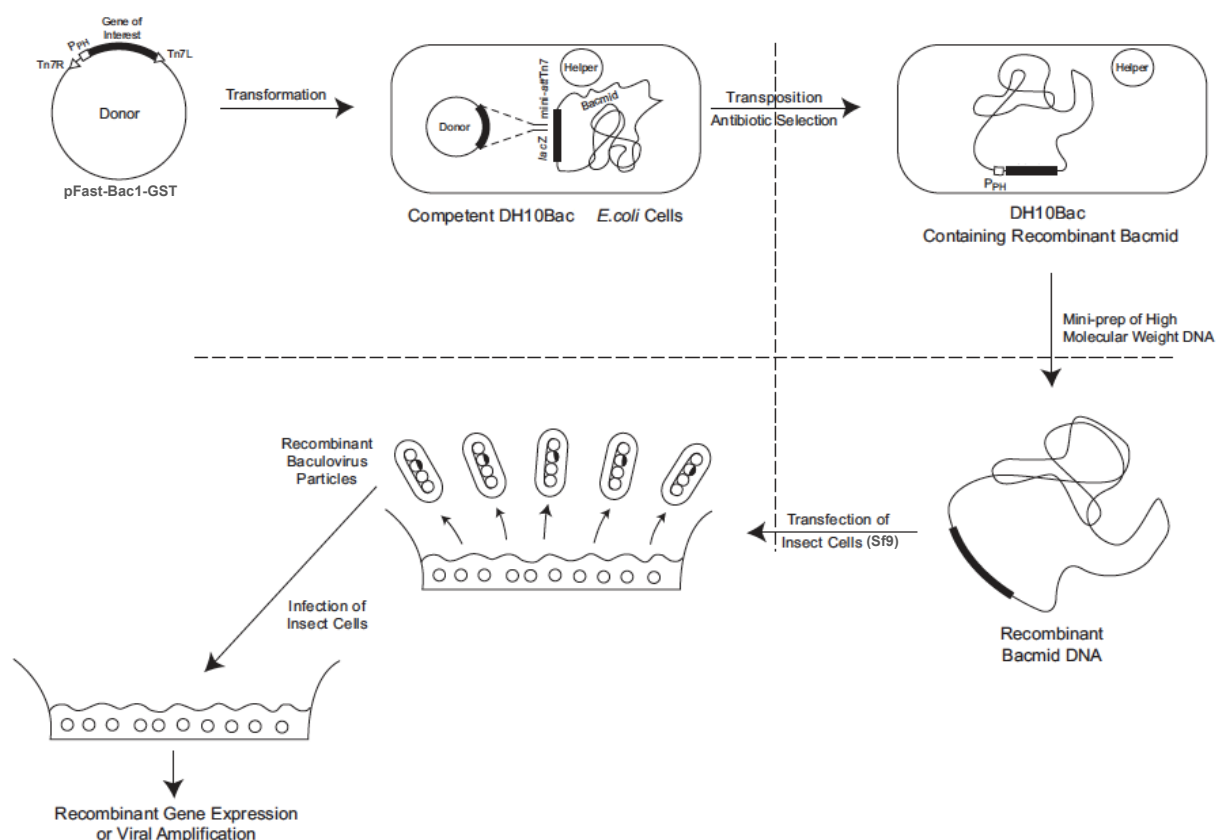


Figure 8.10. Generation of recombinant baculoviruses and protein expression via Bac-to-Bac Expression System. The gene of interest under polyhedron promoter (P_{PH}) is cloned into a pFast-Bac-based donor plasmid (pFast-Bac1-GST, in this study), and the recombinant plasmid is transformed into DH10Bac *E. coli* cells that contain the bacmid with a mini-att-Tn7 site and the helper plasmid. The mini-Tn7 region on the pFast-Bac1 donor plasmid transposes to the mini-att-Tn7 site on the bacmid DNA in the presence of transposase provided by the helper plasmid. Bacterial colonies containing recombinant bacmids are identified by disruption of the *lacZ* gene. Mini-prep for high molecular weight DNA is prepared from selected white *E. coli* clones containing the recombinant bacmid. This DNA is then used to transfect insect cells. After viral amplification the supernatant from the cultured cells will contain viral particles which can be used to infect more fresh insect cells for protein expression. Figure modified from Invitrogen User Guide (Bac-to-Bac Baculovirus Expression System)

8.1.4.2. Generation of recombinant NLK baculoviruses

Recombinant baculovirus encoding NLK_{WT} or NLK_{T219G} were generated according to Invitrogen Bac-to-Bac manual (Bac-to-Bac Baculovirus Expression System, 10359). The donor pFast-Bac1-GST-NLK plasmids containing NLK_{WT} or NLK_{T219G} flanked between mini-Tn7L and mini-Tn7R sites, was transformed into DH10Bac. The cells were cultured under kanamycin (bacmid resistance marker), tetracycline (helper plasmid resistance marker) and gentamicin (pFast-Bac1-GST-NLK resistance marker) selection in the presence of a chromogenic substrate X-gal and an IPTG inducer for LacZ visualisation. 36 hours later the white bacterial colonies were inoculated and cultured in liquid media for 24 more hours. The

recombinant NLK_WT and NLK_T219G bacmids were purified using a mini-prep protocol adopted for high-molecular weight plasmids (Invitrogen manual).

Since recombinant bacmid DNA is greater than 135 kb in size, restriction analysis is difficult to analyse. Thus PCR analysis is used instead to confirm that *NLK* has transposed to the bacmid. PCR of purified bacmids was performed using pUC/M13 Forward and Reverse primers (Table 8.6) that hybridise to sites flanking the mini-att-TN7 fragment within the bacmid DNA (Figure 8.11(A)). Several polymerases (GoTaq G2, Q5 high-Fidelity, Phusion High-Fidelity or *PfuUltra* II Fusion HS) and several thermocycling conditions were tested and those giving the best result are shown in Table 8.6.

Table 8.6. Thermocycling conditions and primers for amplifying bacmids transposed by NLK constructs. F and R – Forward and Reverse primers. Primers are shown in 5' -> 3' orientation.

	Specific primers		T melting, °C	Bacmid only	Bacmid+NLK-GST
pUC/M13	F: 5'-CCCAGTCACGACGTTGTAAAACG-3'	R: 5'-AGCGGATAACAATTCACACAGG-3'	69.3 65.8	~ 300 bp	~ 4500 bp

Polymerase	PfuUltra II Fusion HS			PCR composition (20 µl) 15 ng bacmid DNA 1x Reaction Buffer 40 ng each primer 500 µM each dNTP 0.4 µl polymerase
	T, °C	Duration	No of cycles	
Initial denaturation	93	3 min	1	
Denaturation	94	45 s	30	
Annealing	55	45 s		
Extension	72	5 min		
Final extension	72	7 min	1	
Hold	4	Indefinite	-	

The primers would amplify the PCR product of ~300 bp if the transposition was not successful and ~4500 bp if GST-NLK sequences were transposed into the bacmid DNA (Figure 8.11(A)). The agarose gel with PCR amplicons demonstrated that both NLK_WT and NLK_T219G genes (fused with GST) were successfully incorporated within the recombinant bacmids (Figure 8.11(B)).

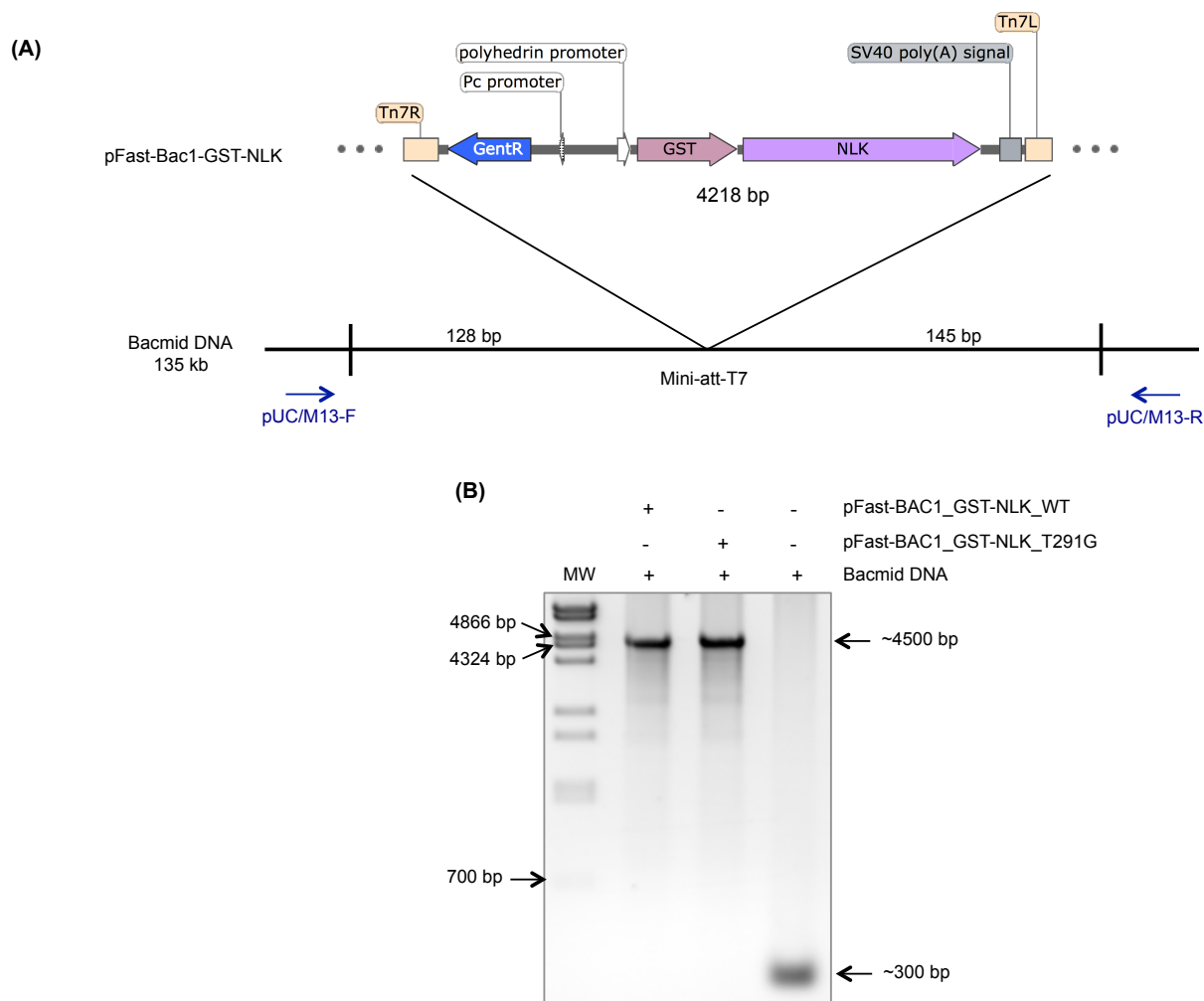


Figure 8.11. PCR amplification of NLK recombinant bacmid. (A) Schematic representative of PCR product (~4500 bp) in case if NLK transposition into the bacmid was successful. pUC/M13-F and pUC/M13-R primers were used to initiate the PCR reaction (blue arrows). (B) 1% Agarose gel with PCR amplicons from bacmids transposed with pFAST-BAC1_GST-NLK_WT, pFAST-BAC1_GST-NLK_T291G or bacmid alone. Bands representing successful transposons into bacmid (~4500 bp) and empty backbone (~300 bp) are indicated with arrows. MW-DNA ladder.

GST-NLK_WT and GST-NLK_T219G bacmid DNA (~ 5 µg) were transfected into Sf9 insect cells using Lipofectamin 2000 reagent. Several ratios of DNA:Lipofetamine 2000 were tested (1:3-1:5) and all conditions demonstrated successful transfection of cells. 7-8 days after transfection, the supernatant containing baculovirus (P1 stock) was harvested and high-titer stocks (P2 and P3) were generated by sequential infecting fresh Sf9 cells for 6-7 more days for each passage. The efficiency of NLK protein expression was assessed via western blotting after infecting fresh Sf9 cells for 72 hours. For this, Sf9 lysates were subject to immunoblotting against GST tag using anti-GST antibody. Both P2 and P3 baculovirus stocks caused high expression of the desired NLK-GST fusion proteins (Figure 8.12(A)). Interestingly, anti-NLK antibodies (Abcam more so than SantaCruz antibodies) also demonstrated specific binding in

Sf9 cells (Figure 8.12(B)), suggesting that non-specific binding observed in HCT116 cells may have been cell-specific (or species-specific).

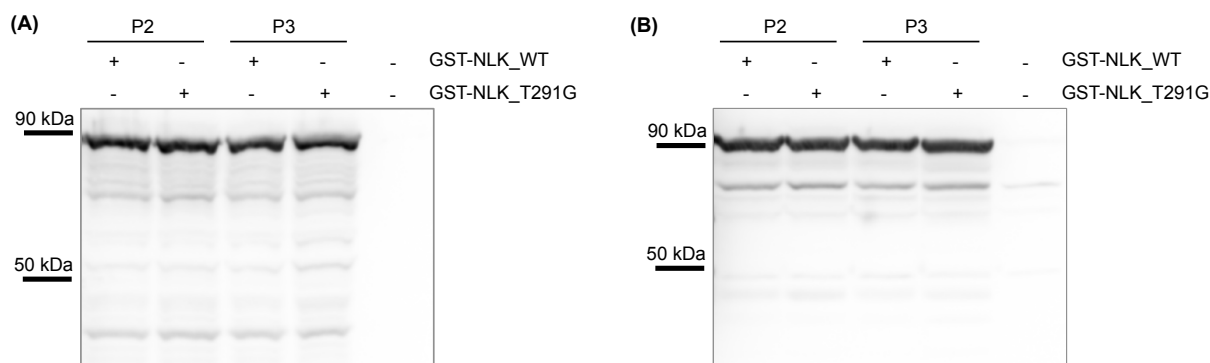


Figure 8.12. NLK_WT and NLK_T291G expression levels in Sf9 cells infected with baculovirus stocks (P2 and P3). Sf9 cells were infected with NLK_WT or NLK_T291G baculoviruses and incubated for 6 days with two different passages (P2, P3). Non infected cells were included as control of non-specific binding. After incubation cells were lysed and subject to western blotting and staining with **(A)** anti-GST or **(B)** anti-NLK antibodies. The NLK-GST fusion proteins are expected to be 83 kDa.

8.1.4.3. Expression of recombinant NLK proteins

Both NLK_WT and NLK_T291G baculoviruses were used for expression and purification of recombinant GST-tagged proteins. For this Sf9 cells were infected with each virus for 3 days following by lysis and purification. NLK_WT and NLK_T291G proteins were immobilised on glutathione Sepharose beads and used as 50% glycerol suspension for further characterisation and inhibition experiments. NLK kinase activity was assessed by detecting phosphorylation of its commercially available substrate, recombinant Myelin Basic Protein (MBP).

8.1.5. NLK^{WT} and NLK^{AS} characterisation and inhibition *in vitro*

8.1.5.1. Introduction

In order to determine if the NLK_T291G gatekeeper mutation enlarged the NLK active site enough to accept the bulky monospecific kinase inhibitor 3-MBPP1 compared to NLK_WT site, kinase activity of NLK and its analogue were assayed in the presence of the inhibitor. Initially, kinase activity of both recombinant NLK proteins was detected in the MBP assay. MBP (24 kDa) was chosen as a NLK substrate because it is a commercially available artificial substrate that is phosphorylated at multiple Ser and Thr sites (Martenson, Law & Deibler 1983, Harauz et al. 2004) by many kinases such as MAPK (Dudley et al. 1995, Kinoshita et al. 2006), ERK2 (Kinoshita et al. 2014), MEK1 (Dudley et al. 1995, Pirman et al. 2015), PINK1 (Kane et al. 2014), TTK (Hewitt et al. 2010). MBP is commonly used as a substrate *in vitro* for kinase assays and there is one commercially available recombinant NLK assay kit supplied with MBP

as a substrate of choice for activity assays (N15-10G SignalChem, USA). Based on this information MBP was chosen as a substrate for checking NLK_WT and NLK_T210G activity. Knowing that MBP has multiple phosphorylation sites and none of them are known to be specific for NLK kinase, it was not possible to use a phospho-specific antibodies to monitor the reaction. Thus, an alternative method had to be chosen. Historically radiolabelling of ATP by [γ - ^{32}P] is common for kinase assays. However, in 2002 Prof. Koike's laboratory (Hiroshima University, Japan) reported a label-free method for visualising protein phosphorylation by a selective phosphate-binding tag molecule, Phos-tag (Takeda et al. 2003). Since then, different analytical methods have been developed using Phos-tag analogues, for instance Mn-Phos-tag SDS-PAGE method that incorporates a dinuclear Mn^{2+} Phos-tag complex into polyacrylamide gel structure (Figure 8.13). Such gel modification provides characteristic separation patterns for non-phosphorylated proteins from phosphorylated according to the number and site of phosphate groups (Kinoshita et al. 2006, Kinoshita-Kikuta et al. 2007, Kinoshita et al. 2008, Kinoshita, Kinoshita-Kikuta & Koike 2009, Kinoshita et al. 2014). This method is becoming widely used for detecting phosphorylation (including of MBP) both *in vitro* and *in vivo* (Barbieri, Stock 2008, Hewitt et al. 2010, Kane et al. 2014, Pirman et al. 2015, Sala et al. 2014). Thus, Mn-Phos-tag SDS-PAGE was chosen to detect MBP phosphorylation by NLK proteins.

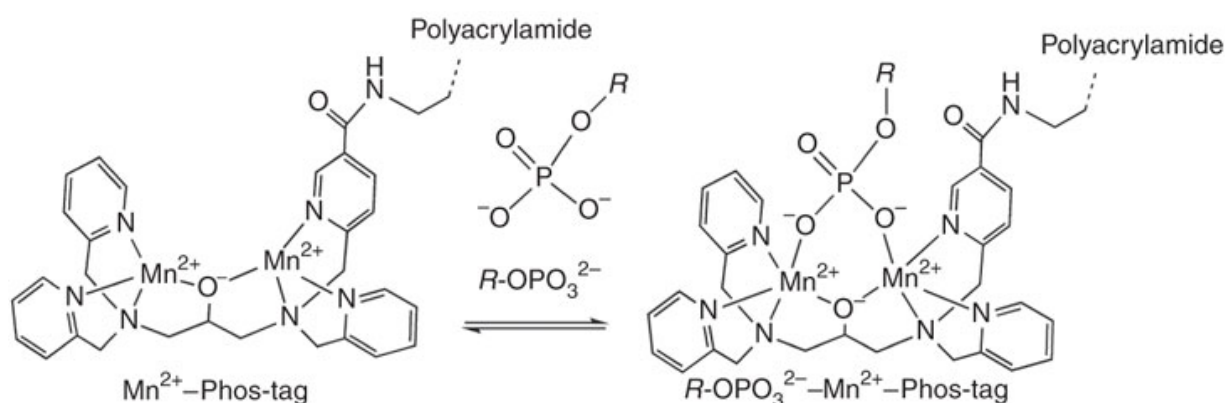


Figure 8.13. Schematic representative of Phos-Tag SDS-PAGE. Phosphorylated proteins migrate in the gel slower than non-phosphorylated proteins due to specific binding of phosphate groups to Phos-Tag polyacrylamide. Scheme adopted from (Kinoshita, Kinoshita-Kikuta & Koike 2009).

8.1.5.2. Kinase assay

The protocol for the kinase assay was performed similar to that reported earlier (Elphick et al. 2009) and also based on commercially available NLK kits (from Sigma, SignalChem and Sino Biological Inc). Immobilised NLK_WT and NLK_T219G were incubated with MBP in the presence or absence of ATP as a phosphate donor (Figure 8.14). Two different kinase buffers (TRIS-HCl and MOPS-based) were compared. Purified MAPK was used as a positive control as a well-characterised MBP kinase.

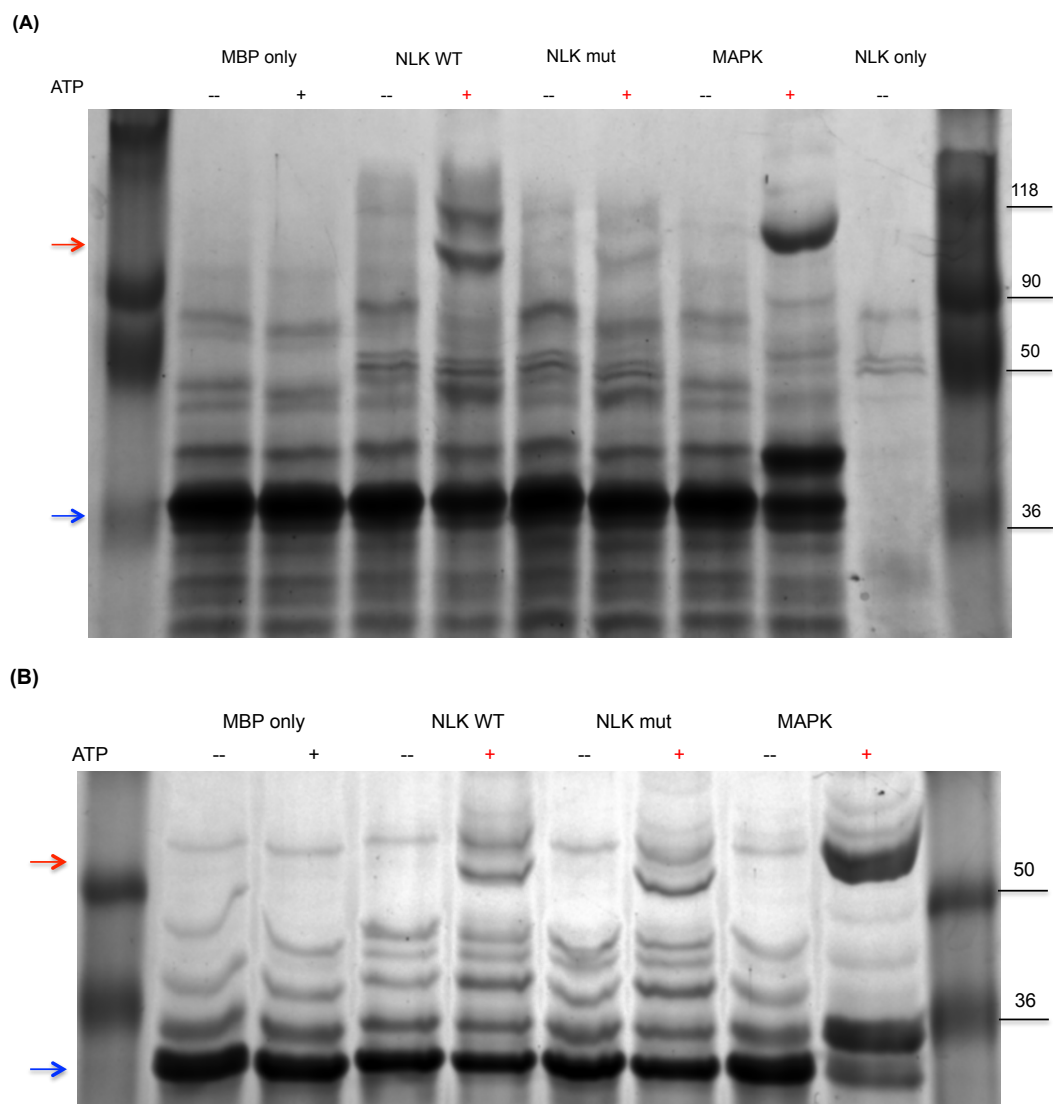


Figure 8.14. Assay of purified NLK proteins. MBP (4 μ g) was incubated with the indicated kinase in the presence or absence of ATP (1 mM) for 30 min at 30° C in **(A)** TRIS-HCl or **(B)** MOPS-based kinase buffers. Phosphorylated and non-phosphorylated MBP were separated on 12.5% Phos-Tag SDS-PAGE. MBP samples treated with no kinases or no phosphate donors were included as controls of non-phosphorylated MBP levels. Red arrows and red font indicate bands assigned to the phosphorylated MBP (red arrows) and non-phosphorylated MBP (blue arrows).

If kinases triggered MBP phosphorylation, the Phos-tag polyacrylamide complex would specifically retard the migration of the phosphorylated MBP causing an upward mobility shift on the gel. Phosphorylated MBP would appear as a slower migrated band compared with corresponding non-phosphorylated substrate, which would be possible to visualise using a generic anti-MBP antibody. Slower migrating bands were clearly detected in MBP samples incubated with all tested kinases in the presence but not the absence of ATP. The MOPS-based kinase buffer demonstrated a better result, thus it was chosen for the next inhibition

experiments. Interestingly, both NLK_WT and NLK_T219G kinases phosphorylated MBP with an equal intensity (in MOPS kinase buffer), indicating that the gate keeper mutation appeared not to affect the kinase activity of the mutant with wild type and mutant versions of NLK phosphorylating similar amounts of MBP in the assay. This result was in a good correlation with another serine-threonine kinase, Cdk2: no substantial decrease in Cdk2 enzymatic activity was detected upon substitution of the gatekeeper residue F80 with glycine lacking a side chain (Kraybill et al. 2002, Elphick et al. 2009). The gate-keeper residue is usually located deep within the ATP-binding pocket of the kinase, distant from their interaction and catalytic surfaces, thus it would be unlikely that the mutation of this residue would affect the recognition, binding and phosphorylation of substrates.

Next, the enlargement of the ATP binding site by the mutation of the gatekeeper was tested to determine if it enabled the NLK_T219G to bind the bulky ATP-competitive inhibitor, 3-MBPP1, or N⁶-modified ATP analogue, N⁶-cyclohexyl-ATP (CxATP) compared to the wild type NLK. Previously Elphick *et al.* showed that enlarged ATP binding site of Cdk2 was able to utilise both endogenous wild type and CxATP with a bulky side chain as a phosphate donor, while wild type Cdk2 was unable to utilise the ATP analogue as a phosphate donor (Elphick et al. 2009). Similarly, bulky purine analogues such as 1-NMPP1 and 3-MBPP1 are commonly used inhibitors for analogue-sensitive kinases because they specifically block enlarged but not wild type ATP binding sites in a variety of kinases including Cdk1 (Bishop et al. 2000), Cdk2 (Bishop et al. 2000, Kraybill et al. 2002, Elphick et al. 2009), Cdk7 (Merrick et al. 2008), Plk1 (Burkard et al. 2007, Burkard, Santamaria & Jallepalli 2012) and v-Src (Kraybill et al. 2002) among others. 3-MBPP1 was reported to exhibit a higher specificity analogue-sensitive kinases compared to the originally developed 1-NMPP1 inhibitor (Merrick et al. 2008).

For this analogue-sensitive experiment, MBP was incubated in the presence of kinases with or without 150 nM of 3-MBPP1. This inhibitor concentration was chosen to be slightly higher than the IC₅₀ for analogue sensitive MAPK (78 nM) determined in our laboratory (unpublished work). The aim was to use an optimal inhibitor concentration that would be enough to inhibit the NLK_T219G mutant but would not be too high for non-specific activity. Both ATP and CxATP were used as phosphate donors, MAPK^{WT} and MAPK^{AS} previously studied in the laboratory (Dr Charlotte Allen, unpublished work) were used as a positive control for this experiment. As expected, the large cyclic adduct CxATP was potent phosphate donor for gate-keeper mutants of both MAPK and NLK, whereas wild type counterparts of these kinases could utilise only endogenous ATP (Figure 8.15). Interestingly, the gate-keeper NLK mutant utilised the wild type ATP and its analogue with the same specificity, while MAPK^{AS} phosphorylated MBP with the reduced activity when exposed to ATP analogue. Likewise, 3-MBPP1 significantly inhibited MAPK^{AS} activity and completely abolished NLK_T219G activity, whereas wild type kinases were not affected by this compound.

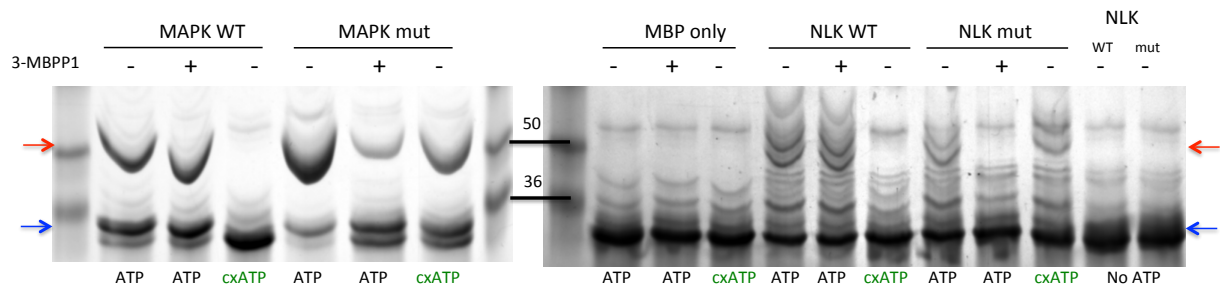


Figure 8.15. Analogue sensitive inhibition of **(A)** MAPK^{WT} and MAPK^{AS} and **(B)** NLK^{WT} and NLK^{AS} proteins in a kinase assay upon treatment with ATP or CxATP +/- 3-MBPP1. 4 µg MBP was incubated with each kinase individually in the presence or absence of MBPP1 (150 nM) and with 1 mM ATP or CxATP as phosphate donors for 30 min at 30° C. Phosphorylated and non-phosphorylated MBP were separated on 12.5% Phos-Tag SDS-PAGE. MBP samples treated with no kinases or no phosphate donors were included as controls of non-phosphorylated MBP levels. Arrows indicate bands assigned to the phosphorylated MBP (red arrows) and non-phosphorylated MBP (blue arrows).

This result illustrates that the mutation of the gate-keeper T219 residue in NLK to an amino acid with a smaller side chain generated an analogue-sensitive kinase that can be utilised as a highly specific tool for the unique inhibition of this kinase *in vitro*.

Based on the above results it was expected that if the T219G mutant NLK was introduced into NLK^{-/-} cells, NLK activity should be exclusively inhibited by 3-MBPP1 with minimal effect on endogenous kinases. Previously 3-MBPP1 treatment was reported to specifically inhibit analogue sensitive mutants of Plk1 (Burkard et al. 2007, Burkard, Santamaria & Jallepalli 2012) and Cdk7 (Merrick et al. 2008) *in vivo* in RPE1 and HCT116 cells respectively.

8.1.6. NLK knock-in via Sleeping Beauty Transposons

NLK^{-/-} HCT116 cells derived in both PTEN^{-/-} and PTEN^{+/+} (from TW and this study) were co-transfected with the pSBbi-GB-NLK (WT or T219G mutant) and pCMV(CAT)T7-SB100 encoding the SB100X transposase in the presence of Lipofectamine 2000 according to Table 8.7.

Table 8.7. The composition of SB transfection mixture for NLK knock-in.

	DNA	Lipofectamine 2000	pSBbi-GB-NLK	pCMV(CAT)T7-SB100
ratio	1	3	19	1
Amounts, µg	4	12	3.8	0.2

After transfection cells were split at different dilutions and grown in media supplemented with blasticidin (10 µg/ml) to allow selection of cells with successful transposon integration. Resistant clones were analysed via flow cytometry to detect the GFP and Western blotted for the FLAG tagged NLK.

pSBbi-GB-NLK_WT and pSBbi-GB-NLK_T219G plasmids encode GFP, which was utilised for the initial screening of the blasticidin-resistant cell clones. Fluorescence was compared for pools and 3-6 individual knock-in clones from each parental cell line with various PTEN and NLK statuses (PTEN^{+/+} NLK^{-/-G} and PTEN^{-/-} NLK^{-/-D} from HCT116 cells derived from Todd Waldman's cells and PTEN^{+/+} NLK^{-/-E} and PTEN^{-/-Φ} NLK^{-/-A} from HCT116 cells developed within this study). All screened colonies and pools were GFP-positive. The flow cytometry data showed that the pools expressed higher GFP levels than most of the clones and, like the PTEN knock-in experiments, similar GFP fluorescence intensity was detected in all knock-in pools. However, a range of distinct intensities (varying up to 40 times) was observed in individual clones, suggesting different integration efficiencies upon transposition in different cells. Similar to PTEN knock-in screening, those clones emitting GFP levels of a similar intensity were chosen for further *in vivo* experiments. As mentioned earlier in Chapter 5.4.2 it is expected that the colonies expressing comparable GFP levels would express similar PTEN levels as well, an important parameter for comparing the effect of NLK inhibition on cell viability.

Individual clones expressing comparable GFP levels from pSBbi-GB-NLK_WT and pSBbi-GB-NLK_T219G transposition (one for each *PTEN/NLK* genotype) were selected for further Western Blot analysis.

All knock-in clones expressed similar levels of FLAG confirming flow cytometry data (Figure 8.16). Interestingly an anti-NLK antibody (AbCam) demonstrated detectable levels of exogenous NLK only, indicating that the endogenous NLK signal in HCT116 cells was too low to detect, further clarifying earlier results (Figure 8.4).

To test the efficacy of the NLK knock-out and NLK knock-in clones, the intracellular pathways that are influenced by NLK localisation and activity were assessed. NLK is known to negatively regulate Forkhead box O (FOXO1, FOXO3a and FOXO4) transcription factors by binding and phosphorylation at multiple Ser/Thr residues of their transactivation domain (Kim et al. 2010, Szypowska et al. 2011, Eijkelenboom, Burgering 2013). Kim *et al.* reported that FOXO1 phosphorylation by NLK inhibited its transcription activity and excluded it from the nucleus (Kim et al. 2010). This process was shown to be independent of the PIP3K/Akt pathway. In contrast, NLK knock-down with siRNA leads to dephosphorylation of FOXO1 and causes its translocation into the nucleus in mammalian HEK293T and COS1 cells. At the same time, FOXO proteins modulate the levels of target genes involved in apoptosis, stress response, DNA repair, glucose metabolism and cell cycle, for instance up-regulation of p27 and down-regulation of Cyclin D1 that is associated with phosphorylation and inactivation of the pRb (Schmidt et al. 2002, Myatt et al. 2010, Liu et al. 2014). Consistent with this, exogenous expression of NLK reduced p27 protein levels while NLK knock-down with siRNA increased p27 expression in HEK293T cells (Szypowska et al. 2011).

On the other hand, NLK down-regulates Notch signalling (Ishitani et al. 2010), and Notch1 was shown to increase expression levels of Skp2 by binding to its promoter and thus decrease p27 levels in T-cell acute lymphoblastic leukemia cells (Dohda et al. 2007). Taking together, these studies indicate that depending on the cell type, NLK can either up-regulate or down-regulate p27 levels.

Jung *et al.* reported that NLK knock-down via siRNA in Hep3B cells elicited a decrease in Cyclin D1 and CDK2 expression, and triggered hypophosphorylation of p130, a pRb protein, which was in a good correlation with NLK mitogenic function in hepatocellular carcinoma cells suggested by the authors (Jung et al. 2010). Quite the opposite, Sa *et al.* demonstrated that Cyclin D1 expression was down-regulated in glioblastoma cells overexpressing NLK (Sa et al. 2015). These contradictory findings also indicate that depending on the cell type and its endogenous function, NLK can either up-regulate or down-regulate cell cycle regulators (such as p27 and Cyclin D1) and thus play either pro- or anti-proliferative functions.

It was interesting therefore to check what function NLK plays in HCT116 cells and what influence PTEN would have. Because AKT inactivates FOXO1 by phosphorylation and thus decreases p27 protein levels in MEFs (Schmidt et al. 2002) and rat INS-1 cells (Fang et al. 2012), one would expect that up-regulation of phosphorylated FOXO1 in PTEN KO HCT116 cells as was observed in (Mendes-Pereira, Lord & Ashworth 2012), would also decrease p27 levels.

The Western blot results (Figure 8.16) demonstrated that (i) knock-out of NLK causes *in vivo* effects such as up-regulation of p27, p53 and to some extent p21 levels primarily in HCT116 cells developed in this study; and (ii) both exogenous NLK_WT and NLK_T219G exhibited identical *in vivo* activity, partially or fully rescuing NLK KO effects in both sets of cells (that is returning p53 and p27 levels back to NLK^{+/+} levels). These results indicate that NLK_WT and NLK_T219G act equally validating the gate keeper mutation as something that does not seem to alter *in vivo* function, which is very critical for further analogue-sensitive mutant use for *in vivo* kinase inhibition experiments.

Interestingly, the subtle differences in cell cycle regulator expression patterns between two sets of HCT116 cell lines were observed (perhaps due to passaging differences). Nevertheless, NLK expression was demonstrating overall pro-proliferative effects in both cells as p53 and Cdk inhibitors (p21 and p27) were down-regulated (both sets), whilst Cyclin D1 levels were up-regulated (this study's only cells).

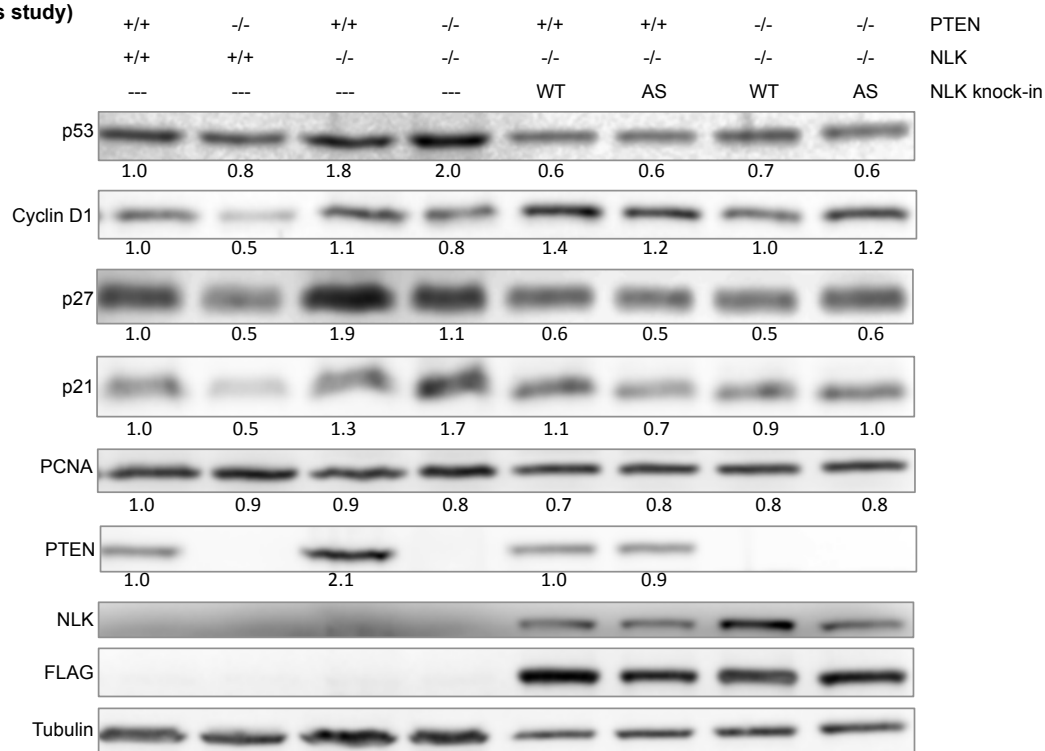
(A) TW HCT116**(B) HCT116 (this study)**

Figure 8.16. Effects of NLK knock-out (shown as NLK $-/-$) and knock-in (shown as WT for NLK_{WT} and AS for NLK_{T219G} knock-ins) in HCT116 clones with different PTEN status (shown as PTEN $+/+$ or $-/-$) on intracellular cell cycle regulators: **(A)** TW HCT116 cells; **(B)** this study's HCT116 cells. Cells were lysed with RIPA buffer and subject to western blotting for the indicated proteins. Tubulin was blotted as a loading control. The representative data of three independent experiments are shown. The numbers under the bands represent the ratio of

protein expression with control (PTEN^{+/+}NLK^{+/+}) samples arbitrary set as 1.0 for each protein and determined by ImageStudioLite (LI-COR Biosciences).

In conclusion, HCT116 cells developed in this study and to lesser extent in TW's group behaved comparable to HCT116 cells in (Mendes-Pereira, Lord & Ashworth 2012) and hepatocellular carcinoma cells in (Jung et al. 2010).

P53 data showing NLK-mediated down-regulation of p53 levels, were more consistent between tested cell lines but contradictory to published data (Zhang *et al.* 2014). Zhang *et al.* demonstrated that NLK interacted and stabilised p53 by blocking the MDM2-mediated ubiquitylation and degradation of p53 in NLK^{+/+} HCT116 cells compared to NLK^{-/-} cells, suggesting that NLK could enhance p53 activity in response to DNA damage (Zhang et al. 2014).

Most importantly, NLK^{WT} and NLK^{AS} knock-in cells exhibited the same phenotype and behaviour, indicating that gate keeper mutation did not affect NLK activity *in vivo* (at least in those pathways screened by Western blotting).

8.1.7. Synthetic lethality to NLK inhibition

The inhibition of NLK_WT and NLK_T219G knock-in cells was performed via MBPPP1 inhibition in cell viability assay to test for synthetic lethality between NLK and PTEN. For this NLK knock-in clones and pools with different PTEN status were incubated with increasing concentrations of 3-MBPPP1 for six days with the compound being refreshed after three days. Unfortunately, no difference in cell viability was detected in any pools or clones based on TW HCT116 cells (Figure 8.17).

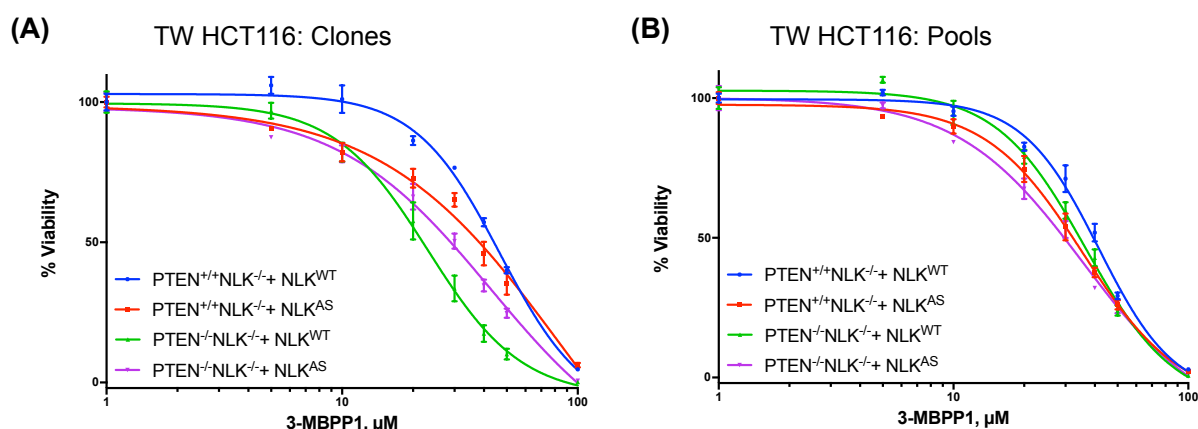


Figure 8.17. Sensitivity of PTEN deficient cells based on TW HCT116 to NLK^{AS} inhibition by 3-MBPPP1: (A) Clones; (B) Pools. PTEN^{+/+}NLK^{WT}, PTEN^{-/-}NLK^{WT}, PTEN^{+/+}NLK^{AS} and PTEN^{-/-}NLK^{WT} cells were plated into 96-well plates (0.05x10⁴) and treated with increasing concentrations of 3-MBPPP1 (5-100 μM) for six days. The inhibitor and media were changed on day three. The cell viability was determined by MTS assay. Control cells were treated with 0.5% DMSO. Each experiment was performed at least two times in triplicates. Each value represents the mean ± SD out of three independent repeats.

However, more in line with predicted results, when using the PTEN-engineered cells created here, 3-MBPP1 inhibition was significantly more toxic to PTEN^{-/-}NLK^{AS} clones and pools but not to PTEN^{-/-}NLK^{WT} pools and clones, and not to any PTEN^{+/+} cells (Figure 8.18).

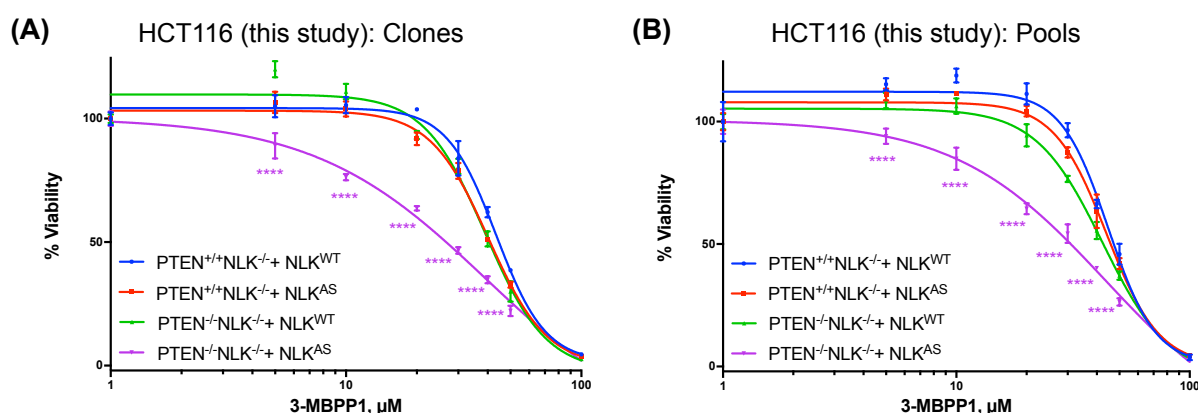


Figure 8.18. Sensitivity of PTEN deficient cells based on this study's HCT116 to NLK^{AS} inhibition by 3-MBPP1: **(A)** Clones; **(B)** Pools. PTEN^{+/+}NLK^{WT}, PTEN^{-/-}NLK^{WT}, PTEN^{+/+}NLK^{AS} and PTEN^{-/-}NLK^{WT} cells were plated into 96-well plates (0.05×10^4) and treated with increasing concentrations of 3-MBPP1 (5-100 μ M) for six days. The inhibitor and media were changed on day three. The cell viability was determined by MTS assay. Control cells were treated with 0.5% DMSO. Each experiment was performed at least two times in triplicates. Each value represents the mean $\pm$ SD out of three independent repeats. A significant difference in cell viability was calculated and compared to the PTEN^{+/+}NLK^{WT} lines on survival curves (**** $p < 0.0001$, two-way ANOVA).

Such exclusive inhibition indicates that bulky 3-MBPP1 inhibitor was only able to bind to the enlarged NLK pocket within NLK^{AS} knock-in cells, creating a system in which the biological functions of NLK can be directly tested. In the context of synthetic lethality with PTEN, NLK inhibition clearly demonstrated specificity being present only in the NLK^{AS} cells and not the NLK wild type cells (and only in PTEN^{-/-} but not PTEN^{+/+} cells). These data establish the specificity of the 3-MBPP1 in this system and validate the existence of synthetic lethality between PTEN and NLK. Interestingly, NLK KO in PTEN^{-/-} cells did not cause synthetic lethality during generation of this genotype. This indicates that NLK activity might be compensated for by other kinases upon complete NLK knock-out allowing PTEN^{-/-}NLK^{-/-} cells to be viable. Acute chemical inhibition of exogenous NLK however did demonstrate synthetic lethality in PTEN^{-/-} cells (as the presence of inactive exogenous NLK still would not trigger the compensation effect). Alternatively synthetic lethality could be due to NLK binding activity in combination with kinase activity. In this case inhibition of NLK^{AS} would work as a dominant-negative protein that binds to a substrate, blocks access to it and thereby prevents its NLK-dependent phosphorylation. This binding of substrate(s) by inhibited NLK would prevent access of other proteins that may act as compensatory regulators in whereas in NLK KO cells, this blocking of access would not occur, allowing compensation. Indeed, a non-kinase activity of NLK was

previously reported to be important for p53 stabilisation and activation in response to DNA damage (Zhang et al. 2014).

8.1.8. Conclusions

This result confirms Mendes-Pereira *et al.* (2012) NLK siRNA findings and suggests that NLK inhibition indeed could be potentially beneficial for PTEN deficient cancers. However, the difference between this study's HCT116 cells and TW cells, as well as listed earlier glioblastoma or colorectal cells where NLK functions as tumour suppressor (Yasuda et al. 2003, Sa et al. 2015), suggest that such synthetic lethality therapy is likely to be extremely cell type dependent and several markers (such as p27 and p21) should be measured in advance to help predict the outcome of this therapy. However, there is a definite interest in development of small molecule inhibitors against NLK considering that two independent techniques (siRNA (Mendes-Pereira, Lord & Ashworth 2012) and chemical genetic inhibition of NLK (this study)) demonstrated comparable results.

Consequently it would be interesting to knock-in NLK_WT and NLK_T219G constructs into HCT116 cells expressing PTEN mutants to study PTEN structural requirements for this lethal sensitivity pair. It would be also beneficial to analyse all NLK knock-in clones and pools upon treatment with 3-MBPP1 by western blotting as an additional *in vivo* control of NLK inhibition with 3-MBPP1. Several more intracellular targets could be analysed as well together with analysed earlier cell cycle regulators (such as P-FOXO1, nucleus FOXO1, Cdk2, P-Rb).

Moreover, the analogue-sensitive NLK_T219G developed within this project can be potentially used as an effective genetic tool to discover more intracellular substrates for this kinase. Such methods were shown to be effective for Cdk1, Cdk2, Plk1 kinases among others kinases (Bishop et al. 2000, Burkard et al. 2007, Merrick et al. 2008, Burkard, Santamaria & Jallepalli 2012). Time constraints prevented further study of these exciting areas in the work described here.

9. PTEN inhibition

9.1. VO-OHpic immobilisation

9.1.1. Introduction

The specificity of VO-OHpic in human cells and its mode of action, as well as putative side effects and affected signalling pathways, have to be assessed by identifying the intracellular targets for this compound. It is known that VO-OHpic directly interacts with PTEN and inhibits its activity *in vitro* (Rosivatz et al. 2006). However, it is not known whether PTEN is the only target for this complex in human cells. Thus, it is important to define the cellular specificity of VO-OHpic, i.e. to identify all intracellular proteins that are direct targets for VO-OHpic.

Affinity chromatography is a widely used method for determination of the binding specificity of small molecules towards biomolecules (Hermanson, Mallia & Smith 1992, Katayama, Oda 2007, Terstappen et al. 2007, Lomenick, Olsen & Huang 2011, Lomenick et al. 2009, Kuramochi et al. 2008, Sato et al. 2010). Therefore, at the beginning of this project one of the aims of the project was to incorporate the VO-OHpic molecule onto a solid support and use it for affinity chromatography. In order to perform affinity chromatography using VO-OHpic it is first necessary to covalently couple this compound to a suitable solid support (Figure 9.1).

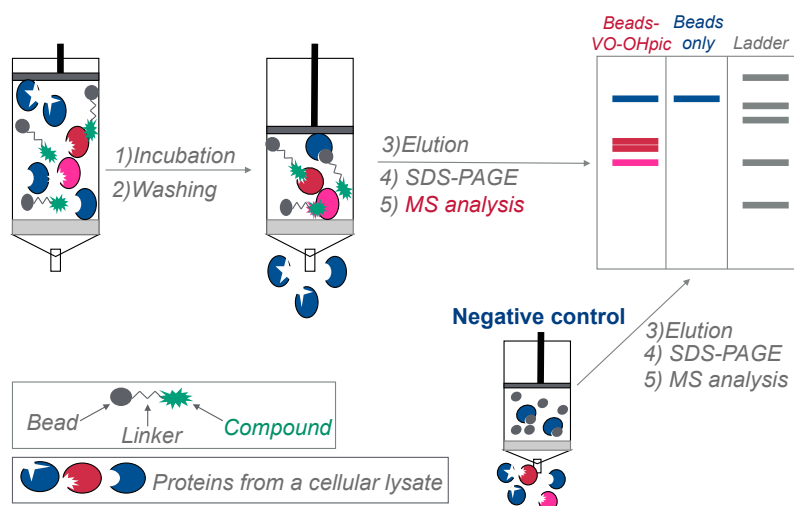
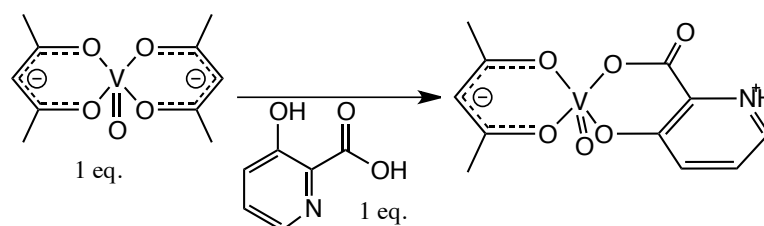


Figure 9.1. Affinity chromatography schematic. Coupled biologically active small molecules (VO-OHpic in this study) can be used for determination of their specificity and intracellular targets. Compounds covalently bound to the solid matrix should be incubated with a cellular lysate and after washing separated by SDS-PAGE. The proteins bound specifically to the immobilised compound can be identified by mass spectrometry analysis.

One of the main difficulties operating with VO-OHpic is how to purify and then characterise the product of coupling. Indeed, there would be no direct methods to prove the chemical structure

Prior to using the biotinylated ligand, non-modified picolinic acid (H-pic) together with 3-hydroxypicolinic acid (OH-pic) was used to test conditions for the synthesis and characterisation of an asymmetrical vanadyl complex. Several starting molecules were tested for the indirect complex building. VOSO_4 was used as a reagent previously (Fedorova et al. 2012) and gave a mixture of product complexes, which was impossible to purify. The next attempt was based on the building of the complex from the better chelating agents (i) $\text{VO}(\text{AcAc})_2$ and (ii) $[\text{VO}(\text{OHpic})(\text{H}_2\text{O})]_4$ under various conditions (Figure 9.2.C).

9.1.2.1. Asymmetric vanadium complex formation from $\text{VO}(\text{AcAc})_2$ with OH-pic



$\text{VO}(\text{AcAc})_2$ was chosen due to its wide use for asymmetric vanadium complexes syntheses from other ligand molecules (Galeffi et al. 1987, Asgedom et al. 1997, Chakravarty et al. 1993, Jarrais, Silva & Freire 2005, Maurya et al. 2006, Koo, Jang & Lee 2003a, Cornman et al. 1992, Smith et al. 2000, Aggarwal, Rai & Rao 1981, Guo et al. 2012, Smrečki, Kukovec & Popović 2012, Li, Lah & Pecoraro 1988, Ghosh et al. 2002, Fish et al. 1987, Cooper, Koh & Raymond 1982, Bolm, Bienewald 1996, Rehder et al. 2002, Amin et al. 2000, Crans 2000, Pereira et al. 2009). The idea was to carry out two step synthesis by adding our two ligands sequentially to $\text{VO}(\text{AcAc})_2$. The first attempt was made on the basis of hydroxypicolinic acid being the first ligand. The protocols and various conditions employed for the similar compounds were tried (Table 9.1, (Galeffi et al. 1987, Koo, Jang & Lee 2003a, Deflon et al. 2002, Melchior et al. 1999, Koo, Jang & Lee 2003b)).

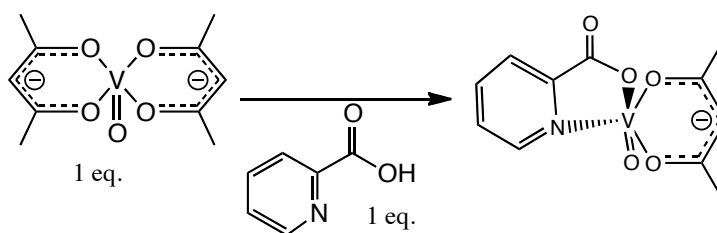
Table 9.1. Reaction conditions for attempted synthesis of $[\text{VO}(\text{OH-pic})(\text{AcAc})]$ (RT, room temperature)

Solvent for OH-pic	Solvent for $\text{VO}(\text{acac})_2$	Reaction conditions	Product	Composition (by MS-ESI ⁺)
Methanol	Methanol	2h reflux	Green-yellow precipitate	Mixture of $[\text{VO}(\text{OH-pic})(\text{AcAc})]$, $[\text{VO}(\text{OH-pic})(\text{H}_2\text{O})]_4$ and VO-OHpic
DCM	DCM	24h RT	Green-brown precipitate	Mixture of $[\text{VO}(\text{OH-pic})(\text{AcAc})]$ and VO-OHpic
Water	Methanol	NaOH to pH=7.0, 1h reflux	Dark-green precipitate	No characteristic mass was observed
CH_3CN	CH_3CN	NaOH to pH=7.0, 3h RT	Dark-green precipitate	No characteristic mass was observed

In each condition, resulting precipitate was analysed by ESI⁺-MS indicating the mixture of vanadyl complexes which was difficult to separate. The solids were set up for recrystallisation from: methanol or ethanol with various co-solvents (THF, EtOAc, DCM, diethyl ether, acetonitrile, toluene, or water) at both +4° C and room temperature. No crystals were observed. The filtrates were left for further crystallisation to yield either a) light-brown crystals that were characterized as [VO(OH-pic)(H₂O)]₄·8H₂O by ESI⁺-MS and X-ray crystallography or b) light blue-green crystals that were characterized as VO(AcAc)₂ by ESI⁺-MS and IR.

Thus, the desired complex has not been purified from the mixture in the experiments with OH-pic being the first ligand.

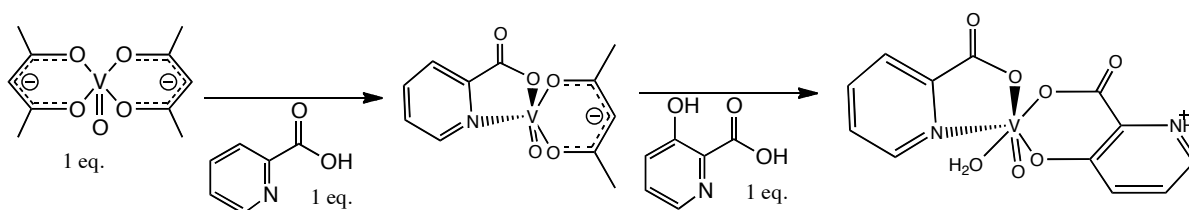
9.1.2.2. Asymmetric vanadium complex formation from VO(AcAc)₂ with H-pic



The next attempt was based on VO(AcAc)₂ reaction with picolinic acid as a first ligand instead of OH-pic. This change of order would potentially limit the formation of possible side complexes via the hydroxyl group that was the main reactive group for the side products formation in reaction with OH-pic.

The reaction was carried out similar to the previously reported procedure (Melchior et al. 1999). Resulting light-blue precipitate and filtrate were characterised by ESI⁺-MS and IR. Spectra showed a mixture of two products: desired [VO(AcAc)(H-pic)] and VO(H-pic)₂ as a main side product. The precipitate was set up for recrystallisation from methanol or water-methanol solution. Filtrate was set up for crystallisation from water, 50% acetonitrile, 50% THF or 50% dioxan (at both +4° C and room temperature). No crystals were observed. Thus, using H-pic instead of OH-pic did not improve product purity.

9.1.2.3. Asymmetric vanadium complex formation from VO(AcAc)₂ with H-pic and OH-pic sequential addition

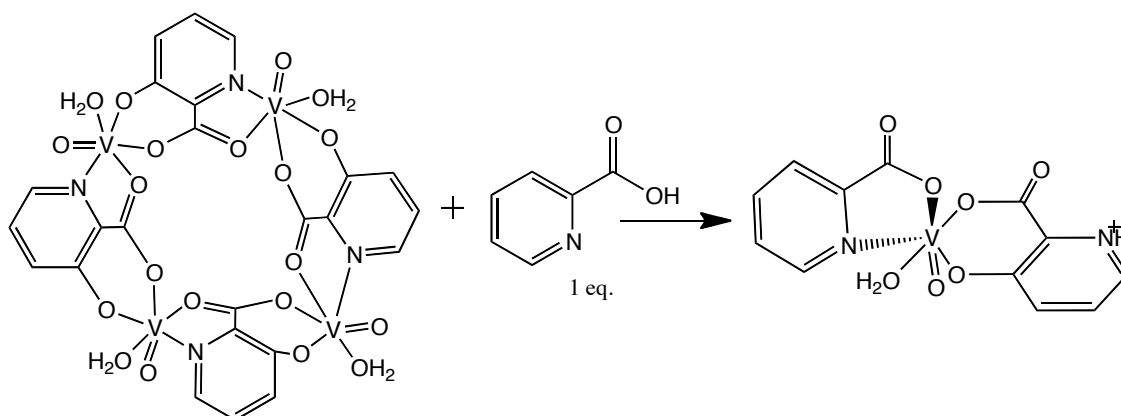


Two-step synthesis attempts from VO(AcAc)₂ were not successful due to difficulties with the purification of the half-substituted product. So it was decided to skip this intermediate purification step and carry on with the addition of the second ligand straight away. H-pic was

chosen as a first ligand as it showed less side products than the OH-pic at the previous attempts. ESI⁺-MS and IR spectra of the resulting product indicated that it was a mixture of following products: VO(AcAc)₂, [VO(AcAc)(OH-pic)], [VO(AcAc)(H-pic)], VO(OH-pic)₂ (i.e. VO-OHpic) and VO(H-pic)₂. Recrystallisation of the filtrate (at both +4 °C and room temperature) didn't result in any crystals formation.

Thus, it was not possible to purify the desired asymmetric vanadium complex from VO(AcAc)₂ reactions.

9.1.2.4. Asymmetric vanadium complex formation from [VO(OHpic)(H₂O)]₄



The above work showed that the asymmetric complex building from VO(AcAc) didn't give the desired results. Similar problems (mixture of product complexes) were observed when VOSO₄ was used as a reagent (during MRes part of this project). At the next stage [VO(OHpic)(H₂O)]₄·8H₂O was used as a starting molecule. It was previously reported that this tetranuclear complex can be transformed back to the mono-vanadium complex with 11% yield upon adding more 3-hydroxypicolinic acid (Yano et al. 2002). The aim of this work was to improve it and to use picolinic acid ligand on the last step in order to create an asymmetric complex.

At first, [VO(OHpic)(H₂O)]₄ complex was synthesised and characterised according to the literature recommendations (Yano et al. 2002, Nakai et al. 2004). Picolinic acid ligand was used at the next step in order to synthesise an asymmetric complex. Building the desired complex from [VO(OHpic)(H₂O)]₄ gave more promising results: only one complex was present in the final solution. ESI⁺-MS spectrum (Figure 9.3) of the solution confirmed the presence of the desired product mass with no indication of other possible complexes. Elemental analysis also proved the purity of the desired product.

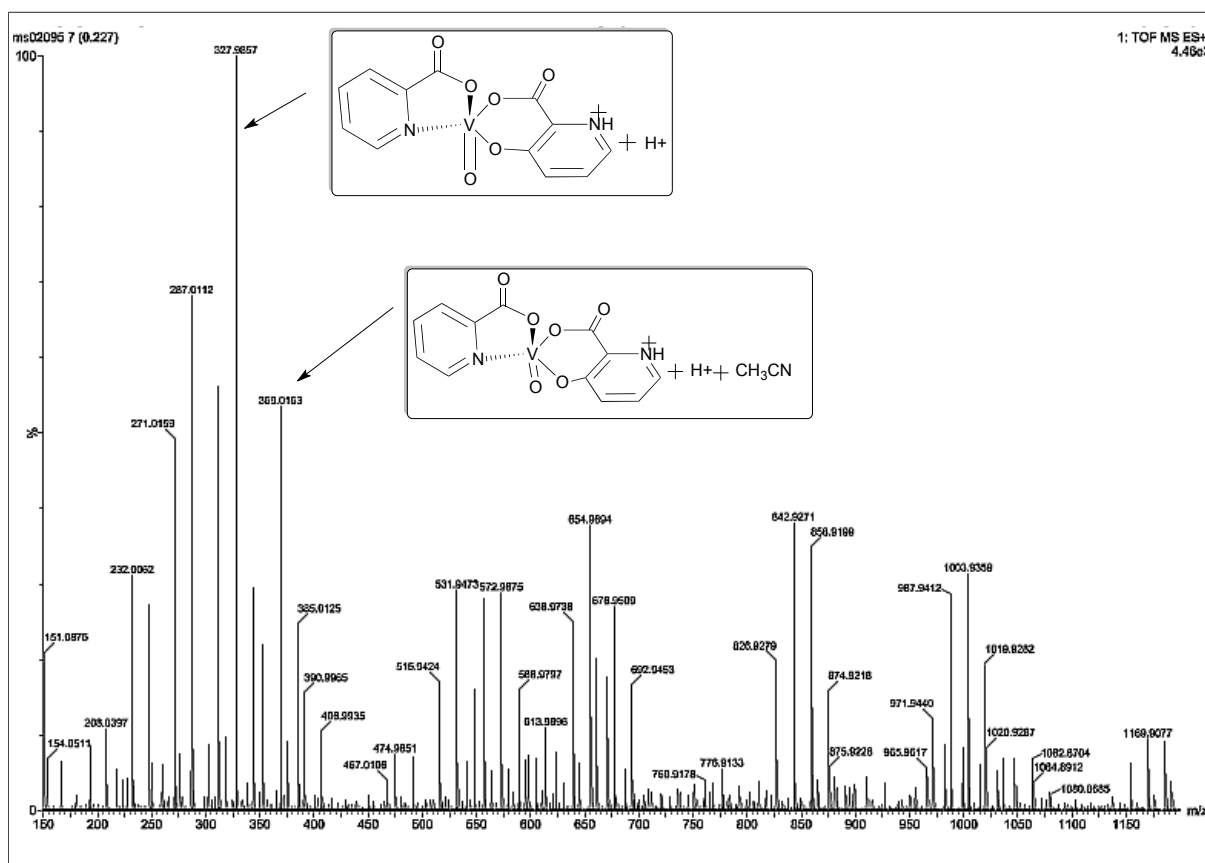


Figure 9.3. ESI⁺-MS spectrum of [VO(OH-pic)(H-pic)] complex synthesised from [VO(OH-pic)(H₂O)]₄. Two peaks at m/z 328 and 369 are due to the desired complex product [M+H]⁺ and [M+CH₃CN+H]⁺ respectively. There are no peaks corresponding to the reagent complex or other possible side products.

Crystallisation attempts were performed with the final material. A range of solvents and crystallisation conditions were tested but didn't result in any crystal formation.

This asymmetric complex indicated the proof of the concept and reaction conditions for further synthesis of asymmetric biotinylated complex. However this work was not continued due to lack of time for this part of the project.

9.2. Determination of VO-OHpic specificity without immobilisation: Metalloproteomics

9.2.1. Introduction

Affinity chromatography methods have greatly contributed towards identification of the intracellular targets of a compound. However, such methods are based on immobilisation of the compound, which requires a chemical modification step. As a result of that chemical modification, the biological activity of the compound can be reduced or even lost. This can

happen if the immobilisation matrix operates as a shield between the molecule and its cellular targets, or if the compound is derivatised through a chemical group that is essential for its activity (Guiffant et al. 2007). In order to avoid this problem, several functionality sites of the compound should be tested for potential matrix attachment points to eliminate the chance of missing biological targets. However, having more than one position for immobilisation is often chemically challenging.

To overcome the limitations of affinity chromatography, an alternative target screening method that does not require compound immobilisation can be used. *Drug Affinity Responsive Target Stability* (DARTS) (Lomenick, Olsen & Huang 2011, Lomenick et al. 2009) or *Cellular Thermal Shift Assay* (CETSA) (Savitski et al. 2014) are examples of such alternative methods. These methods are based on higher stability of protein targets when bound to a compound compared to non-bound proteins: DARTS uses stability to proteases and CETSA assesses thermal stability. These new techniques in label-free target identification require assay optimisation and were too time consuming for this project so another non-affinity based method called *metalloproteomics* was chosen for comparing intracellular targets with those discovered by affinity chromatography.

Metallomic and metalloproteomic approaches have become more commonly used in the recent years for studying pharmacological profiles, functional pathways and potential targets of metallodrugs (Wang et al. 2015b, Mounicou, Szpunar & Lobinski 2009, Sun, Tsang & Sun 2009, Sun, Chai 2010, Maret 2010). Metallomic methods are based on the detection of intracellular putative biological targets via quantitative determination of trace metals associated with them. *Inductively Coupled Plasma Mass Spectrometry* (ICP-MS) is the most utilised technique for metal detection in biological systems due to its high sensitivity and robustness (Wang et al. 2015b, Sun, Tsang & Sun 2009). Metallomic approaches have to be coupled to well-established proteomics in order to separate and identify proteins and other biological targets. *Two-Dimensional Gel Electrophoresis* (2D-GE) and *Liquid Chromatography* (LC) are the most powerful techniques for protein separation allowing resolution of hundreds of proteins in a single sample (Sun, Chai 2010, Sun, Tsang & Sun 2009, Witzmann, Grant 2003, Mounicou, Szpunar & Lobinski 2009).

In this work 1D-GE (Tsang et al. 2012) on a mixture of recombinant proteins was used to try to establish a proof of the concept.

9.2.2. Non-denaturing native gels

PolyAcrylamide Gel Electrophoresis (PAGE) has commonly been used for protein separation from complex mixtures. Unfortunately, it is commonly carried out in the presence of denaturing agents (such as SDS) or chelating agents (such as EDTA) causing likely loss of compound binding and/or associated metals during the separation process (Sevcenco et al. 2009). Thus,

a non-denaturing technique called “*Blue Native PolyAcrylamide Gel Electrophoresis*” (BN-PAGE) is widely used in metalloproteomics for protein separation under physiological-like conditions (Wittig, Braun & Schägger 2006). It is based on the specific binding and staining of separated proteins with a dye (Coomassie brilliant blue G250). Then again, this method was not suitable for VO-OHpic-containing proteins due to acidic pH of the Coomassie brilliant solution (pH ~ 3.0) as it is known that VO-OHpic is stable only at neutral pH conditions (pH 6.0 – 8.0) (Kiss et al. 2000). All alternative staining solutions either also have acidic pH or contain SDS. Hence, in this work various native gel conditions were tested and optimised for recombinant proteins (including PTEN) initially stained with InstantBlue (Sigma) whilst for the final optimised gels with the proteins pre-incubated with VO-OHpic no staining step was performed.

9.2.2.1. PAGE native gels

At first, a simple model system was created in order to mimic a minimal cellular lysate environment. For this a recombinant PTEN (47 kDa) (as a positive control for VO-OHpic binding) and recombinant proteins of distinct molecular weights were chosen for a trial separation mixture: bovine serum albumin (BSA, 66 kDa), ovalbumin (OVA, 42.7 kDa), carbonic anhydrase (30 kDa), lysozyme (14.3 kDa). Initially, these proteins were separated individually on a 10% denaturing SDS-PAGE. A denaturing gel demonstrated a nice ladder of separation (Figure 9.4(A)). Further, the Ornstein and Davis formulation ((Ornstein 1964, Institute of Molecular Development LLC 2001) was chosen for running the proteins on the native PAGE. It is based on Tris-Glycine buffer compositions of neutral to slightly basic pH (pH 6.8 – 8.8) and contains no denaturing or reducing agents. Several polyacrylamide percentages of the resolving gel were tested (10, 8, 6, 5 and 4%). Some proteins (BSA, OVA and carbonic anhydrase) were nicely resolved under these native gel conditions but PTEN was stuck between the stacking and resolving gel in all cases (Figure 9.4(B)).

These results can be explained by two facts. Firstly PTEN can dimerise *in vivo* and *in vitro* (Papa et al. 2014, Heinrich et al. 2015) making its apparent molecular weight substantially greater than that of the monomeric protein. Secondly PTEN is predicted to have a very small native charge (pI = 5.94 by ProtParam (ExPASy) or 6.37 by ProtCalc (sourceforge.net)): if the net charge is close to zero, then the protein will not migrate in the electric field. In order to address this issue proteins samples were dissolved in TRIS pH 8.6 instead of Tris (pH 6.8), which would improve a net charge of PTEN to -16.5 (at pH 8.6) from -3.7 (at pH 6.8). Also a lower percentage stacking gel (2.5% instead of 4%) or no stacking gel were tested (Figure 9.4(C,D)). 6% resolving gel without a stacking gel and TRIS pH 8.6 sample buffer showed the best result on control proteins (BSA and carbonic anhydrase) but none of these attempts provided any improvement for PTEN.

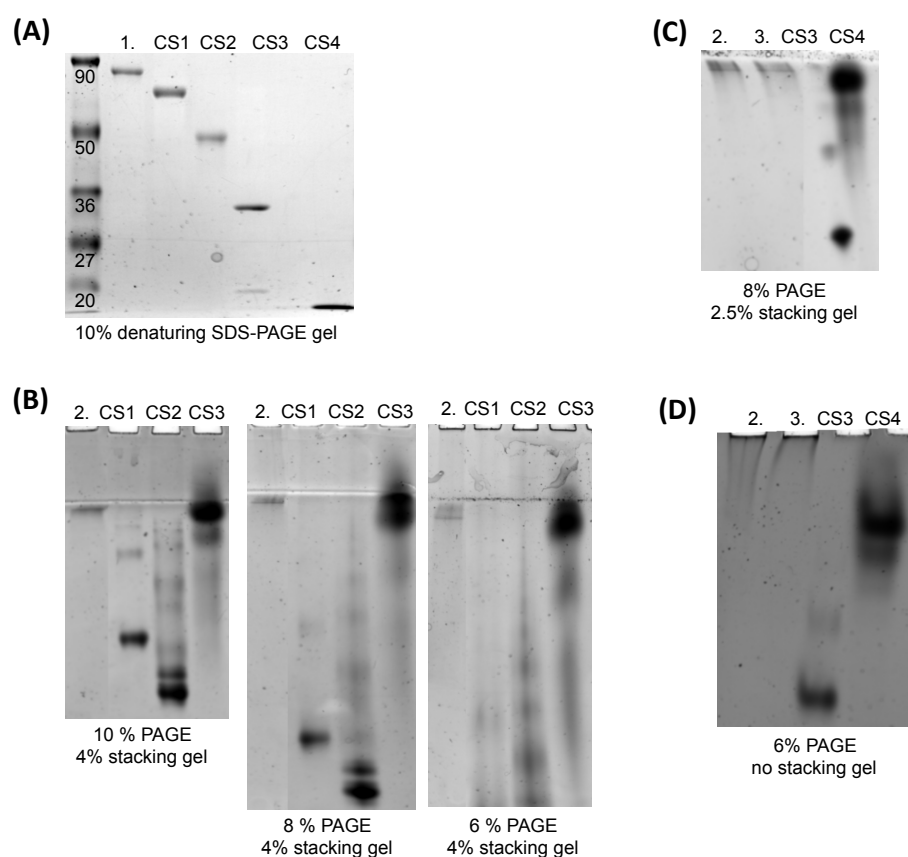


Figure 9.4. Native PAGE gels. **(A)** 10% denaturing SDS-PAGE. Nice separation can be seen for all proteins tested. **(B)** Native PAGE gel examples based on Ornstein and David formulation. Control sample (CS) proteins were resolved well on 6-10% gels but PTEN was stuck on the edge of stacking gel under all conditions. **(C)** Native PAGE gel with a smaller acrylamide concentration in the stacking gel or **(D)** no stacking gel at all did not help PTEN moving into the resolving gel. Neither did pH increase of the sample loading buffer. Samples: **1.** PTEN-GST (71 kDa, 1 μ g); **2.** PTEN (47 kDa, 2 μ g), pH 6.8; **3.** PTEN (2 μ g), pH 8.6; **CS1.** BSA (66 kDa, 1 μ g); **CS2.** OVA (42.7 kDa, 1 μ g); **CS3.** Carbonic anhydrase (30 kDa, 1 μ g); **CS4.** Lysozyme (14.3 kDa, 1 μ g).

Buffer and gel composition were then prepared according to Electrophoretic Mobility-Shift Assay (EMSA) protocols (Carey, Peterson & Smale 2013). 4% native PAGE resolving gel in a TRIS/Borate/EDTA (TBE) buffer is used for this technique. Herein, EDTA as a chelating agent was removed (as this could sequester the vanadium from VO-OHpic). However, using the borate gradient system instead of glycine did not increase PTEN migration in the gels (negative data not shown).

9.2.2.2. Agarose native gels

Finally, a native agarose gel electrophoresis was performed following the previously reported protocol (Kim, Yokota & Kim 2000, Kim 2011). This method can be used to separate proteins with either a positive or a negative pI as it is performed on a horizontal platform with the wells positioned in the centre of a 0.8% agarose gel. This allows proteins to migrate in both

directions: proteins with a pI higher than the buffer pH carry a net positive charge and migrate towards the cathode, while proteins with a pI lower than the buffer pH and have a negative charge – migrate towards the anode. This potentially provides longer separation distance for protein mixture or cellular lysate. At first, PTEN and other recombinant proteins were run in individual lanes and stained. Very good separation for all proteins was observed including PTEN and PTPMT1 (another PTP that strongly binds VO-OHpic *in vitro* and can be used as a positive control) (Figure 9.5(A)).

However, considerable loss of sample occurred during loading. Decreasing the Tris concentration and increasing the salt concentration in the sample loading buffer and mixing glycerol with ficoll (type 400) solved this problem. Based on the migration distances observed, PTEN was mixed with those control proteins that were most distinct from it (carbonic anhydrase, lysozyme and RNase A) and resolved on agarose gels (Figure 9.5(B)). Unfortunately, almost no protein migration was observed in a mixture lane compared to the lanes with these proteins individually. This indicated that proteins probably formed aggregates within their mixture, which changed their migration speed.

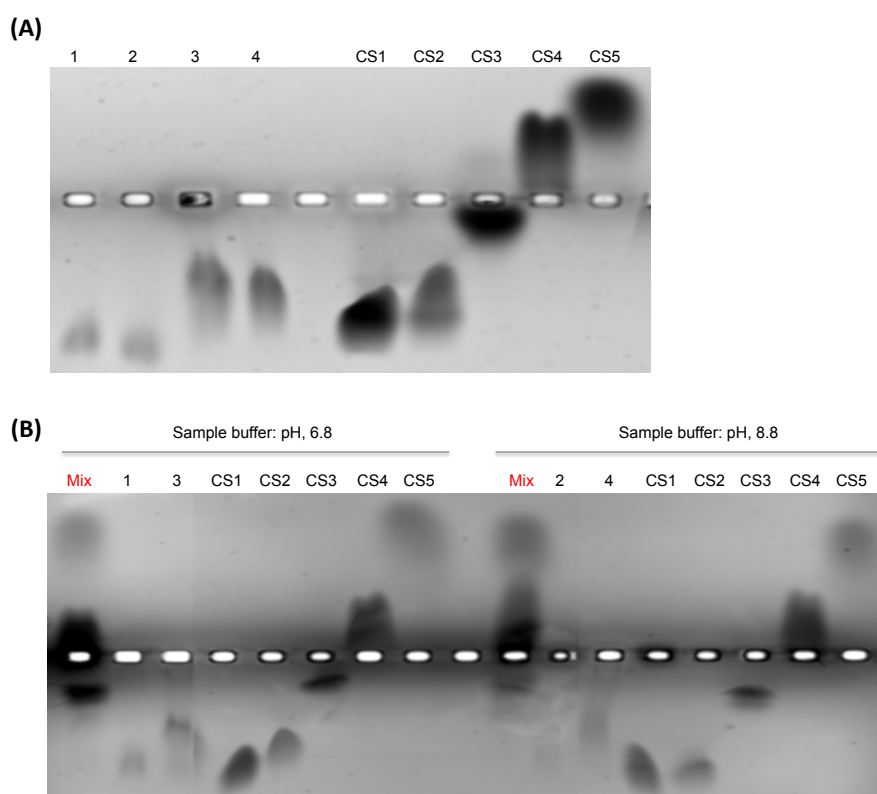


Figure 9.5. Native agarose gels. **(A)** Horizontal agarose gel provided nice separation for all proteins tested (including PTEN and PTPMT1). Both pH conditions in the sample buffer worked well. **(B)** Protein mixture was run alongside individual proteins. Proteins in the mixture stuck all together giving no separate picks for individual proteins at any pH. Agarose gel was run at 50 V for 1 hour and stained with InstantBlue solution. Samples: **1.** PTEN (47 kDa, 4 μ g), pH 6.8; **2.** PTEN (4 μ g), pH 8.6; **3.** PTPMT1 (23 kDa, 4 μ g), pH 6.8; **4.** PTPMT1 (4 μ g), pH 8.6; **CS1.** BSA

(66 kDa, 2 µg); **CS2**. OVA (42.7 kDa, 4 µg); **CS3**. Carbonic anhydrase (30 kDa, 4 µg); **CS4**. Lysozyme (14.3 kDa, 4 µg); **CS5**. RNase A (13.7 kDa, 4 µg)

In order to proceed with the following binding experiments it was decided to run proteins that had been pre-incubated with VO-OHpic on separate lanes in a gel, excise the appropriate protein-containing regions of the gel and then compare amount of VO-OHpic bound by analysing vanadium levels in each band using ICP-MS, with the expectation of elevated levels of vanadium recovery from the PTEN and PTPMT1 samples only.

9.2.3. ICP-MS

For the first ICP-MS analysis, PTEN (10 µg) and BSA (10 µg and 20 µg) were pre-incubated with VO-OHpic (1 µM) and loaded in duplicates on adjacent lanes of an agarose native gel. The protein position was determined by splitting and staining one of two duplicate lanes and using it as a guide for choosing the fragment on the unstained lane that corresponded to the protein (Figure 9.6(A)). Several gel fragments above and below the fragment that corresponded to the protein position were used as control samples (Figure 9.6(B)). All unstained gel fragments were dissolved in nitric acid and submitted for the ICP-MS analysis.

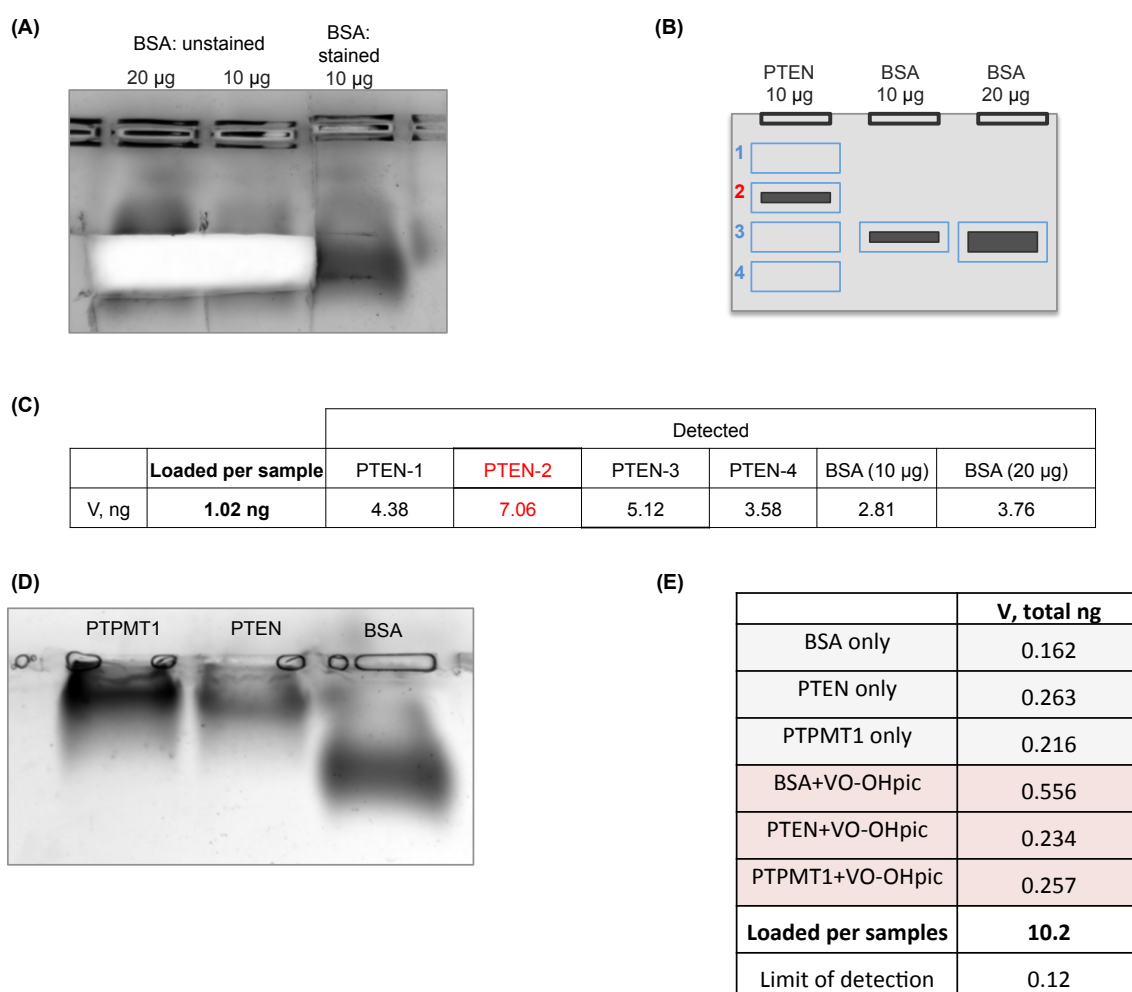


Figure 9.6. Sample preparation and ICP-MS data. **(A)** Native agarose gel electrophoresis was performed with samples in duplicate lines in order to determine protein position on the unstained lane by comparison with the stained lane. Here, BSA samples are shown as an example: One BSA lane was cut out, stained separately and placed adjacent to an unstained lane; fragments on unstained BSA lane (10 µg and 20 µg) judged to contain BSA band were excised for ICP-MS analysis (empty cut-out spot is shown on the figure as the bands were removed for further analysis). **(B)** Schematic picture of the six gel fragments submitted for the first ICP-MS analysis. All samples were pre-incubated with 1 µM VO-OHpic. Several fragments above and below the main PTEN band (band 2) were submitted as negative controls. **(C)** ICP-MS results (first analysis) demonstrated higher vanadium levels in the PTEN-fragment 2 compared to all other samples but these levels were significantly higher than loaded vanadium levels from 1 µM VO-OHpic. **(D)** Example of a stained gel for the samples submitted for the second ICP-MS analysis. **(E)** ICP-MS results (second analysis) showed very low vanadium levels in all samples and no increase for the samples pre-incubated with 10 µM VO-OHpic.

The results demonstrated a good trend of higher vanadium levels in the sample that contained the PTEN protein (fragment 2 on Figure 9.6(B)) compared to all other samples (Figure 9.6(C)). Despite this positive result vanadium levels in all samples were several times higher than the calculated total amount of vanadium from VO-OHpic. This could be due to contamination from buffers, nitric acid and/or from the gel tank.

For the next experiment ultra-pure double-distilled nitric acid was used and all solutions were prepared in high-purity 18mOhm water. Also gel tanks were assessed for potential sources of vanadium: electrodes were made either from a noble metal (usually platinum), metal/metal ion (for example, Cu/Cu²⁺) or metal/insoluble metal salt (for example, Ag/AgI/Cl⁻) (Neihof, Schuldiner 1960) indicating that no vanadium contamination could arise from them.

Duplicate samples of PTEN, PTPMT1 and BSA (10 µg) pre-incubated with or without VO-OHpic (10 µM) were separated on an agarose gel and non-stained fragments corresponding to protein bands were analysed by ICP-MS (Figure 9.6(D)). Vanadium levels in these samples were close to the limit of detection and ten times lower than in the previous experiment (even though ten times higher VO-OHpic concentration was used in this experiment) (Figure 9.6(E)). No increase in vanadium levels was observed in proteins pre-incubated with VO-OHpic compared to free proteins except for BSA. This indicated that these low vanadium levels possibly correspond to some contamination that was reduced in the second experiment compared to the first one. To support this hypothesis, levels of other metals were measured in all samples. The results indeed showed very high levels of Cr, Ni, Cu and Zn. Thus, in order to proceed with these experiments further optimisation and much cleaner conditions have to be utilised to eliminate contaminating metals.

Moreover, no increase in vanadium signal in VO-OHpic samples can be a sign of **(1)** the complex decomposition under electrophoretic conditions; or **(2)** vanadium levels in proteins being lower than the limit of detection by this ICP-MS method. The first reason can be resolved by changing the running buffer composition (Tris-Glycine running buffer used for

these experiments changed pH from pH 8.5 to a very acidic pH (below pH 3.5) within minutes so the buffer had to be replaced repeatedly but this may still have caused pH-dependent VO-OHpic decomposition). Alternatively other methods can be used instead of gel electrophoresis – such as capillary electrophoresis or liquid chromatography (Mounicou, Szpunar & Lobinski 2009). The second problem can be addressed by improving the methods of metal detection: coupling ICP-MS to Laser Ablation (LA-ICP-MS) can provide higher sensitivity (Sun, Tsang & Sun 2009, Sun, Chai 2010, Wang et al. 2015b). Unfortunately within this project there was no time to perform all these possible modifications. However, the results in this chapter can be used as a starting point for further work in this direction.

10. Conclusions

This work aimed to study the intracellular roles of the tumour suppressor PTEN in cancer cells with a view to further validating pro-senescence and synthetic lethality therapies. PTEN is often disrupted in a range of human cancers via mutations or deletions. In order to connect different genetic alterations of PTEN to potential therapeutic options, we have created isogenic PTEN knock-out (homozygous or heterozygous) and knock-in cell lines expressing wild type or site-specific mutant variants of PTEN. These lines alongside cancer cells with PTEN loss were studied with respect to senescence and synthetic lethality phenotypes.

Initially, the newly developed CRISPR/Cas9 technology was successfully utilised for PTEN KO in three human immortalised diploid cell lines. As a result heterozygous ($PTEN^{+/-}$) and homozygous ($PTEN^{-/-}$) lines were derived from HCT116, MRC-5 and PNT1A cells. We showed that this technique could be used as a reliable and robust alternative to the well-established homology recombination or site-specific endonuclease technologies. We also demonstrated that using multiple gRNAs per reaction could increase the likelihood of generating complete KO of the target gene although this also led to complications with the clone characterisation and allele assignment.

Further to making isogenic cells with $PTEN^{+/+}$, $PTEN^{+/-}$ and $PTEN^{-/-}$ genotypes, we aimed to generate $PTEN^{mut/-}$ cells by expression of appropriate constructs in $PTEN^{-/-}$ cells. This would allow us to study structure/function relationships of PTEN for pro-senescence and synthetic lethality phenotypes and make comparisons to the heterozygous $PTEN^{+/-}$ lines expressing WT PTEN with the same genetic background. Two methods allowing the precise insertion of one target gene copy into the genome were chosen for these knock-in experiments. Unfortunately, the first method attempted, FRT-Flp recombination, did not provide us with successful clones whilst the second method, CRISPR/Cas9 knock-in, allowed the knock-in of only one out of five desired mutants. Since these experiments were performed within this study, more literature has been published on the ways of improving the HDR-mediated genome editing rate: (i) Combining paired gRNAs with Cas9 Nickase mutants that cut only one DNA target strand (either complementary or non-complementary) can in some cases induce HDR while reducing levels of NHEJ-mediated indels (Mali et al. 2013b, Ran et al. 2013, Sander, Joung 2014) ; (ii) Cell transfection with Cas9 recombinant protein and gRNA in the RNA form rather than plasmids potentially to increase the yield of successful integrants and to reduce off-target effects (Liang et al. 2015) ; (iii) Use of longer homology recombination arms (up to 1 kb) (Irion, Krauss & Nusslein-Volhard 2014) ; (iv) Single strand DNA donors (Lin et al. 2014a) or endogenous linearisation of dsDNA donors with I-SceI enzymes (Gratz et al. 2014) ; (v) Inclusion of a fluorescent reporter with the target gene for easier screening of the successful

HDR-mediated clones (Yang et al. 2013) ; (vii) Inhibition of DNA ligase IV, a key enzyme in the NHEJ pathway, via use of the inhibitor Scr7, to increase the efficiency of HDR up to 19 fold (Maruyama et al. 2015).

Unfortunately, the time frames of this project did not allow us to exploit with these approaches, but instead a Sleeping Beauty transposition system co-expressing GFP was utilised for PTEN knock-in. Knocked-in clones emitting GFP signals of a similar intensity were selected for further studies; clones expressing similar GFP levels expressed similar PTEN levels, allowing comparison of the effects of PTEN mutations on synthetic lethality and senescence phenotypes between cell lines.

The generated cell lines were used to study the mechanisms underlying the PTEN-induced cellular senescence (PICS) and synthetic lethality. Although Alimonti *et al.* demonstrated that PTEN inhibition with VO-OHpic triggered PICS in PTEN^{+/-} MEFs (Alimonti et al. 2010) and in hepatocellular carcinoma cell line Hep3B which expresses low levels of PTEN (Augello et al. 2016), we did not observe the senescence response in any of our PTEN^{+/-} human diploid cells upon inhibition with VO-OHpic or another PTEN inhibitor, BpV(HOpic). It is possible that the PICS phenotype is not displayed by all human cells. This may be because of some compensation effect from alternative Akt regulators, for instance PHLPP1 phosphatase. Also, recent work of Augello *et al.* demonstrated that VO-OHpic treatment also significantly activated the ERK1/2 pathway (Augello et al. 2016). This agrees with earlier studies showing PTEN to be a negative regulator of the ERK1/2 pathway (Gu, Tamura & Yamada 1998, Chetram, Hinton 2012). Over-activation of the ERK pathway has been shown to trigger a senescence response (Cagnol, Chambard 2010, Steelman et al. 2011, Deschenes-Simard et al. 2014). Taken together, these findings indicate that VO-OHpic activation of both the Akt and ERK pathways could induce cellular senescence in some cell types. Within this study only the Akt pathway was studied, so it would be interesting to analyse MEK-ERK1/2 pathway upon PTEN inhibition in our cells in this context.

The second part of this project focused on studying the sensitisation of cancer cells to PTEN inhibition in the presence of other reagents or inhibition strategies. Some reports demonstrated that treatment with curcumin (Chen et al. 2013) or inhibition of PARP (Mendes-Pereira et al. 2009), TTK (Brough et al. 2011b), Skp2 (Lin et al. 2010, Chan et al. 2013) or NLK (Mendes-Pereira, Lord & Ashworth 2012) could trigger synthetic lethality in PTEN^{-/-} cells suggesting potential therapeutic applications. However, our data demonstrated that curcumin treatment, PARP or TTK inhibition did not cause significant synthetic lethality in any of our isogenic cell lines regardless of PTEN genotype. In addition, Skp2 and NLK inhibition caused a selective lethal sensitivity response in PTEN null cells only.

We attempted to define the structure/function attributes of PTEN that were required for Skp2 sensitivity in isogenic human cell lines. Higher sensitivity to Skp2 inhibition with Skp2-25 in

HCT116 PTEN^{-/-} cells was dependent on a lack of PTEN phosphatase activity (responsible for PTEN catalytic and tumour suppressor activity) and/or PDZ domain (which is thought to facilitate PTEN protein-protein interactions and PTEN stabilisation at particular subcellular localisations), but independent of PTEN nuclear localisation. We did not find that this lethal sensitivity was caused via a senescence response as was previously suggested by Chan *et al.* (2013) and this work would benefit from further studies towards the mechanism of this lethality. Chemical genetic inhibition of NLK also triggered synthetic lethality in PTEN null HCT116 cells suggesting that development of a small molecule inhibitor against NLK could be potentially beneficial for PTEN deficient cancers. However, such synthetic lethality therapy is likely to be cell type dependent as NLK can play either pro- or anti-proliferative functions in different cell types and can either up-regulate or down-regulate cell cycle regulators such as p27, p21, Cyclin D1 (Dohda et al. 2007, Jung et al. 2010, Szypowska et al. 2011, Sa et al. 2015). Further knock-in and structural experiments would be interesting for the biological characteristic of this therapy.

Overall, our data support and broaden some published studies while contradicting others. There are numerous examples of contradictory results in the PTEN literature: for instance, Kao *et al.* (Kao et al. 2007) showed that PTEN re-expression in PTEN deficient U251 cells reduced DSBs and sensitised cells to ionising radiation, suggesting that PTEN promotes radiosensitivity. In contrast, other reports (Lee, Kim & Waldman 2004, Fraser et al. 2012) demonstrated that loss of PTEN increased sensitivity to ionising radiation. Likewise, the broad literature had no consensus regarding sensitivity of PTEN disrupted models to PARP inhibition, TTK inhibition and curcumin treatment (discussed in details in Chapters 7.2-7.4). Similarly, both PTEN inhibition in MEFs and mouse models (Chen et al. 2005, Alimonti et al. 2010) and PTEN overexpression in human fibroblasts (Courtois-Cox et al. 2006) were shown to trigger cellular senescence, indicating that the PTEN/Akt pathway must be very finely tuned (Acosta, Gil 2012). This indicates potential limitations in the likely effectiveness of PICS and pro-senescence therapies in general as being highly dependent on genetic context, cell/tissue types and other pathways.

The published data (Fraser et al. 2012) and the results from this study support the hypothesis that experimental down-regulation or expression of PTEN does not phenocopy the PTEN loss that occurs within the context of tumorigenesis. Moreover, the phenotype of each cancer type and sub-type is complex and cell specific and cannot be combined only by PTEN status. Thus, Yoo *et al.* demonstrated that loss of PTEN activated distinct downstream signalling pathways in a tissue-specific manner based on a mouse line that conditionally disrupted PTEN in the urogenital epithelium. In their study, initially high cellular proliferation in the PTEN null bladder was inhibited within days via p21, while cell growth in PTEN^{-/-} prostate remained elevated with no signs of p21 induction. Furthermore, the Akt/mTOR pathway was activated in PTEN null

prostate but not in bladder (Yoo et al. 2006). This suggests that alternative downstream signalling pathways could be activated by loss of PTEN in different cell and tissue types. This range of contradictory data may be exacerbated by the fact that PTEN loss appears to contribute to genomic instability (Bunz 2002, Toulany et al. 2008, Hunt et al. 2012). Thus, isogenic lines with different levels of PTEN expression or expressing different mutant versions of PTEN may not be stable over time making comparisons difficult. In support of this, our data indicated that the cells with manipulated PTEN genotypes were not producing a stable response over time to a specific agent making it very difficult to draw conclusions. To sum up, this potential genomic instability presents major problems for doing the types of assays that we set out to do in this study. This conclusion has important implications for the study of PTEN function in cancer and suggests that experimental models of PTEN status need to be chosen very carefully and monitored rigorously in order to generate robust findings of potential therapeutic significance.

11. Bibliography

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12. Appendix

12.1. DNA ladders

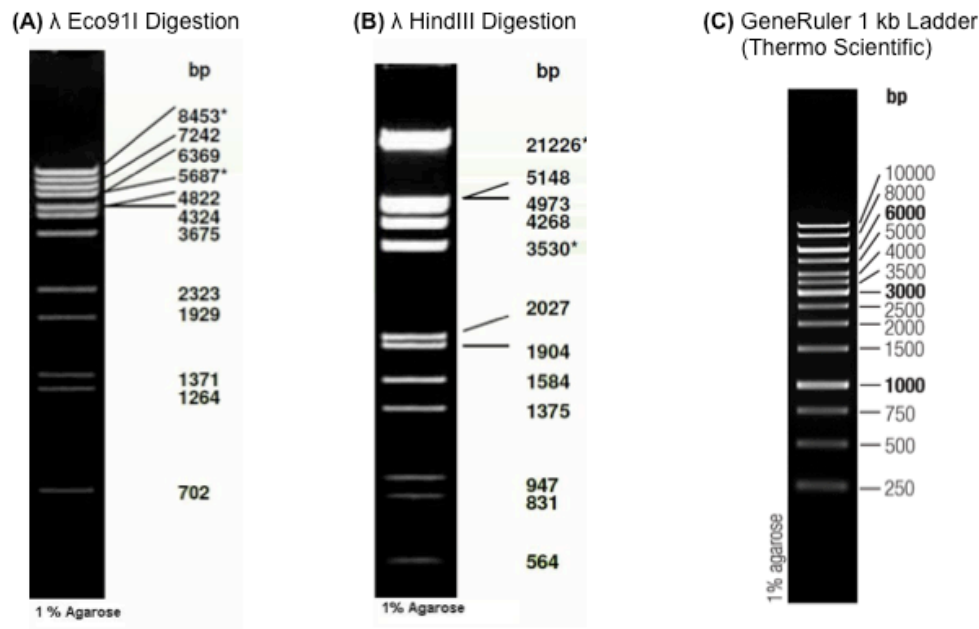


Figure 12.1. Agarose gels to show the molecular weights (bp) of the bands of the digestion products used as DNA ladders in this study. **(A)** Lambda DNA Eco91I digestion; **(B)** Lambda DNA HindIII digestion; **(C)** GeneRuler 1 kb Ladder (Thermo Scientific).

12.2. Sequencing data\$\$\$\$\$\$

12.2.1. PTEN-WT_pGEM-T

5' sequencing primer: T7P (TAATACGACTCACTATAGGG)

AGTTNGCTCCGGCNGCNGGCCGCGGNATTAGCTTATGACAGCCATCATCAAAGAGATCGTTAGCAGAAACAAAAGGAGAT
ATCAAGAGGATGGATTGCTAGACTTGACCTATATTTATCCAAACATTATTGCTATGGGATTTCTGCAGAAAGACTT
GAAGGCGTATACAGGAACAATATTGATGATGTAGTAAGGTTTTGGATTCAAAGCATAAAACCATTACAAGATATACAA
TCTTTGTGCTGAAAGACATTATGACACCGCCAAATTTAATTGCAGAGTTGCACAATATCCTTTGAAGACCATAACCCAC
CACAGCTAGAAGCTTATCAAACCTTTTGTGAAGATCTTGACCAATGGCTAAGTGAAGATGACAATCATGTTGCAGCAATT
CACTGTAAAGCTGGAAGGGACGAAGTGGTGAATGATATGTGCATATTTATTACATCGGGGCAAATTTTAAAGGCACA
AGAGGCCCTAGATTTCTATGGGGAAGTAAGGACCAGAGACAAAAAGGGAGTAAGTATTCCAGTCAGAGGCGCTATGTGT
ATTATTATAGCTACCTGTTAAAGAATCATCTGGATTATAGACCAGTGGCACTGTTGTTTCACAAGATGATGTTTGAAACT
ATTCCAATGTTCACTGGCGGAAGTTCGAACTTCCTCAGTTTGTGGTCTGCCAGCTAAAGGTGAAGATATATTCCTCCAATTC
AGGACCCACACGACGGGAAGACAAGTTCATGTACTTTGAGTTCCTCAGCCGTTACCTGTGTGTGGTGATATCAAAGTAG
AGTTCTTCCACAAACAGAAAGATGCTAAAAAAGGACAAAATGTTTCACTTTTGGGTAAATACATTCTTCATACCAGGA
CCAGAGGAAAACCTCAGAAAAAGTAGAAAATGGAAGTCTATGTGATCAAGAAATCGATAGCATTTGCAGTATAGAGCGTGC
AGATAATGACAAGGAATATCTAGTACTTACTTTAACAAAAAATGATCTTGACAAAGCAAATAAAGACAAAGCCAACCGAT
ACTTTTCTCCAAATTTTAAAGGGTGAAGCTGTACTTCACAAAAACAGTAAAAGGAGCCGTCAAATCCAGAGGCTAGCAGTT
CAACTTCTGTAAACACCAGATGTTAGTGACAATGAACCTGATCATTATAGATATTCTGACACCNCTGACTCTGATCCAAAA
ANTGAACCTTTTTGATGAAGATCAGCA

3' sequencing primer: SP6 (ATTTAGGTGACACTATAG)

GTTGGGNNCNCNTCCATATGGTCGACCTGCAGGCGGCCGCACTAGTGATTCTCGAGTCAGACTTTTGTAAATTTGTGTATGC

\$\$\$\$\$\$ All primers are in 5' -> 3' orientation

TGATCTTCATCAAAAGGTTTCATTCTCTGGATCAGAGTCAGTGGTGTGAGAATATCTATAATGATCAGGTTTCATTGTCACT
AACATCTGGTGTTACAGAAGTTGAACTGCTAGCCTCTGGATTTGACGGCTCCTCTACTGTTTTGTGAAGTACAGCTTCA
CCTTAAAAATTTGGAGAAAAGTACGTTGGCTTTGTCTTTATTTGCTTTGTCAAGATCATTTTTGTAAAGTAAGTACT
AGATATTTCTTGCTCATTATGTCACGCTCTATCTGCAAAATGCTATCGATTTCTTGATCAGATAGCTTCCATTTTCTAC
TTTTCTGAGGTTTCTCTGGTCTGGTATGAAGAATGATTTACCCAAAAGTGAACATTTTGCCTTTTTTAGCATCT
TGTTCTGTTTGTGAAGAAGTCTACTTTGATATCACCACACACAGGTAACGGCTGAGGGAACTCAAAGTACATGAAGTTG
TCTTCCCGTCGTGTGGTCTGAATTGGAGGAATATATCTTACCTTTAGCTGGCAGACCACAACTGAGGATTGCAAGT
TCCGCCACTGAACATTGGAATAGTTTCAAACATCATCTTGTGAAACAACAGTGCCACTGGTCTATAATCCAGATGATTCT
TTAACAGGTAGCTATAATAACACATAGCGCCTCTGACTGGGAATAGTTACTCCCTTTTTGTCTCTGGTCTTACTTCC
CCATAGAAATCTAGGGCCTCTTGTGCCTTTAAAAATTTGCCCGCATGTAATAAATATGCACATATCATTACACCAGTTCCG
TCCCTTTCCAGCTTACAGTGAATTGCTGCAACATGATTGTCATCTTCACTTAGCCATTGGTCAAGATCTTCACAAAAGG
GTTTGATAAGTTCTAGCTGTGGTGGGTTATGGTCTTCAAAGGATATTGTGCAACTCTGCAATTAATTTGGCGGTGCA
TAATGTCTTTCAGCACAAAGATTGTATATCTTGAATGGTTTTATGCTTTGAATCCAAAACCTTACTACATCATCAAT
ATTGTTCTGGATACGCTTCAAGTCTTTCTGCAGGNANCCCATAGCAATAATGTTTGNTAANNNTAGGTCAAGTCTAA
GTCGAATCCATCCTCTTGAANTCCCTTTNNNTTNTGGTAACGATCCNTTTGAAGAAGGCTGGCNTAAGCTTAATCCCN
GGCCTGGGGNCGGGANNATGNAANNCCGGGCCAATCCCCNT

12.2.2. PTEN-H93A_pGEM-T

First 5' sequencing primer: T7P (TAATACGACTCACTATAGGG)

TCTATANNACGGATCTCACTGGCACTCGACNGTAACCTTCATCCTTACTGTATTGTCATGGTTAATACCANTCAANATA
CGGAGACGCGTATACAGGAACAATATTGATGATGTAATAAGGTTTTGGATTCAAAGCATAAAAACCATTACAAGATATA
CAATCTTTGTGCTGAAAGACATTATGACACCGCCAAATTTAATTGCAGAGTTGCACAATATCCTTTTGAAGATGCTAATC
CACCACAGCTAGAACCTTATCAAACCCCTTTGTGAAGATCTTGACCAATGGCTAAGTGAAGATGACAATCATGTTGCAGCA
ATTCAGTGAAAGCTGGAAGGGACGAAGTGGTGAATGATATGTGCATATTTATTACATCGGGGCAAATTTTTAAAGGC
ACAAGAGGCCCTAGATTTCTATGGGGAAGTAAGGACCAGAGACAAAAAGGGAGTAAGTATTTCCAGTCAGAGGCGCTATG
TGATTATTATAGCTACCTGTTAAAGAATCATCTGGATTATAGACCAGTGGCACTGTTGTTTACAAGATGATGTTTGAA
ACTATTCCAATGTTCAGTGGCGGAACCTTGAATCCTCAGTTTGTGGTCTGCCAGCTAAAGGTGAAGATATATTCTCCAA
TTCAGGACCCACACGACGGGAAGACAAGTTTCATGTACTTTGAGTTCCCTCAGCCGTTACCTGTGTGGTGATATCAAAG
TAGAGTTCTTCCACAAACAGAACAGATGCTAANAAAGGACAAAATGTTTCACTTTTGGGTAAAACATTCTCATACCAG
GACCAGAGGAACNTCAGANAGTANAAATGGAAGTCTATGTGATCAGAAATCGATAGCTTTGCAGTATAGAGCGTGACAG
TATGACAGCAATATCTAGTACTTACTTNACAAAATGACTTGNAAAGCAATAAAGANACGCCAACGATACTTTCTCCAAT
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NTGACCTGATCATATAAANTCTGAACNTGATCTGATCAAAGATGACCTTTGNGAANNAGNTAACATTACAAAGCTGAT
CGACCTGCACTAAGGNCCTATCTTAGGNCCNTAATCTAANTCGCGATACCTCACGGNCTCTATTGCAANTCNATTGGNCC
NTGCAGCCACATTGGAANNACGGTGGACGAAGTGAATCAANGGCCNAANNNTGGCGACAGGCAACTGTCCGGNAATG
TTCCNNAATCCNAANGACCGAAAAANTAANCGGGNC

Second 5' sequencing primer: GTTTGAAACTATTCCAATGTTTCAGTG

GCTAAGGTGAAGAATATTCTCCAATTCAGGACCCACACGACGGGAAGACAAGTTTCATGTACTTTGAGTTCCCTCAGCCG
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TTGGGTAAATACATTCTTCATACCAGGACAGAGGAAACCTCAGAAAAAGTAGAAAATGGAAGTCTATGTGATCAAGAAA
TCGATAGCATTTCAGTATAGAGCGTGACGATAATGACAAGGAATATCTAGTACTTACTTTAACAAAAAATGATCTTGAC
AAAGCAAAATAAGACAAAGCCAACCGATACTTTTCTCCAAATTTTAAAGGTGAAGCTGTACTTCACAAAAACAGTAGAGGA
GCCGTCAAATCCAGAGGCTAGCAGTTCAACTTCTGTAACACCAGATGTTAGTGACAATGAACCTGATCATTATAGATATT
CTGACACCACTGACTCTGATCCAGAGAATGAACCTTTTGTGAAGATCAGCATACACAAATTACAAAAGTCTGACTCGAG
CATGCATCTAGAGGGCCCTATTCTATAGTGTACCTAAATGCTAGAGCTCGCTGATCAGCCTCGACTGTGCCTTCTAGTT
GCCAGATCACTAGTGCGGCCGCTGCAAGTGCAGCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATT
CTATAGTGTACCTAAATAGCTTGGCGTAATCATGGTCTAGCTGTTTCTGTGTGAAATTGTTATCCGCTCACAAATTC
ACACAACATCAGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAAGTCACTAATTAATTGCGTTGC
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GTTTGCATATTGGGGCGCTCTTCCCGTTNCTCGGCTCACTTGACTCGCTTGCNNCGGGCCGTTCCGGGCTGCGGCCNAA
GGGNTATCAA

12.2.3. PTEN-C124S_pGEM-T

First 5' sequencing primer: T7P (TAATACGACTCACTATAGGG)

ATTCGAGATAACGTCAAGATGGATTAGATCTCTAGTTGACTAAAATATATCCTNCTTATAGTGTATCGAATTTCCAGCA
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AGATATACAATCTTTGTGCTGAAAGACATTATGACACCGCCAAATTTAATTGCAGAGTTGCACAATATCCTTTTGAAGAC
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CCTCCAATTCAGGACCCACACGACGGGAAGACGAAGTTCATGTACTTTGAGTTCCCTCAGCCGTTACCTGTGTGGTGAT
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CATACCAGGACAGAGGAAACCTCAGAAAAAGTAGAAAATGGAAGTCTATGTGATCAAGAAATCGATAGCATTTCAGTA
TAGAGCGTGACGATAATGACAAGGAATATCTAGTACTTACTTTAACAAAAAATGATCTTGACAAAGCAATAAAGACAAA

GCCAACCGATACTTTTCTCCAAATTTTAAGGTGAAGCTGTACTTCACAAAAACAGTAGAGGAGCCGTCAAATCCAGAGGC
TAGCAGTTCAACTTCTGTAACCCNAGATGTTAGTGACAAATGAACCTGATCATTATAGATATTCTGACCCNACTGACTCTG
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TATTCTAANGTGGC

Second 5' sequencing primer: GTTTGAAACTATTCCAATGTTTCAGTG

TGAGGTCNCCAGCTAAGGTGAAGAATATTCCTCCAATTCAGGACCCACACGACGGAAGACAAGTTTCATGTACTTTGAGT
TCCCTCAGCCGTTACCTGTGTGTGGTGATATCAAAGTAGAGTTCTTCCACAAACAGAAACAGATGCTAAAAAGGACAAA
ATGTTTCACTTTGGGTAAATACATTCTTCATACCAGGACCAGAGGAAACCTCAGAAAAAGTAGAAAAATGGAAGTCTATG
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GCCTTCTAGTTGCCAGAATCACTAGTGCAGCGCCCTGCAGGTGCACCATATGGGAGAGCTCCCAACGCGTTGGATGCATA
GCTTGAGTATTCTATAGTGTACCTAAATAGCTTGGCGTAACCATGGTTCATAGCTGTTTCCCTGTGTGAAATTTTATCCG
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GCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAAATCANNNGGNATAACGCAGGAAAGAACTTGGNAGCAA
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3' sequencing primer: CACTGAACATTGGAATAGTTTCAAAC

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TTGTTCCAACTGGAACAACNCTCAACCTATCTCGGTCTATTCTTTGATTNAAANGGGATTNNGCCAATTNCGGCCTA
TTGGTTAAAAANTGAGCTGATT

12.2.4. PTEN-G129E_pGEM-T

First 5' sequencing primer: T7P (TAATACGACTCACTATAGGG)

GAGATGTCTATANNACGGATTTCACTGACACTTGACNTAACCCNANGCTTACTGTATTGTCATGGTTATTACCANTCA
NNATACNTGAGACGCGTATACAGGAACAATATTGATGATGTAATAAGGTTTTTGGATTCAAAGCATAAAAACATTACAA
GATATACAATCTTTGTGCTGAAAGACATTATGACACCCGCAATTTAATTGCAGAGTTGCACAATATCCTTTTGAAGACC
ATAACCCACCACAGCTAGAATTATCAACCCCTTTGTGAAGATCTTGACCAATGGCTAAGTGAAGATGAACATCATGTT
GCAGCAATTCAGTGTAAAGCCGGCAAGGAACGAACCTGGTGAATGATATGTGCATATTTATTACATCGGGGCAATTTTT
AAAGGCACAAGAGGCCCTAGATTTCTATGGGGAAGTAAGGACCAGAGACAAAAAGGGAGTAACATTCCCAGTCAGAGGC
GCTATGTGTATTATTATAGCTACCTGTTAAAGAATCATCTGGATTATAGACCAGTGGCACTGTTGTTTCACAAGATGATG
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AGCAGTTCAACTTCTGTAACACCAGATGTTAGTGACAATGAACCTGATCATTATAGATATTCTGAANCCNTTGACTCTGA
TCCAANGAATGAACCTTTTGTGAAANTCAGCATACCNAAATTTACAAAAGTCTGGACTCGAGCATGGATCTTAGAGGG
CCCNNTTCTATAAGGGNCACCTAAAAANNCTAAAA

Second 5' sequencing primer: GTTTGAAACTATTCCAATGTTTCAGTG

GGTCTGCCGCTAAAGGTGAAGAATATTCCTCCAATTCAGGACCCACACGACGGAAGACAAGTTTCATGTACTTTGAGTTCT
CCTCAGCCGTTACCTGTGTGTGGTGATATCAAAGTAGAGTTCTTCCACAAACAGAAACAGATGCTAAAAAGGACAAAAT
GTTTCACTTTGGGTAAATACATTCTTCATACCAGGACCAGAGGAAACCTCAGAAAAAGTAGAAAAATGGAAGTCTATGTG
ATCAAGAAATCGATAGCATTGTCAGTATAGAGCGTGCAGATAATGACAAGGAATATCTAGTACTTACTTTAACAAAAAAT
GATCTTGACAAAGCAAATAAAGACAAAGCCAAACCGATACTTTTCTCCAAATTTTAAGGTGAAGCTGTACTTCACAAAAAC
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TGACTCGAGCATGCATCTAGAGGGCCCTATTCTATAGTGTACCTAAATGCTAGAGCTCGCTGATCAGCCTCGACTGTGC

CTTCTAGTTGCCAGAATCACTAGTGC GGCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGC
TTGAGTATTCTATAGTGTACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCGTGTGAAATTGTTATCCGCT
CACAAATCCACACACATACGAGCCGGAAGCATAAAGTGTAAGCCCTGGGTGCCTAATGAGTGAGCTAACTCACATTAA
TTGCGTTGCGCTCACTGCCCCGCTTTCCAGTCGGGAAACCTGTCTGCGCAGCTGCATTAATGAATCGGCCAACGCGCGGGG
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GGTATCAGCTCACTCAAAGGCGGTAAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAATGTGAGCAAAAG
GCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTCCATAGGCTCCGCCCCNTGACAAGCATCAC
AAAAATCGACGCTCAAGTCAAANGNNGNNGAAACCNANANGANNAAANGATACCAGGCGTTNCCCCCTGGAANCNCNTC
GGGGNNNNCCNTNTNCCAACCTGGCCNTTNAACCGGA

3' sequencing primer: CACTGAACATTGGAATAGTTTCAAAC

GTCATAATCCAGATGATTCTTTACAGGTAGCTATAATAATACACATAGCGCCTCTGACTGGGAATAGTTACTCCCTTTT
GTCTCTGGTCCTTACTTCCCATAGAAATCTAGGGCCTCTTGTGCCTTTAAAAATTTGCCCGATGTAATAAATATGCAC
ATATCATTACACCAAGTTCGTTCCCTTGGCGGCTTTACAGTGAATTGCTGCAACATGATTGTCATCTTCACTTAGCCATTGG
TCAAGATCTTCAAAAAGGGTTTGATAAGTTCTAGCTGTGGTGGGTTATGGTCTTCAAAAGGATATTGTGCAACTCTGCA
ATTAATTTTGGCGGTGTCAATAATGCTTTTCAGCACAAAGATTGTATATCTTGTAAATGGTTTTATGCTTTGAATCCAAAA
ACCTTACTACATCATCAATATTGTTCTGTATACGCTTCAAGTCTTTCTGCAGGAAATCCCATAGCAATAATGTTTGGA
TAAATATAGGTCAAGTCAAGTCAATCCATCCTCTTGATATCTCCTTTTGTCTGCTAACGATCTCTTGATGATGGC
TGTCATAAGCTTGGTGTCCCTATAGTGAGTCGTATTAATTTGATAAGCCAGTAAGCAGTGGGTTCTAGTTAGCCAG
AGAGCTCTGCTTATATAGCTCCCAATCCCGCGCCATGGCGCGGGAGCATGCGACGTGCGGGCCCAATTCGCCCTA
TAGTGAGTCGTATTACAATCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCTGGCGTTACCCAACCTTAATC
GCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGC
AGCCTGAATGGCGAATGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCT
ACACTTGCAGCGCCCTAGCGCCCGCTCTTTCGCTTCTTCCCTTCCCTTTCGCGCACGTGCGCGGCTTCCCGCTCA
AGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTAGTGCTTTACGGCACCTCGACCCCAAAAAAATTGATTAGGGTG
ATGGTTACGCTAGTGGGCCATCGCCCTGATAGACGGTTTTNCGCCCTTGACGTTGGAATCCACGTTCTTTAATAGTGGA
CTCTTGNTCCAACTGGAA

12.2.5. PTEN-V403A_pGEM-T

First 5' sequencing primer: T7P (TAATACGACTCACTATAGGG)

GNCGNTGNTCCGGCCNCTGGCCGCGNNAATTTGGAGANGTCTATAATNGACGGANCCNCTGGCNCTCNACCGTAACCC
TCATGCCTTACTGNCTTGNCTNNGGTTANTNCCNGCCANTAGANGGGAGANGCGTANACTAGGANCANNATTGANGATGN
ANTNAGGTTTTTGGATTCAAAGCATAAANACCATTACNAGATNTACATCTTTGTGCTGAAAGACATTATGACACCGCCG
AATTTAATTGCNNAGTTGCACAATATCCTTTTGAAGACCATAACCCACCNCAGCTAGAATTATCAAACCCCTTTGTGAA
GATCTTGACCAATGGCTAAGTGAAGATGACAATCATGTTGCGACGAATTCATCTGTAAGCTGGAAGGGACGAAGTGCNGT
AATGATATGTGCATATTTATTACATCGGGGCAATTTTTAAAGGCACAAGAGGCCCTAGATTTCTATGGGGAAGTAAGGA
CCAGAGACAAAAAGGGAGTAACCTATCCAGTCAGAGGCGCTATGTGTATTATTATAGCTACCTGTTAAGAATCATCTG
GATTATAGACCAGTGGCACTGTTGTTTCAAGATGATGTTTGAACCTATTCCAATGTTCACTGGCGGAACCTTGCAATCC
TCAGTTTGTGGTCTGCCAGCTAAAGGTGAAGATATATTCCCAATTCAGGACCCACNCGACGGGAAGACAAGTTTCATGT
ACTTTGAGTTCCCTCAGCCGTACCTGTGTGTGGTGATATCAAAGTAGAGTTCTTCCACAAACAGAACAGATGCTAAAA
AAGGACAAAAATGTTTCACTTTTGGGTAATACNTTCTTCATACCAGGACCAGAGGAAACCTCAGAAAAACGTANAANATG
GGACNTCTATGTGATCAAGACATCGATAGCATTTGCGNGTATANAGCGTGCGAGATCATGACAAGGAATATCNTANTACTTT
CCTTTGACAAAAATGATCTTGACNAAGCCAAATAAAGGACAAAGCCCAACCGATACTTTTTCTCAAATTTTAAAGNGAA
GCTGNTNCCCTCNAAAAACGNTAAAGGAGCCGTCNANTCCAGAGGCTGCAGTTCCACTTCTNNAAACCCCNAAAGGTTAG
TGANAATGAACCGGATCNTTAAATATTCGNNCCCCCTGACTCNGATCCNAAAAATGAACCTTTTTTGAANATCNNTA
ANNAAATTTCAANGGCCTGACNCGANNCAATCCCTTTNNGGNNCCCNNGNGTTACNNTTGGGGAAACNCCNAGCGGTTG
NNGGCACCTNGNTTCTNAAGGGCCCCCAANTGTNNGGNANCCNNGGCCAACTGTTNCGGGGGAANTTTTTCCNNCNN
TNCCCNCAATNAANCNGAAAAAANGTAACNCGGGGGCCAAGGGGNCNCCCNCTNTGNGGGGGCCCCCTTCNNGGNACN

Second 5' sequencing primer: GTTTGAAACTATTCCAATGTTTCAGTG

AGCTAAGGTGAAGAATATTCCTCCAATTCAGGACCCACACGACGGGAAGACAAGTTTCATGTACTTTGAGTTCCCTCAGCC
GTTACCTGTGTGTGGTGATATCAAAGTAGAGTTCTTCCACAAACAGAACAGATGCTAAAAAAGGACAAAAATGTTTCACT
TTTGGGTAAATACATTCTTCATACCAGGACCAGAGGAAACCTCAGAAAAAGTAGAAAATGGAAGTCTATGTGATCAAGAA
ATCGATAGCATTTGCAGTATAGAGCGTGCAGATAATGACAAGGAATATCTAGTACTTACTTTAAACAAAAATGATCTTGA
CAAAGCAAAATAAAGACAAAGCCAACCGATACTTTTCTCAAATTTTAAAGGTGAAGCTGTACTTCAAAAAACAGTAGAGG
AGCCGTCAAATCCAGAGGCTAGCAGTTCAACTTCTGTAACACCAGATGTTAGTGACAAATGAACCTGATCATTATAGATAT
TCTGACACCACTGACTCTGATCCAGAGAATGAACCTTTTGTGAAGATCAGCATACACAAATTACAAAGGCCTGACTCGA
GCAATCACTAGTGCGGCGCGCTGCAGGTGCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTA
TAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCTGTGTGAAATTGTTATCCGCTCACAATTCCACA
CAACATACGAGCCGGAAGCATAAAGTGTAAGCCCTGGGGTGCCCTAATGAGTGAGCTAACTACATTAATTGCGTTGCGCT
CACTGCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTG
CGTATTGGGCGCTCTTCCGCTTCTCGCTCACTGACTCGCTGCGCTCGGTGCTTGGCTGCGGCGAGCGGTANCAGCTCA

3' sequencing primer: CACTGAACATTGGAATAGTTTCAAAC

GTGNTAATCCAGATGATTCTTTACAGGTAGCTATAATAATACACATAGCGCCTCTGACTGGGAATAGTTACTCCCTTTT
GTCTCTGGTCCTTACTTCCCATAGAAATCTAGGGCCTCTTGTGCCTTTAAAAATTTGCCCGATGTAATAAATATGCAC
ATATCATTACCACTTCTGCTCCCTTCCAGCTTTCAGTGTGAGTGTGCAACATGATTGTCATCTTAGCATTTAGCCATTGG
TCAAGATCTTCAAAAAGGGTTTGATAAGTTCTAGCTGTGGTGGGTTATGGTCTTCAAAAGGATATTGTGCAACTCTGCA
ATTAATTTTGGCGGTGTCAATAATGCTTTTCAGCACAAAGATTGTATATCTTGTAAATGGTTTTATGCTTTGAATCCAAAA

ACCTTACTACATCATCAATATTGTTCTGTATACGCCTTCAAGTCTTTCTGCAGGAAATCCCATAGCAATAATGTTTGA
TAAATATAGGTCAAGTCTAAGTCAATCCATCCTCTTGATATCTCCTTTTGTCTGCTAACGATCTCTTGATGATGGC
TGTCATAAGCTTGGGTCTCCCTATAGTGTAGTCTGATTAATTTTCGATAAGCCAGTAAGCAGTGGGTCTCTAGTTAGCCAG
AGAGCTCTGCTTATATAGACCTCCCAATCCCGCGGCCATGGCGGCCGGGAGCATGCGACGTGGGGCCCAATTCCGCCCTA
TAGTGAGTCTGATTACAATTCAGTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCTGGCGTTACCCAACTTAATC
GCCTTGCAGCACATCCCCCTTTGCGCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGC
AGCCTGAATGGCGAATGGACGCGCCCTGTAGCGGCGCATTAAAGCGCGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCT
ACACTTGCAGCGCCCTAGCGCCCGCTCCTTTTCGCTTTCTTCCCTTCTTCTCGCCACGTTGCGCCGCTTTCCCGCTCA
AGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTNAGTGCTTTACGGCACCTCGACCCNNAANCTTGATTAGGGTG
ATGGTTACGTAAGTGGGCCATCGCCCTGATAGACGGTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTANNANNGGAA
TCTNNTNCCAACCTGGAACAANNNTCAACCCNTNCGGNTNTTCTTTNGATTNAANGGGATTTGCCAATTCCGGCC

12.2.6. PTEN-K13E&K289E_pGEM-T

5' sequencing primer: T7P (TAATACGACTCACTATAGGG)

CTCCGGCCGCTGGCCGCGGATTCAAGCTTATGACAGCCATCATCAAAGAGATCGTCTCGAGAAACGAAAGGAGATATC
AAGAGGATGGATTGACCTTAGACTTGACCTATATTTATCAAACATTATTGCTATGGGATTTCTGCAGAAAGACTTGAA
GGCGTATACAGGAACAATATTGATGATGTAGTAAGGTTTTGGATTCAAAGCATAAAAACCATTAACAAGATATAAATCT
TTGTGCTGAAAGACATTATGACACCGCCAAATTTAATTGCAGAGTTGCACAATATCCTTTGAAGACCATAACCCACCAC
AGCTGAACTTTATCAAACCTTTTGTGAAGATCTTGACCAATGGCTAAGTGAAGATGACAATCATGTTGCGACCAATTCAC
TGTAAGCTGGAAGGGACGAACTGGTGAATGATATGTGCATATTTATTACATCGGGGCAAATTTTAAAGGCACAAGA
GGCCCTAGATTTCTATGGGGAAGTAAGGACCAGAGACAAAAGGGAGTAACATTTCCAGTCAGAGGCGCTATGTGTATT
ATTATAGCTACCTGTTAAGAATCATCTGGATTATAGACAGTGGCACTGTTGTTTCACAAGATGATGTTTGAAGTATT
CCAAATGTTCAAGTGGCGGAACTTGCAATCCTCAGTTTGTGGTCTGCCAGCTAAAGGTGAAGATATATTCTCCAATTCAGG
ACCCACACGACGGAAGACAAGTTCATGTACTTTGAGTTCCCTCAGCCGTTACCTGTGTGTGGTGATATCAAAGTAGAGT
TCTTCCACAAACAGAACAGATGCTAAAAAAGGACAAAATGTTTCACTTTTGGGTAAATACATTCTTCATACCAGGACCA
GAGGAGACGTCAGAAGAAGTAGAAAATGGAAGTCTATGTGATCAAGAAATCGATAGCATTTCAGTATAGAGCGTGCAGA
TAATGACAAGGAATATCTAGTACTTACTTTAACAAAAANTGATCTTGACAAAGCAAATAAAGACAAAGCCAAACCGATACT
TTTCTCAAATTTAAGGTGAAGCTGTACTTCACAAAACAGTAGAGGAGCCGTCAAATCCAGAGGCTAGCAGTTCAACT
TCTGTAACACCAGATGTTAGTGACAATGAACCTGATCATTATAGATATTCTGACACCACTGACTCTGATCCAGANAATGA
ACCTTTTGATGAAGATCAGCTTACCNAANTTACAAAAGTCTGACTCGAGAATCCNTAATGCGGNCCGCTG

3' sequencing primer: SP6 (ATTTAGGTGACACTATAG)

ACACGCGTNNNGNCTCTCCATATGGTGCAGCTGCAGGCGGCCGCACTAGTGATTCTCGAGTCAGACTTTTGTAAATTTGT
TATGCTGATCTTCATCAAAGGTTCACTTCTGGATCAGAGTCAGTGGTGTGAGAATATCTATAATGATCAGGTTTCATTG
TCACTAACATCTGGTGTTACAGAAGTTGAAGTCTAGCCCTCTGGATTGACGGCTCCTCTACTGTTTTGTGAAGTACAG
CTTCACCTTAAATTTGGAGAAAAGTATCGGTTGGCTTTGTCTTTATTTGCTTTGTCAAGATCATTTTTTGTAAAGTAA
GTACTAGATATTCCTTGTCAATTATCTGCACGCTCTATACTGCAAATGCTATCGATTCTTGATCAGATAGACTTCCATTT
TCTACTTCTCTGACGCTCCTCTGGTCTGGTATGAAGAATGATTTACCCAAAAGTGAAACATTTTGTCTTTTTTAG
CATCTTGTCTGTTTGTGGAAGAAGTCTACTTTGATATCACCACACACAGGTAACGGCTGAGGGAACCTCAAAGTACATGA
ACTTGTCTTCCCGTCGTGTGGGTCTGAATTGGAGGAATATATCTTCACCTTTAGCTGGCAGACCACAACTGAGGATTG
CAAGTTCGCCCACTGAACATTGGAATAGTTTCAAACATCATCTTGTGAAACAACAGTGCCACTGGTCTATAATCCAGATG
ATTCTTTAACAGGTAGCTATAATAACACATAGCGCCTCTGACTGGGAATAGTTACTCCCTTTTTGTCTCTGGTCTTTA
CTTCCCATAGAAATAGGGCCTCTTGTGCCCTTTGCCCGATGTAATAAATATGCACATCATTACACCA
GTTGCTCCCTTTCCAGCTTTACAGTGAATTGCTGCAACATGATTGTCTTCACTTACGCTTGGTCAAGATCTTCACA
AAAGGTTTGATAAGTTCTAGCTGTGGTGGGTATGGTCTTCAAAGGATATTGTGCAACTCTGCAATTAAATTTGGCGG
TGTGATAATGCTTTTCAGCACAAAGATTGTATATCTTGAATGGTTTTATGCTTTGAATCCAAAACCTTACTACATCA
TCAATATTGTTCTGGATACGCCTTCAAGTCTTTCTGCAGGAAATCCCATAGCAATAATGTTTGGATAANTATAGGTCAA
GTCTAAATCCAANTCCATCCNTTGAANTCTCCT

12.2.7. pGEM-T_{short} PTEN

5' sequencing primer: T7P (TAATACGACTCACTATAGGG)

CCGGCCGCCNNGCCGCGGGATTGAATTCGAGAGAAGTGAAGCGCAGTCGCGTCCCAGCGCCGAGCGGTATCCTGCCGCA
GCGCATAAAGAGTCCCAGCACATCACCGCCCGCCGGCCTGCCCGCCCCCTCCGCCGCCGCGCGGGAGCCCGGGCGCCTC
GGAAGACCCGAGGGGAGGCGGGAGGCGAGCGAGGCGGACGGACCGCGCGGGCGAGGGGAGGGCAGGGCAGGGCA
GGGGGCGGTAGGAGGGGGCAGAGCGGTAGCTCTGGGTGCGAGCGCAGAGTCCCCAAGCCGAGGCTCTACTGAGCATGCC
CAG
GTAGCTGCTGGGGCTTGCTCGGGCCGGTTCCAGCCGCCAGCCTGCAGCTGCACTTGCTGCGGCTTTTGCAGCAACGC
GAGGCGAGGATAACGAGCTAAGCCTCGGCCTCTGCCAGAAACCCAGCCGAGGAGGCTAGGCTGTTGTGGGGCGGGG
TGGAGGACTGATGATGAAAGCTGAGATGGGTGCGTTGAGCAGTGTCACTGACTCAGATCTAATCACTAGTGCGGCGCCCT
GCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTACCTAAATAGCTTGG
CGTAATCATGGTCATAGCTGTTTCTGTGTGAAATTTGTTATCCGCTCACAAATCCACACAACATACGAGCCGGAAGCATA
AAGTGTAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGG
AAACCTGTGTCGCGAGCTGATTAATGAATCGGCCAACGCGGGGAGAGGCGGTTTGCATATTGGGCGCTCTTCCGCTT
CCTCGCTCACTGACTCGCTGCGCTCGGTGCTTGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTA
TCCACAGAATCAGGGGATAACGCGAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCG
GTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAATCGACGCTCAAGTCAGAAGTGGCGAANCC
CGACGGGACTATAAAGATACCAGGCGTTTCCCCCTGGAANCTCCTCGTGCGCTCTCCTGTTCCGACCTGGCGGT

CGCTNNGGANCNCTCCATATGGTCGACCTGCAGGCGGCCGCACTAGTGATTAGATCTGAGTCAGTGACACTGCTCAACGC
ACCCATCTCAGCTTTCATCATCAGTCCTCCACCCCGCCCCACAACAGCCTACCCTGCCTCCGGCTGGGTTTCTGGGCAG
AGGCCGAGGCTTAGCTCGTTATCCTCGCCTCGCGTTTGTGCAAAAGCCGACGCAAGTGACAGCTGCAGGCTGGCGGCTGGG
AACCGGCCCGAGCAAGCCCCAGGACGACTACACTGGGCATCTCAGTAGAGCCTCGGGCTTGGGGACTCTGCGCTCGCAC
CAGAGCTACCGCTCTGCCCCCTCCTACCGCCCCCTGCCCTGCCCTGCCCTCCCCCTCGCCCGCGCGGCTCCCGCTCCGCCTC
TCGCTCGCCTCCCGCCTCCCCCTCGGTCTTCCGAGGCGCCCGGGCTCCCGGCGCGGCGCGGAGGGGGCGGGCAGGCCGG
CGGGCGGTGATGTGGCGGGGACTCTTTATCGCTCGCGGAGGATACGCGCTCGGCGCTGGGACGCGACTGCGCTCAGTTCTC
TCGAATTCAAATCCCGCGCCATGCGCGCGCGGAGCATGCGACGTCTCGGCGCAATTCGCCCTATAGTAGCGTATTACA
ATTTACTGGCCGTCGTTTTTACAACGTCGTGACTGGGAAAACCCCTGGCGTTACCCAACTTAATCGCCTTGCGACATCCC
CCTTTCCGACAGTGCGCGTAATAGCGAAGAGCGCCGACGATCGCCCTTCCCAACAGGTCGCGAGCCTGAATGGCGAATG
GACGCGCCCTGTAGCGGCGCATTAAGCGCGCGGGTGTGGTGTTACGCGACAGTGTACCGCTACACTTGGCCAGCGCCCT
AGCGCCCGCTCCTTTTCGTTTCTTCCCTTCTTTCTCGCCACGTTTCGCCGGCTTCCCCGTCAAGCTCTAAATCGGGGG
TCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGG
CCATCGCCCTGATGACAGCTGTTTTTCGCCCTTTTGACGTTGAAAGTGCCAGTTCTTTAATAGTGGACTCTGTGTTCCAAACTGG
AACACACACTGACACCTACTGTTTCTATTTTGATTNAAAGAGTTCGCCCAATTNCGGCTATTGGTTAAAAAATG
NCTTGATTTAACAAAAATTTNNACCGGAATTTTAACAANTATTAAACGCTTAAAAATTCNNGGAANNNGGGAATTTT

CCGGCCGCCNNGCCGCGGGATTAGATCTCTCCGGAGCTATCACTGGGGAGTGGGAATTTGGAAAGTTCGCCAACTAGGGA
CACACGTGACCTCCTTCGGAAGAGTAGTTCGCAGCTGTGGCCCGTGTATCCTTCCACCTCCTTTTGAACCCCTCCTAGGTC
CTCGCCCCCGCCACTCGCTGGGCTGCAGCTTCTACCGTTTCCGTACTTTCACCTAACCCCGTAACCCCAACGTCGACG
TCCGGCCCGGGGCGCGCGAGGCTTGGCCCCGGGCGCATCATCTGCCGGTTTTCACGGCGGCCAAGGGGGGGCGGGGC
TAGGTGGTCTCTGAGAACCGAGCTTGACTCCGACGCCGCGAACCGACCTGGAGCCCCGAGGGGAAAGATGCTCGACTCTCTT
GGGGGACCCGAGGCGGGCGCAGGAGAGGCTCGGGGTGCTGCCCACTACAGGGATCCTCTTCAGTTTCATTATGATAG
GTGCCCTTTGGGCCCTTGAATTAACCGGCTATGTGTTCACGTTTCAGCAGCGTCGGCTGAGAGCTTTCATTTTAGGGCA
AACGAGCCGAGTTACCGGGGAAGCGAGAGGTGGGGCGCTGCAAGGGAGCCGGATGAGGTGATACACGCTGGCGACACAAT
AGCAGGTTGCTCTTTGTGCTAAGACTGACACCTAGGACACAGATTTGGGGGAAGGGGGAATCTCTAGGCAAAGGCTGT
TACAGTCAAATCTCTGCAACGATTGTGATCCGACAGCGGTGCAAAAGGAAAGCGAATGCAGTCCACGCCCGGAAAT
CTAGGGTAGAGGCAAGGGGGGAGGGTATTCCTTGCAGGGACCGCTGCAATTTCCCTTACACTGAGCAGCGGTG
TCACCTGGTCTTTTACCTGTGCACAGGTAACCTCAGACTCGAGTCAGTGACACTGCTCAACGCACCCATCTCAGCTTT
CATCATCAGTCTCTCCACCCCGCCCCACAACGCTACCCGCGCTCCGGCTGGGTTTCTGGGCAAAAGGCNAAGGCTTAA
CCTCGTTATCTCGCCCTCGCGTTGCTGCAAAAGCC

GTTGGGAGCTCTCCATATGGTCGACCTGCAGGCGGCCGCACTAGTGATTGAATTCGAGAGAACTGAGCGCAGTCGCGTCC
 CAGCGCCGAGCGCGTATCTCTGCCGACGCGCATAAAGAGTCCCGCCACATACCCGCCCGCCGGCCTGCCCGCCCCCTCCGC
 CGCCGCGCCGGAGCCCGGCCCTCGGAAGACCGGAGGGGAGGCGAGGAGCGAGAGGCGGAGCCGAGCCCGCCCGCC
 GCGGAGGGGAGGGCAGGGCAGGGCAGGGCGGCTAGGAGGGGGCAGAGCGTAGCTCTGGGTGCGAGCGCAGTAGTCC
 CAAGCCGAGGCTCTACTGAGCATGCCAGTGTAGCTGCTGGGGCTTGCTCGGGCCGGTTCACGACCGCCAGCCTGCAGC
 TGCATCTGCTGCGGCTTTTGACGAACGCGAGGCGAGGATAACGAGCTAAGCCTCGGCCTCTGCCAGAAACCCAGCCGA
 GGCAGGGTAGGCTGTTGGGGGGGGGGAGGACTGATGATGAAGCTGAGATGGGTGCGTGTAGACGTGTGCTACTGACT
 CGAGTCTGAGGTTACCTGTGCACAGGTGAAAGGACCAAGGTGACCACGCTGCTAGTGTAGAGGGAATCAGTGACGAGCGT
 CCTGCAAGGGGAATACCCCTCCCCCTTGCTCTACCCCTAGATTTCCGCGGCGTGGACTGCATTCGCTCTTTCTTTTGC
 ACCGCTGTGCGATCACAACTGTTGCGAGAGATTTGACTGTAAACAGCTTTGCCCTAGAGATCCCCCTTCCCCCAAATCTG
 TGTCTCATGGTGTGAGTCTTAGCACAAGAACCACTGCTATTGCTCGCCAGCGTGATCACCTCATCCGGTCCCTT
 GCAGCGCCCCACCTCTCGCTTCCCCGGTAACTCGGCTCGTTTGCCTAAAAATGAAAGCTCTCAGCCGAGCGTGTGAAC
 GTGAACACATAGCCGTTGAATTTCAAGGGCCCCAAAGGGCACCTATCTAAATGAACTGAAAGAGGATCCCTGTGAGTGGGA
 CGACACCCCGCAGGCCTCTCCTGCGCCCGCTCCGGTGCCCCAAGAAAGTCGAGCATCTTCCCCCTCGGGCTCCAGGTGCG
 TTCGCGGCGTCGGAATCAAGCTCGGTTCTCAAAGACCACCTAGCCCCNCCCCCCTTGGCCGCCGTGAAACCCGGCAGG
 ATGGATCG

AAAGCGGCTNNTNAACGACGGCCAGTAACATAACGGTCCTAAGGTAGCGAGGCCCTGGGTGGCGAATTCGGCACGAGGCT
CTCGGAAGCTGCAGCCATGATGGAAGTTTGAGAGTTGAGCCCTGTGAGGCCGAGGCCGGGGCTCAGGCCAGGGAGATGAGA
GACGGCGGGCGGCCGCGCCCGGAGCCCTCTCAGCGCTGTGAGCAGCCGCGGGGGCAGCCCTCGGGGAGCGCGGCC
GGCCTGCGGCGGGCGGACGCGCGGCCGCTTCTCGCCTCCTCTTCGTCTTTCTAACCGTGACAGCTCTTCTCGGCTTCTCT
GAAAGGGAAGGTGGAAGCCGTGGGCTCGGGCGGGAGCCGGCTGAGGCGCGGCGGCGGCGGCGGCACCTCCCGCTCCTGG
AGCGGGGGGGGAGAAGCGGGCGGGCGGCGCGGCCGCGCGGCTGCAGCTCAAGGAGGGGGTCTGAGTCGCCTGTACCAT
TTCCAGGGCTGGGAACGCGCGGAGAGTTGGTCTCTCCCTCTACTCGCTCCCAACGCGCGGGCGGCGGCGGCACATCCA

GGGACCCGGGCGGTTTAAACCTCCCGTCCGCCGCCGCCGCACCCCCCGTGGCCCGGGCTCCGGAGGCCGCCGGCGGA
GGCAGCCGTTTCGGAGGATTATTCGTCTTCTCCCCATTCCGCTGCCGCCCGCTGCCAGGCCTCTGGCTGCTGAGGAGAAGCAG
CCCCAGTCGCTGCAACCATCCAGCAGCCGCCGACGACGCTTACCCGGCTCGGTTCCAGAGCCAAGCGCGCGCAGAGCG
AGGGGCACTCAGTACCGCCAAGTCCAGAGCCATTTCATCTCAGAGAAGCCCCGCCACCAGCAGCTTCTGCCATCTCT
CTCTCTCTTTTCTTTCAGCCACAGGCTCCAGACATGACAGCCATCATCAAAGAGATCGTTAGCAGAAACAAAGGGAGAT
ATCAAGAGGATGGATTCCGACTTAACTTGACCTATATTTATCCAAACATTATTGCTATGGGATTCCTGCAAAAAGACTT
GAAGGCGTATACCGG

12.2.10. pcFRT-PTEN^{short}

First 5' sequencing primer: GGCGACACGGAAATGTTG

CTTTNAATATTGACATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAAT
AGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTGACGGATCGGGAGATCTCTCCGGAGCTATCACTGGGG
AGTGGGAATTTGGAAAGTTCCCCAACTAGGGACACAGTGACCTCCTTCGGAAGTAGTTCCGACTGTGGCCCGTGTATC
CTCCACCTCCTTTTGAACCTCCTAGGTCTCTCGCCCCGCCCACTCGCTGGGCTGCAGCTTCCTACCGTTCCGTACTT
TCCACTCAACCCGGTAACCCCAAACGTGCACGGTCCGGCCGGGGCGCGCGGAGCCTGGCCCCGGGCGATCCATCCTGCCG
GGTTTTACGGCGGCCAAGGGGGGGCGGGGCTAGGTGGTCTCTGAGAACCGAGCTTGAAGTCCGACGCCGCGAACCGACCT
GGAGCCCCGAGGGGAAAGATGCTCGACTCTCTGGGGGACCCGAGCGGGCGCAGGAGAGGGCCTGCGGGGTGCGTCCCACT
CACAGGGATCCTCTTTCAGTTCAATTTAGATAGGTGCCCTTTGGGCCCTTGAATTCACCGCTATGTGTTACGTTTCAGC
ACGTGCGGCTGAGAGCTTTCAATTTTAGGGCAAACGAGCGAGTTACCGGGGAAGCGAGAGGTGGGGCGCTGCAAGGGAG
CCGGATGAGGTGATACAGCTGGCGACACAATAGCAGGTTGCTCTTTGTGCTAAGACTGACACCATGAGGACACAGATTT
GGGGGAAGGGGGAATCTCTAGGCAAAGGCTGTACAGTCAAATCTCTGCGAACGATTGTGATCCGACAGCGGTGCAAAAG
GAAAGAGCGAATGCAGTCCACGCCGCGGAAATCTAGGGGTAGAGGCAAGGGGGGAGGGTATTCCCTTGCAGGGACCGT
CCCTGCATTTCCCTCTACACTGAGCAGCGTGGTCACTGCTCTTTTACCTGTGACAGGTAACCTCAAACCTCNAAGTCA
GTGACACTGCTCAACGCACCCATCTCAGCTTTCATCATCAGTCTCCACCCCGCCCNACAANNNGCTAACCTGGCCTCC
GGCTGGGTT

Second 5' sequencing primer: GACCAATCTTGTTTTACC

GTCAGTTGAAAAAGGTTGTGTAGCTGTGTATATACCTTTTTGTGTCAAAGGACATTTAAATTCATTAGGATT
AATAAGATGGCACTTTCCCGTTTTAAAAAAGGACATGCTGGGGATGCGGTGGGCTCTAT
GGCTTCTGAGCGGAAAGAACCAGCTGGGGCTCTAGGGGGTATCCCCACGCGCCCTGTAGCGGCGCATTAAAGCGCGCGG
GTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCAGCGCCCTAGCGCCCGCTCCTTTTCGCTTTCTTCCCTTCTCTT
CTCGCCACGTTTCGCCGGCTTTCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCGATTTAGTCTTTACGGCA
CCTCGACCCCCAAAAAATTTGATTAGGGTGATGGTTACGTACCTAGAAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAA
GTATAGGAACTTCCTTGGCCAAAAAGCCTGAATCACCAGCGACGTCTGTGAGAAGTTTCTGATCGAAAAGTTCGACAGC
GTCTCCGAGCTGGTTCGGGCCATTTCGGAGGGCGAAGAATCGGTCAATACACTACATGGGCGTGATTTTATATGCGGATT
GCGGGTAAATAGCTGCGCCGATGGTTTCTACAAAGATCGTTATGTTTATCGGCACTTTGCATCGGCCGCGCTCCCGATT
CGGAAGTGCTTGACATTGGGGAATTCAGCGAGAGCCTGACCTATTGCATCTCCCGCCGTGCACAGGGTGTCACGTTGCAA
GACCTGCCTGAAACCGAATGCCCGCTGTTCTGCAGCCGTCGCGGAGGCCATGGATGCGATCGCTGCGGCCGATCTTAG
CCAGCGAGCGGGTTCGGCCATTTCGGACCGCAAGGAATCGGTCAATACACTACATGGGCGTGATTTTATATGCGGATT
GCTGATCCCCATGTGTATCACTGGCAAATGTGATGGACGACACCGTCAGTGCCTCGCTCGCGCAGGCTCTCGATGAGCT
GATGCTTTGGCCGAGGACTGCCCCGAAGTCCGGCACCTCGTGGCACGCGGATTTCGGCTCAACAATGTCTGACGGAC
AATGGC

3' sequencing primer: CGAGAAACGCCGCGCTG

GCGCGGCTCCNAAGGCGCNCNCCCCGCGGCNNTCACAGGCGCTGAGAGGGGCTCCGGGCCGCGGCCGCCGCTCTCTC
ATCTCCCTCGCTGAGCCCGGCTCGCCTCACAGCGGCTCAACTCTCAAATTCATCATGGTGCAGCTTCCGAGAGCCT
CGTGCCGAATTCGAGAGAACTGAGCGAGTCGCTCCCAGCCGAGCGCGTATCCTGCCGAGCGCATAAAGAGTCCCCG
CCACATCACCGCCCCGCCGCTGCCGCCGCCCTCCGCCGCCGCGCCGGAGCCCGGGCGCCTCGGAAGACCGAGGGGAG
GCGGGAGGCGAGCGAGAGGCGGACGGGACCGCGCGGGCGAGGGGAGGGGAGGGGAGGGGCGGTAGGAGGG
GGCAGAGCGGTAGCTCTGGGTGCGAGCGCAGAGTCCCCAAGCCGACGGCTCTACTGAGCATGCCAGTGTAGCTGCCTGG
GGCTTGCTCGGGCCGGTTCAGCCGCCAGCCTGCAGCTGCACTTGCTGCGGCTTTTGCAGCAACGCGAGGCGAGGATAAC
GAG
CTAAGCCTCGGCCTCTGCCAGAAACCCAGCCGAGGCGAGGAGGCTAGGCTGTTGTGGGCGGGGGTGGAGGACTGATGATGA
AAGCTGAGATGGGTGCGTTGAGCAGTGTCACTGACTCGAGTGTGAGGTTACCTGTGCACAGGTGAAAAGGACCAGGTGAC
CACGTGCTCAGTGATAGAGGAAATGCAGGGGACGGTCCCTGCAAGGGGAATACCCTCCCCCTTGCTCTACCCCTAGAT
TTCCGCGGCGTGGAATGCACTTCTCTTTCTTTTGCACCGCTGTCCGATCACAATCGTTCCGAGAGATTTGACTGTAAC
AGCCTTTGCTAGAGATTCCCCCTTCCCCAAATCTGTGTCCTCATGGTGTGAGTCTTAGCACAAGAGCAACCTGCTAT
TGTGTGCCAGCGTGTATCACCTCATCCGGCTCCCTTGACGCGCCCCACCTCTCGCTTCCCCGGTAACCTCGGCTCGTTT
CCCTAAAAATGAAAGCTCTCAGCCGAGCGTGTGAACGTGAACACATAAGCCGTTGAATTTCAAGGGCCCCAAGGGCACC
TATCTAATTTGAATGAANGAGGATCCCTGGGGAGGGGGACGNCNCCCCGGAAGGCTCTTCTGGGGCCNGCTCCGGTGGCCC
CAAAAANGTCAAGGATTTTTCNNTTCGGGGTCCAGGNNGGTTNCCGGGGNTCGGNNNNAANNNGGTTNCAAAAACCC
CNTAAACCCCCCCCCC

12.2.11. pcFRT-PTEN^{long}

First 5' sequencing primer: GGCGACACGGAAATGTTG

AATATTGACATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGG
TTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTGACGGATCGGGAGATCTGAGTCAGTGACACTGCTCAACGCAC

CCATCTCAGCTTTTCATCATCAGTCTCCACCCCCGCCCCACAACAGCCTACCCTGCCTCCGGCTGGGTTTCTGGGCAGAG
 GCGGAGGCTTAGCTCGTTATCCTCGCCTCGCGTTGCTGCAAAAGCCGAGCAAGTGCAGCTGCAGGCTGGCGGCTGGGAA
 CCGGCCGAGCAAGCCCCAGGCAGCTACACTGGGCATGCTCAGTAGAGCCTGCGGCTTGGGACTCTGCCTCGCACCCA
 GAGCTGAGCTCTGCCCCCTCCTACCGCCCCCTGCCCTGCCCTGCCCTCCCCCTCGCCCCGCGCGGTCCCGTCCGCTCTC
 GCTCGCCTCCCGCTCCCTCGGTCTTCCGAGGCGCCCGGGCTCCCGGCGCGCGGCGGAGGGGGCGGGCAGGCCGCGC
 GGCGGTGATGTGGCGGACTCTTTATGCGCTGCGGCAGGATACGCGCTCGGCGCTGGGACGCGACTGCGCTCAGTTCTCTC
 GAATTCGGCACGAGGCTCTCGGAAGCTGCAGCCATGATGGAAGTTTGAGAGTTGAGCCGCTGTGAGGCGAGGCCGGGCTC
 AGGCGAGGGAGATGAGAGACGGCGGCGGCGCGGCCGCGGCCGAGCCCTCTCAGCGCCTGTGAGCAGCCGCGGGGGCAGCGC
 CCTCGGGAGCCGCGGCGCTGCGGCGGCGGCGAGCGGCGGCTTTCTCGCCTCCTCTTCTGCTTTTCTAACCGTGCAGCCT
 CTTCTCGGCTTCTCCTGAAAGGGAAGGTGGAAGCCGTGGGCTCGGCGGGAGCCGGCTGAGGCGCGGCGGCGGCGGCG
 GCACCTCCCGCTCCTGGAACGGGGGGGAGAAGCGGCGGCGGCGGCGGCGGCGGCTGCAGCTCCAGGNNAGGGGT
 CTGAGTCGCTGTACCATTTCCAGGGCTGGGAACGCCGA

Second 5' sequencing primer: GACCAATCTTGTTTTACC

GTCAGTTGAAAAGGTTGTGTAGCTGTGTATATACCTTTTTGTGTCAAAAGGACATTTAAAATTCAATTAGGATTA
 ATAAAGATGGCACTTTCCGTTTTAAAAAAGGACATTTGAGGATGCTGGGGATGCGGTGGGCTCTATG
 GCTTCTGAGGCGGAAAGAACCAGCTGGGGCTTAGGGGATATCCCCACGCGCCCTGAGCGGCGCATTAAAGCGCGGCGGG
 TGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTTCGCTTTCTTCCCTTCTTTT
 TCGCCACGTTGCGCGGCTTTCCCGTCAAGCTCTAACTCGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCAC
 CTCGACCCCAAAAACTTGATTAGGGTGATGGTTACAGTACCTAGAAAGTTCTATTCCGAAGTTCCATTCTCTAGAAAG
 TATAGGAACCTTCTTGCCAAAAAGCCTGAACCTACCGCGACGCTCTGTCGAGAAGTTTCTGATCGAAAAGTTCGACAGCG
 TCTCCGACCTGATGCAGCTCTCGGAGGGCGAAGAATCTCGTGCTTTAGCTTCGATGTAGGAGGGCGTGATATGTCCTG
 CGGGTAAATAGCTGCGCCGATGGTTTCTACAAAGATCGTTATGTTTATCGGCACCTTTCATCGGCCGCGCTCCCGATTCC
 GGAAGTGCTTGACATTTGGGAATTCAGCGAGAGCCTGACCTATTGTCATCTCCCGCCGTGCACAGGGTGTCACGTTGCAAG
 ACCTGCCTGAAACCGAACTGCCCCGCTGTTCTGCAGCCGGTTCGCGGAGGCCATGGATGCGATCGCTGCGGCCGATCTTAGC
 CAGACGAGCGGGTTCGGCCCATTCGGACCGCAAGGAATCGGTCAATACACTACATGGCGTGATTTTATATGCGGATTGCG
 TGATCCCCATGTGTATCACTGGCAAAGTGTGATGGACGACACCGCTCAGTGCGTCCGTCGCGCAGGCTCTCGATGAGCTGA
 TGCTTTGGCCGAGGATGCCCGAAGTCCGGACCTCGTGACGCGGATTTCGGCTCCAACATGTCCTGACGGACAAT
 GGCCGCATAACAGCGGTCAATTGACTGGAANNAGGCGATGTTGCGGGATTCCCAATACAAAGTCGCCAACATCTTCTTCT
 GGAAGGCC

3' sequencing primer: CGAGAAACGCCGCGCGTG

CCCCGCGGCTGCTACAGGCGCTGAGAGGGGCTCCGGGCGCGGCGCGCGCGCTCTCTCATCTCCCTCGCCTGAGCCCGG
 CCTCGCCTCACAGCGGCTCAACTCTCAAACTTCCATCATGGCTGCAGCTTCCGAGAGCCTCGTGCCGAATTCGAGAGAAC
 TGAGCGCAGTCGCGTCCCAGCGCCGAGCGCGTATCCTGCCGAGCGCATAAAGAGTCCCGCCACATACCCGCCCGCGGC
 CTGCCCGCCCCCTCCGCCCGCGCGCCGGGAGCCCGGCGCTCGGAAGACCGAGGGGAGGCGGAGGCGAGCGAGAGG
 CGGACGGGACCGCGCCGGGCGAGGGGAGGGCAGGGCAGGGGCGGAGGAGGGGCGGATAGGAGGGGCGAGCGGTAGCTCTGGG
 TGCGAGCGCAGAGTCCCCAAGCCGAGGCTCTACTGAGCATGCCAGTGATGCTGCCTGGGGCTTGCTCGGGCCGGTCCC
 AGC
 CGCCAGCCTGCAGCTGCACTTGCTGCGGCTTTTGACGCAACGCGAGGCGAGGATAACGAGCTAAGCCTCGGCCTCTGCC
 AGAAACCCAGCCGAGGCGAGGCTAGGCTGTTGTGGGGCGGGGGTGGAGGACTGATGATGAAAGCTGAGATGGGTGCGTTG
 AGCAGTGCTACTGACTCAGATCTCCCGATCCGTCGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATT
 TGTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCCTGATAAATGCTTCAATAATATTGAAA
 AAGGAAGAGTATGATATTCAACATTTCCGTGTCCGCTTATTTCTTTTTCGGGCAATTTGCTTCTCTGTTTTGCTC
 ACCCAGAAACGCTGGTGAAGTAAAGATGCTGAAGATCGTTGGGTGACGAGTGGGTTACATCGAACTGGATCTCAA
 CAGCGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAAGCTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGGC
 GCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTGCGCGCATACNTATTCTCAGAA

12.2.12. pEGFP-puro-FRT

5' sequencing primer: CMV-F (CGCAAATGGGCGGTAGGCGTG)

GGGCTTAGCAACNNGTANTGACCGTCAGATCCGCTAGCATGGAAGTTCTATTCCGAAGTTCTATTCTCTAGAAAGTA
 TAGGAACTTCACCGGTGCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTACCGGGGTGGTGCCCATCCTGGTTCGAGCTG
 GACGGCGACGTAACCGGCCACAAGTTACGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCCTGAA
 GTTCATCTGCACCACCGGCAAGCTGCCGTGCCGTGCCACCCCTCGTGACCACCTGACCTACGGCGTGCAGTGCTTCA
 GCCGCTACCCCGACCATGAAGCAGCAGCACTTCTTCAAGTCCGCCATGCCGAAGGCTACGTCCAGGAGCGCACCATC
 TTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCT
 GAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACCTACAACAGCCACAACGTCTATA
 TCATGGCCGACAAGCAGAAGAAGCGCATCAAGGTGAACCTCAAGTCCGCCACAACATCGAGGACGGCAGCGTGCAGCTC
 GCCGACCACTACAGCAGAACACCCCATCGGCGACGCGCCGCTGCTGCTGCCGACAACCACTACCTGAGCAGCCAGTGC
 CGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCTGCTGGAGTTCGTGACCGCCGCGGGATCACTCTCG
 GCATGGACGAGCTGTACAAGTCCGAGCTCAGATCTAGGAGACGACCTTCCATGACCGAGTACAAGCCACGGTGCGCCTC
 GCCACCCGCGACGACGTCCCCCGGGCGGTACGCACCTCGCCGCGCGTTCGCCGACTACCCGCCACGCGCCACACCGT
 CGACCCGAGCCGACGAGCTGAGCGGGTACCGGAGCTGCAAGAACTTCTCAGCGCGCTCGGGCTCGANTCGGCAAGN
 NNGTGGGTGCGGACAACGNNCCCCGGTGCGGCTGAGACACGCCGAAAGCGTCNAAACGGGGGCGGTGTTGCGC
 GAGATCGGCCCGCGCATGGCCNAATTTGAGCGGTTCCCGGCTGGCCCNCGACCAANCA

3' sequencing primer: EGFP-N (CGTCGCCGTCCAGCTCGACCAG)

CGGGAAGCTCCNNGCCTNNCTCCCATGGTGCGACCGGTGAAGTTCTATACTTTCTAGAGAATAGGAACTTCGGAATAG

12.2.13. pcDNA3-PTEN^{short}

First 3' sequencing primer: CGAGAAACGCCGCCGCTG

Second 3' sequencing primer: CTGGCAACTAGAAGGCACAGTCGAGGC (69)

12.2.14. pcDNA3-PTEN^{long}

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Second 5' sequencing primer: GGTGATGTGGCGGGACTC

GCGCNGGGAGCGACTGCGCTCAGTTCTCTCGAATTCGGCAGCAGGCTCTCGGAAGCTGCAGCCATGATGGAAGTTTGAGA
GTTGAGCCGCTGTGAGGCGAGGCCGGGCTCAGGCGAGGGAGATGAGAGACGGCGGGCGGCCGAGCCCTCTC
AGCGCCTGTGAGCAGCCGCGGGGCGAGCGCCCTCGGGGAGCCGGCGGCTGCGGCGGCGGCAGCGGGCGGCTTTCTCG
CCTCCTCTTCTGCTTTTTCTAACCGTGCAGCCTCTTCTCGGCTTCTCCTGAAAGGGAAGGTGGAAGCCGTGGGCTCGGGCGG
GAGCCGGCTGAGGCGCGGCGGCGGCGGCGGCACCTCCCGCTCCTGGAGCGGGGGGAGAAGCGGCGGCGGCGGCGGCGG
GCGGCGGCTGCAGCTCCAGGGAGGGGCTGTAGTCGCCTGTCACCATTTCCAGGGCTGGGAACGCCGAGAGATTGGTCTCT
CCCCTTCTACTGCCTCCACAGGCGGCGGCGGCGGCGGCACATCCAGGGACCCGGGCGGTTTTAAACCTCCCGTCCGCC
GCCGCCGCACCCCCGTGGCCCCGGGCTCCGGAGGCCGCCGGCGGAGGCAGCCGTTCCGAGGATTATTCGTCTTCTCCCA
TTCCGCTGCCGCCGCTGCCAGGCCTCTGGCTGCTGAGGAGAAGCAGGCCAGTCGCTGCAACCATCCAGCAGCCGCCGCA
GCAGCCATTACCCGGCTGCGGTCCAGAGCCAAGCGGCGGCGAGCGAGGGGCATCAGCTACCGCCAAGTCCAGAGCCATTT
CCATCCTGCAGAAGAAGCTCCGCCACCAGCAGCTTCTGACATCTCTCTCCTCTTTTCTTACGCCACAGGCTCCAGAC
ATGACAGCCATCATCTAAGAGATCGTTAGCAGAAACAAAAGGAGATATCAGGAGGATGGATTGACTTAGACTTGACCTA
TATTTATCCAAACATTATTGCTATGGGATTTCTGCAAAAAGACTTGAAGGGG

Third 5' sequencing primer: GACCAATCTTGTTTTACC

GTCAGCTNGANAANGGTTGTGTAGCTGTGTATGTATATACCTTTTTGTGTCAAAAGGACATTTAAAATTCAATTAGGAT
TAATAAAGATGGCACTTTCCCGTTTTAAAAAAGGACATCGAGCATGCATCTAGAGGCCCTATTCTAT
AGTGTCAACCTAAATGCTAGACTCGCTGATCAGCTCGGCTGTGCTTCTAGTTGCCAGCCATCTGTTGTTTGGCCCTCC
CCCGTGCCTTCTTACCCCTGGAAGGTGCCACTCCACTGTCTTCTCTAATAAATGAGGAAATTGCATCGCATTGTCT
GAGTAGGTGTCAATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATG
CTGGGGATGCGGTGGGCTCTATGGCTTCTGAGGCGGAAAGAACAGCTGGGGCTCTAGGGGGTATCCCCACGCGCCCTGT
AGCGCGCATTAAGCGCGGGGTGTGGTGTACGCGCAGCGTACGCTGACCTTACACTTGCAGCGCCCTTAGCGCCGCTCC
TTTCGCTTCTTCCCTTCTTCTCGCCACGTTCCGCCGCTTCCCGCTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGT
TCCGATTTAGTGCTTTACGGCACCTCGACCCAAAAAAGTATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGA
TAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTAAATAGTGGACTCTTGTTCAAACTGGAACAACACTCAA
CCCTATCTCGGTCTATTCTTTTATTATAAGGATTTGCCGATTTCCGCTATTGGTTAAAAANTGAGCTGATTTAAC
AAAAATTTAACCGCAATTAATTCTGTGGAATGTGTGTCAGTTAAGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAA
ATATGCAAAGCATGCATCTCATTTATTAGCAACCAGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAAAANTATGCAA
AGCNTGGANCTCAATTATTAGCAACCATAATCC

Fourth 5' sequencing primer: GACCGTCCCTGCATTTCC

TGCCAGGTAACCTCAGACTCGAGTCAGTGACACTGTCTAACGCACCCATCTCAGCTTTCATCATCAGTCTCCACCCCCG
CCCCACAACAGCCTACCCTGCCTCCGGCTGGGTTTCTGGGCAGAGGCCGAGGCTTAGCTCGTTATCCTCGCCTCGCGTTG
CTGCAAAAGCCGACGCAAGTGCAGCTGCAGGCTGGCGGCTGGGAACCGGCCGAGCAAGCCCCAGGCAGCTACACTGGGC
ATGCTCAGTAGAGCCTGCGGCTTGGGGACTCTGCGCTCGCACCCAGAGCTACCGCTCTGCCCCCTCTACCGCCCCCTGC
CCTGCCCTGCCCTCCCCTCGCCGCGCGGTCCCGTCCGCTCTCGCTCGCCTCCCGCTCCCTCGGTCTTCCGAGGCG
CCCGGGCTCCCGGCGCGGCGGCGGAGGGGGCGGGCAGGCCGCGGGCGGTGATGTGGCGGGACTCTTTATGCGCTGCG
GCAGGATACGCGCTCGGCGCTGGGACGCGACTCGCTCAGTTCTCGAATTCGGCACGAGGCTCTCGGAAGTGCAGCCA
TGATGGAAGTTGAGAGTTGAGCCGCTGTGAGGCGAGGCGGGCTCAGGCGAGGGAGATGAGAGACGGCGCGCGCCGCG
CCCGGAGCCCCCTCAGCGCCTGTGAGCAGCCGCGGGGGCAGCGCCCTCGGGAGCCGCGCGGCTGCGGCGGCGGCGAG
CGGCGGCGTTTCTCGCCTCCTCTTCTGCTTTTCTAACCGTGCAGCCTTCTCCTCGGCTTCTCCTGAAAGGGAAGGTGGAAGC
CGTGGGCTCGGGCGGAGCCGGCTGAGGCGCGGCGGCGGCGGCACCTCCCGCTCCTGGAGCGGGGGGGNAGAAGC
GGCGGCGGCGCGNTCCGCGGCGGCTGC

First 3' sequencing primer: CGAGAAACGCCGCGGCTG

GNGGGGCTCCGAGGGCGCTGCCTCGCGNTTGCTCACAGGGCGCTGACAGGGGNTNCGGGNCGTNGNCGCCGACGTCTCT
CATCTCCCTCGCCATGAGCCCTNGCCTCGCCTCACAGTCGGCTCAACATCTTCAAATCTTGATCAATGAGCTGC

Second 3' sequencing primer: CTGGCAACTAGAAGGCACAGTCGAGGC

CTATAGAATAGGGCCCTCTAGATGCATGCTCGAGTTTTTTTTTTTTTTTTTTTTTAAACGGGAAAGTGCCATCTTTAT
TAATCCTAATTGAATTTAAATGTCCTTTTGACACAAAAAGGTATATACATGACACAGCTACACAACCTTTTTTCAACTG
GACAAACAGTGTCAAAACCTGTGGATGTATAGGGTAAACAAGATTGGTCAGGAAAAGAGAATTGTTCTATAACTGGT
AATCTGACACAATGTCCTATTGCCATTAATAAAGGTCCATTTTCAAGTTTATTCAAGTTTATTTTATGTTGTTTT
ATCCCTCTTGATAAAAAAATTCAGACTTTTGTAAATTTGTGATGCTGATCTTCAATCAAAAGGTTCAATCTCTGGATC
AGAGTCAGTGGTGTGAGAATATCTATAATGATCAGGTTTATTGTCTACTAACATCTGGTGTACAGAAGTTGAAGTCTAG
CCTCTGGATTTGACGGCTCCTCTACTGTTTTGTGAAGTACAGCTTACCTTAAATTTGGAGAAAAGTATCGGTTGGCT
TTGTCTTTATTTGCTTTGTCAAGATCATTTTTTGTAAAGTAAGTACTAGATATTCCTTGTCTATTATCTGCACGCTCTAT
ACTGCAAAAGTATCGATTTCTGATCACATAGACTTCCATTTTCTACTTTTTCTGAGGTTTCTCTGGTCTGGTATGA
AGAATGATTTACCAAAAGTGAACATTTTGTCTTTTATGATCTTGTCTGTTTGTGAAGAAGTCTACTTTTGATA
TCACCACACACAGGTAACGGCTGAGGGAAGTCAAAGTACATGAAGTTGCTTCCCGTCTGTGGGTCTGAATTGGAGGA
ATATATCTTACCTTTAGCTGGCAGACCACAAAGTGAAGTTCCGCCACTGAACATTGGAATAGTTTCAACA
TCATCTTGAAACAACAGTGCCACTGGTCTATAATCCAGATGATTCTTAAACAGGTAGCTATAATAACACATAGCGC
CTCTGACTGGGAANTTACTCCCTTTT

12.2.15. pGEM-PTEN-AAV-noPolyA

5' sequencing primer: M13-40F (GTTTTCCCAGTCACGAC)

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CGACTCACTATAGGNCGAATTGGGCCCCGACGTCGCATGCTCCCGGCCGCCATGGCCGCGGGATTCTGGCCTTATCTCACA
GGTAAACTGACGCACGGAGGAACAATATAAATTGGGGACTAGAAAGGTGAAGAGCCAAAGTTAGAACTCAGGACCAACT
TATTCTGATTTTGTTCCTCTCTCTCTGGGAAGTGAAGGAAGCTGCAGCACCAGGATCAGTGAAACGCA
CCAGACGGCCGCGTCAGAGCAGCTCAGGTTCTGGGAGAGGGTAGCGCAGGGTGGCCACTGAGAACCGGGCAGGTCACGCA
TCCCCCCTTCCCTCCACCCCTGCCAAGCTCTCCCTCCAGGATCCTCTCTGGCTCCATCGTAAGCAAACCTTAGAGG
TTCTGGCAAGGAGAGAGATGGCTCCAGGAAATGGGGGTGTGTACCAGATAAGGAATCTGCCTAACAGGAGGTGGGGGT
AGACCCAATATCAGGAGACTAGGAAGGAGGAGGCCTAAGGATGGGGCTTTCTGTCAACCAATCCTGTCCCTCTAGATGCA
TGCTCGAGTCAGACTTTTGTAAATTTGTATGCTGATCTTCATCAAAGGTTTCTTCTGGATCAGAGTCAGTGGTGTG
AGAATATCTATAATGATCAGGTTTCTTGTCTACTAACATCTGGTGTACAGAAGTTGAACTGCTAGCCTCTGGATTTGACG
GCTCCTCTACTGTTTTGTGAAGTACAGCTTACCTTAAATTTGGAGAAAAGTATCGGTTGGCTTTGTCTTTATTTGCT
TTGTCAAGATCATTTTTGTAAAGTAACTAGATATTCCTTGTCTATTATCTGCACGCTCTATACTGCAATGCTATC
GATTTCTTGATCAGATAGACTTCCATTTTCTACTTTTTCTGAGGTTTCTCTGGTCTGGTATGAAGAATGTATTTACCC
AAAAGTGAAACATTTTGTCTTTTTTAGCATCTTGTCTGTTTGTGGAAGAACTCTACTTTGATATCACCACACACAGGT
AACGGCTGAAGGAACCTCAAAGTACATGAACCTTGTCTCCCGTCGTGTGGNTCCTGAATTGGAGGAATATATCTTCACCT
TAGCTGGCAGACNNNAANCTGAAGATTGCAAGTCCGCCACTGAACATTGGAATAGTTTCAAACATCATCTTGGNAACAA
CAGTGGCNCCTGGTCTANAATCCAGATGATTCTTNAACAGGTAGCTA
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12.2.16. pcPTEN-AAV

5' sequencing primer: M13-40F (GTTTTCCCAGTCACGAC)

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ACGACTCACTATAGGGCGAATTGGGCCCCGACGTCGCATGCTCCCGGCCGCCATGGCCGCGGGATTCTGGCCTTATCTCAC
AGGTAAACTGACGCACGGAGGAACAATATAAATTGGGGACTAGAAAGGTGAAGAGCCAAAGTTAGAACTCAGGACCAAC
TTATTCTGATTTTGTTCCTCTCTCTGGGAAGTGAAGGAAGCTGCAGCACCAGGATCAGTGAAACGC
ACCAGACGGCCGCGTCAGAGCAGCTCAGGTTCTGGGAGAGGGTAGCGCAGGGTGGCCACTGAGAACCGGGCAGGTCACGC
ATCCCCCCTTCCCTCCACCCCTGCCAAGCTCTCCCTCCAGGATCCTCTCTGGCTCCATCGTAAGCAAACCTTAGAG
GTTCTGGCAAGGAGAGAGATGGCTCCAGGAAATGGGGGTGTGTACCAGATAAGGAATCTGCCTAACAGGAGGTGGGGT
TAGACCCAATATCAGGAGACTAGGAAGGAGGAGGCCTAAGGATGGGGCTTTCTGTCAACCAATCCTGTCCCTCTAGTCGA
GCCCCAGCTGTTTCTTCCGCTCAGAAGCCATAGAGCCACCGCATCCCCAGCATGCCTGCTATTGTCTTCCCAATCCT
CCCCCTTGCTGTCTGCCCAACCCACCCCAAGAATAGAATGACACCTACTCAGACAATGCGATGCAATTTCTCTATTT
TATTAGGAAAGGACAGTGGGAGTGGCACCTTCCAGGGTCAAGGAAGGCACGGGGGAGGGGCAAACAACAGATGGCTGGCA
ACTAGAAGGCACAGTCGAGGCTGATCAGCGAGCTCTAGATGCTGCTCGAGTCAGACTTTTGTAAATTTGTGATGCTGAT
CTTCATCAAAGGTTTCTTCTGATCAGAGTCAGTGGTGTGAGAATATCTATAATGATCAGGTTTCTTGTCTACTAACA
TCTGGTGTACAGAAGTTGAAGTCTAGCCTCTGGATTTGACGGCTCCTCTACTGTTTTGTGAAGTACAGCTTACCTT
AAAATTTGGAGAAANGTATCGGTTGGCTTTGTCTTTATTTGCTTTGTCAAGATCATTTTTTGTAAAGTAACTAANT
ATTCTTGTCTATTCTGCACGCTCTATACTGCAATGCTATCGATTTCTTGATCAGATAGACTTCCATTTTCAACTTTTTTC
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12.2.17. pcPTEN_C124S-AAV

3' sequencing primer: CACTGAACATTGGAATAGTTTCAAAC

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TATCCAGATGATTCTTTACAGGTAGCTATAATAATACACATAGCGCCTCTGACTGGGAATAGTTACTCCCTTTTTGTCTC
TGGTCCTTACTTCCCATAGAAATCTAGGGCCTCTTGTGCCTTTAAAAATTTGCCCCGATGTAATAAATATGCACATATC
ATTACACAGTTTCGTCCTTGGCGGCTTTAGAGTGAATTGCTGCAACATGATTGTCATCTTCACTTAGCCATTGGTCAAG
ATCTTCACAAAAGGGTTTGATAAGTTCTAGCTGTGGTGGGTTATGGTCTTCAAAGGATATTGTGCAACTCTGCAATTA
ATTTGGCGGTGTCTAATGTCTTTACGACAAAGATTGTATATCTTGAATGGTTTTATGCTTTGAATCCAAAAACCTT
ACTACATCATCAATATTGTTCTGTATACGCCTTCAAGTCTTCTGCAAGAAATCCCATAGCAATAATGTTTGGATAAAT
ATAGGTCAAGTCAAGTCAAGTCAAGTCAAGTCAAGTCAAGTCAAGTCAAGTCAAGTCAAGTCAAGTCAAGTCAAGTCA
TAAGCTTGGGTCTCCCTATAGTGAGTCGTATTAATTTGATAAGCCAGTAAGCAGTGGGTTCTCTAGTTAGCCAGAGAGC
TCTGCTTATATAGACCTCCACCGTACACGCCTACCGCCCATTTGCGTCAATGGGGCGGAGTTGTTACGACATTTTGGAA
AGTCCCGTTGATTTTGGTGCCAAAACAACTCCCATTTGACGTCAATGGGGTGGAGACTTGGAAATCCCCGTGAGTCAAA
CGCTATCCACGCCCATTGATGTACTGCCAAAACCGCATACCATGGTAATAGCGATGACTAATACGTAGATGTAATGCCA
AGTAGGAAAGTCCCATAGGTCATGTACTGGGCATAATGCCAGGCGGGCCATTTACCGTCATTGACGTCAATAGGGGGCG
TACTTGGCATATGATACACTTGATGTACTGCCAAGTGGGCAGTTTACCGTAAATACTCCACCCATTGACGTCAATGGAAA
GTCCCTATTGGCGTTACTATGGGAACATACGTCATTATTGACGTCAATGGNCCGGGGTCTGTTGGCGGTGAGCCAGGCG
GGCCATTTACCGTAAGTTATGTA
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12.2.18. pcPTEN_G129E-AAV

3' sequencing primer: CACTGAACATTGGAATAGTTTCAAAC

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AATCCAGATGATTCTTACAGGTAGCTATAATAATACACATAGCGCCTCTGACTGGGAATAGTTACTCCCTTTTTGTCTC
TGGTCCTTACTTCCCATAGAAATCTAGGGCCTCTTGTGCCTTTAAAAATTTGCCCCGATGTAATAAATATGCACATATC
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ATTACACCAGTTTCGTTCCCTTGCCGGCTTTACAGTGAATTGCTGCAACATGATTGTCATCTTCACTTAGCCATTGGTCAAG
 ATCTTCACAAAAGGGTTTGATAAGTTCTAGCTGTGGTGGGTTATGGTCTTCAAAAGGATATTGTGCAACTCTGCAATTAA
 ATTTGGCGGTGTCTAATGTCTTTAGCACAAGATTGTATATCTTGAATGGTTTTATGCTTTGAATCCAAAAACCTT
 ACTACATCATCAATATTGTTCTGTATACGCCCTTCAAGTCTTTCTGCAGGAAATCCCATAGCAATAATGTTTGGATAAAT
 ATAGGTCAAGTCTAAGTCAATCCATCCTCTTGATATCTCCTTTTGTCTGCTAACGATCTCTTTGATGATGGCTGTCA
 TAAGCTTGGGTCTCCCTATAGTGAGTCGTATTAATTTGATAAGCCAGTAAGCAGTGGGTTCTCTAGTTAGCCAGAGAGC
 TCTGCTTATATAGACCTCCCACCGTACACGCCTACCGCCCATTTGCGTCAATGGGNGCGGAGTTGTTACGACATTTTGA
 AAGTCCCGTTGATTTTGGTGCCAAAACAACTCCCATTTGACGTCAATGGGGTGGAGACTTGGAATCCCCGTGAGTCAAA
 CCGCTATCCACGCCCATTGATGTAAGTCCGCAATCACCATGGTAATAGCGATGACTAATACGTAGATGTAAGTCC
 AAGTAGGAAAGTCCCATAGGTCATGTAAGTGGGCATAATGCCAGGCGGGCCATTACCGTCATTGACGTCAATAGGGGGC
 GTACTTGGCATATGATACACTTGATGTAAGTGGGCAGTTTACCGTAAATACTCCACCCATTGACGTCAATGGAA
 AGTCCCTATTGGCGTTACTATGGGAACATACGTCAATTATTGACGTCAATGGGCGNGGGNTCGTTGGGCGGTGAGCCAGGC
 GGGCCATTTACCGTAAGTTATGTAACGCGGAACCTCCNTATATGGGCTATGAACTAATGACCCCGTAATTGATTACTAT

12.2.19. pcPTEN_H93A-AAV

3' sequencing primer: CACTGAACATTGGAATAGTTTCAAAC

TCCAGATGATTCTTNACAGGTAGCTATAATAATACACATAGCGCCTCTGACTGGGAATAGTTACTCCCTTTTGTCTCTG
 GTCTTACTTCCCATAGAAATCTAGGGCCTCTTGTCCTTTAAAAATTTGCCCCGATGTAATAAATATGCACATATCAT
 TACACCAGTTTCGTTCCCTTTCCAGCTTTACAGTGAATTGCTGCAACATGATTGTCATCTTCACTTAGCCATTGGTCAAGAT
 CTTCAACAAAAGGGTTTGATAAGTTCTAGCTGTGGTGGATTAGCATCTTCAAAAGGATATTGTGCAACTCTGCAATTAAAT
 TTGGCGGTGTCTAATGTCTTTTCCAGCACAAGATTGTATATCTTGAATGGTTTTATGCTTTGAATCCAAAAACCTTAC
 TACATCATCAATATTGTTCCCTGTATACGCCCTTCAAGTCTTTCTGCAGGAAATCCCATAGCAATAATGTTTGGATAAATAT
 AGGTCAAGTCTAAGTCAATCCATCCTCTTGATATCTCCTTTTGTCTGCTAACGATCTCTTTGATGATGGCTGTCTATA
 AGCTTGGGTCTCCCTATAGTGAGTCGTATTAATTTGATAAGCCAGTAAGCAGTGGGTTCTCTAGTTAGCCAGAGAGCTC
 TGCTTATATAGACCTCCCACCGTACACGCCCTACCGCCCATTTGCGTCAATGGGGCGGAGTTGTTACGACATTTTGAAGG
 TCCCGTTGATTTTGGTGCCAAAACAACTCCCATTTGACGTCAATGGGGTGGAGACTTGGAATCCCCGTGAGTCAAAACCG
 CTATCCACGCCCATTGATGTAAGTCCGCAATCACCATGGTAATAGCGATGACTAATACGTAGATGTAAGTCC
 TAGGAAAGTCCCATAGGTCATGTAAGTGGGCATAATGCCAGGCGGGCCATTACCGTCATTGACGTCAATAGGGGGCGTA
 CTTGGCATATGATACACTTGATGTAAGTGGGCAGTTTACCGTAAATACTCCACCCATTGACGTCAATGGAAAGT
 CCCTATTGGCGTTACTATGGGAACATACGTCAATTATTGACGTCAATGGGCGNGGGNTCGTTGGGCGGTGAGCCAGGCGGG
 CCATTTACCGAA

12.2.20. pcPTEN_V403A-AAV

5' sequencing primer: GTTTGAAACTATTCCAATGTTTCAGTG

TGATCTNGTTNGTGGTCTGCCGCTNAGGTGAAGATATATTCCTCCAATTCAGGACCCACACGACGGGAAGACAAGTTTCA
 GTACTTTGAGTTCCCTCAGCCGTTACCTGTGTGTGGTGATATCAAAGTAGAGTTCTTCCACAAAACAGAACAGATGCTAA
 AAAAGGACAAAATGTTTCACTTTTGGGTAAATACATTCTTCATACCAGGACCAGAGGAAACCTCAGAAAAAGTAGAAAAT
 GGAAGTCTATGTATCAAGAAATCGATAGCAATTTGCAGTATAGAGCGTGAGATAATGACAAGGAATATCTAGTACTTAC
 TTTAACAAAAAATGATCTTGACAAAGCAAATAAAGACAAAGCCAACCGATACTTTTCTCAAATTTAAGGTGAAGCTGT
 ACTTCACAAAAACAGTAGAGGAGCCGTCAAATCCAGAGGCTAGCAGTTCAACTTCTGTAACACCAGATGTTAGTGACAAT
 GAACCTGATCATTATAGATATTCTGACACCACTGACTCTGATCCAGAGAATGAACCTTTTGTGAAGATCAGCATACACA
 AATTACAAAGGCCTGACTCGAGCATGCATCTAGAGCTCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTG
 TTGTTTGGCCCTCCCGCGTGCCTTCTTGAACCTGGAAGGTGCCACTCCCACTGTCTTTCTAATAAAATGAGGAAAT
 GCATCGATTGTCTGAGTAGGTGTCTTATTCTTGGGGGNGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAG
 ACAATAGCAGGCATCTGGGGATGCGGTGGGCTCTATGGCTTCTGAGGCGGAAAGAACCAGCTGGGGCTCGACTAGAGGG
 ACAGGATTGGTGACAGAAAAGCCCCATCCTTAGGCCTCCTCCTTCTAGTCTCCTGATATTGGGTCTAACCCCACTCTC
 GTTAGGCAGATTCTTATCTGGTGACACACCCCATTTCTGGAGCCATCTCTCTCTTGGCAGAACCTTAAGGTTTG
 CTTACGATGGAGCCAGAGAGGATCCTGGGAGGGAGAGCTTGGCAGGGGTGGGAAG

12.2.21. pcPTEN_K13E&K289E-AAV

First 5' sequencing primer: M13-40F (GTTTTCCAGTCACGAC)

TATCGACTNCNATAGGNCGAATTGGGCCGACGTGCGATGCTCCCGGCCGATGGCCGCGGGATTCTGGCCTTATCTCA
 CAGGTAAAAGTACGACGACGGAGGAACAATATAAATTGGGGACTAGAAAGGTGAAGAGCCAAAGTTAGAACTCAGGACCAA
 CTTATTCTGATTTTGTCTTCCAACTGCTTCTCCTCTTGGGAAGGTGAAGGAAGCTGCAGCACCAGGATCAGTGAACG
 CACCAGACGGCCGCGTCAGAGCAGCTCAGGTTCTGGGAGGGTAGCGCAGGGTGGCCACTGAGAACCAGGGCAGGTCAGG
 CATCCCCCTTCCCTCCACCCCTGCCAAGCTCTCCCTCCAGGATCCTCTCTGGCTCCATCGTAAGCAAACCTTAGA
 GGTTCTGGCAAGGAGAGAGATGGCTCCAGGAAATGGGGGTGTGTACCAGATAAGGAATCTGCCTAACAGGAGGTGGGGG
 TTAGACCAATATCAGGAGACTAGGAAGGAGGAGGCCAAGGATGGGGCTTTTCTGTACCAATCCTGTCCCTCTAGTCG
 AGCCCCAGTGGTCTTTCCGCTCAGAAGCCATAGAGCCACCCGATCCCGCATGCCTGATTGCTTTCCCAATCC
 TCCCCCTTGCTGCTGCTGCCCAACCCCAACCCCAAGAAATAGAAATGACACCTACTCAGACAATGCGATGCAATTTCTCATT
 TTATTAGGAAAGGACAGTGGGAGTGGCACCTTCCAGGGTCAAGGAAGGCACGGGGGAGGGGCAAACAACAGATGGCTGGC
 AACTAGAAGGCACAGTCGAGGCTGATCAGCGAGCTCTAGATGCATGCTCGAGTCAGACTTTTGTAAATTTGTGTATGCTGA
 TCTTCATCAAAAGGTTCACTTCTGGATCAGAGTCAGTGGTGTGAGAATATCTATAATGATCAGGTTCAATTGTCACTAAC

ATCTGGTGTTACAGAAGTTGAAGTCTAGCCTCTGGATTTGACGGCTCCTCTACTGTTTTGTGAAGTACAGCTTCACCT
TAAAATTTGGAGAAANGTATCGGTTGGCTTTGTCTTTATTTGCTTTGTCAAGATCATTTTTTGTAAAGTAAGTACTAAN
TATTCCTTGGCATTATCTGGACGCTCTATACTGCAAAATGCTATCGATTCTTGATCACAANGACTTCCATTTTCCACTTC
TTCTGAAGTCCCCNTGGGCCTGGNTATGAAAA

Second 5' sequencing primer: GTTTGAAGTATTCCAATGTTTCAGTG

TGGTCTGCCGCTAAGGTGAAGATTATTCCTCCAATTCAGGACCCACACGACGGGAAGACAAGTTTCATGTACTTTGAGTTC
CCTCAGCCGTTACCTGTGTGTGGTGATATCAAAGTAGAGTTCTTCCACAAACAGAACAGATGCTAAAAAGGACAAAAT
GTTTCACTTTTTGGGTAAATACATTCTTCATACCAGGACCAGAGGAGACGTCAGAAGAGTAGAAAATGGAAGTCTATGTG
ATCAAGAAATCGATAGCATTGTCAGTATAGAGCGTGCAGATAATGACAAGGAATATCTAGTACTTACTTTAACAAAAAT
GATCTTGACAAAGCAAATAAAGACAAAGCCACCGATACCTTTCTCCAAATTTTAAGGTGAAGCTGTACTTCACAAAAAC
AGTAGAGGAGCCGTCAAATCCAGAGGCTAGCAGTTCAACTTCTGTAACACCAGATGTTAGTGACAATGAACCTGATCATT
ATAGATATTCTGACACCACTGACTCTGATCCAGAGAATGAACCTTTTGATGAAGATCAGCATACAAAATTACAAAAGTC
TGAATCGAGCATGCATCTAGAGCTCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCTC
CCCCGTGCCTTCTTGACCCTGGAAGGTGCCACTCCACTGTCTTTCTAATAAAATGAGGAAATTGCATCGCATTGTC
TGAGTAGGTGTCATTATTTCTGGGGGGTGGGGGNGGGGNCAGGACAGCAAGGGGNGAGGATTGGGAAGACAATAGCAG
GCATGCTGGGGATGCGGTGGGCTCTATGGCTTCTGAGGCGGAAAGAACCAGCTGGGGCTCGACTAGAGGGACAGGATTGG
TGACAGAAAAGCCCCATCCTTAGGCCTCCTCCTTAGTCTCCTGATATTGGGTCTAACCCCCACCTCCTGTTAGGCAG
ATTCTTATCTGGTGACACACCCCCATTCTGGAGCCATCTCTCCTTGCCAGAACCTCTAAGGTTTGCTTACGATGG
AGCCNGAGAGGATCCTGGGAGGGAAANCTTGGCAGGG

3' sequencing primer: CACTGAACATTGGAATAGTTTCAAAC

TATCCAGATGATTCTTTACAGGTAGCTATAATAATACACATAGCGCCTCTGACTGGGAATAGTTACTCCCTTTTGTCTC
TGGTCTTACTTCCCCATAGAAATCTAGGGCCTCTTGTGCCTTTAAAAATTTGCCCCGATGTAATAAATATGCACATATC
ATTACACCAGTTCGTCCTTTCCAGCTTTACAGTGAATTGCTGCAACATGATTGTCATCTTCACTTAGCCATTGGTCAAG
ATCTTCACAAAAGGGTTTGATAAGTTCTAGCTGTGGTGGGTATGGTCTTCAAAGGATATTGTGCAACTCTGCAATTAA
ATTTGGCGGTGTCTAATGCTTTTCAGCACAAAGATTGATATCTTGAATGGTTTTATGCTTTGAATCCAAAAACCTT
ACTACATCATCAATATTGTTCTGTATACGCCTTCAAGTCTTCTGCAGGAAATCCCATAGCAATAATGTTTGGATAAAT
ATAGGTCAAGTCTAAGTCAATCCATCCTCTTGATATCTCCTTTGTTTCTCGAGACGATCTCTTTGATGATGGCTGTCA
TAAGCTTGGGTCTCCCTATAGTGAGTCGTATTAATTCGATAAGCCAGTAAGCAGTGGGTTCTCTAGTTAGCCAGAGAGC
TCTGCTATATAGACCTCCACCGTACACGCCTACCGCCCATTTGCGTCAATGGGGCGGAGTTGTTACGACATTTTGAA
AGTCCCGTTGATTTTGGTGCCAAAACAACTCCCATGACGTCATGAGTCAATGGGGTGGAGACTTGGAAATCCCCGTGAGTCAAAC
CGCTATCCACGCCCATTGATGTAAGTCCAAACCGCATCACCATGGTAATAGCGATGACTAATACGTAGATGTAAGTCCCA
AGTAGGAAAGTCCCATAGGTCATGTAAGTGGGCATAATGCCAGGCGGGCCATTACCGTCATTGACGTCAATAGGGGGCG
TACTTGGCATATGATACACTTGATGTAAGTGGCAAGTGGGCGATTTACCGTAAATACTCCACCCATTGACGTCAATGGAAG
GTCCCTATTGGCGTTACTATGGGAACATACGTCATTATTGAC

12.2.22. pSBbi-GB-PTEN

3' sequencing primer: CACTGAACATTGGAATAGTTTCAAAC

TATCCAGATGATTCTTNACAGGTAGCTATAATAATACACATAGCGCCTCTGACTGGGAATAGTTACTCCCTTTTGTCTC
TGGTCTTACTTCCCCATAGAAATCTAGGGCCTCTTGTGCCTTTAAAAATTTGCCCCGATGTAATAAATATGCACATATC
ATTACACCAGTTCGTCCTTTCCAGCTTTACAGTGAATTGCTGCAACATGATTGTCATCTTCACTTAGCCATTGGTCAAG
ATCTTCACAAAAGGGTTTGATAAGTTCTAGCTGTGGTGGGTATGGTCTTCAAAGGATATTGTGCAACTCTGCAATTAA
ATTTGGCGGTGTCTAATGCTTTTCAGCACAAAGATTGATATCTTGAATGGTTTTATGCTTTGAATCCAAAAACCTT
ACTACATCATCAATATTGTTCTGTATACGCCTTCAAGTCTTCTGCAGGAAATCCCATAGCAATAATGTTTGGATAAAT
ATAGGTCAAGTCTAAGTCAATCCATCCTCTTGATATCTCCTTTTGTCTGCTAACGATCTCTTTGATGATGGCTGTCA
TAAGCTTGGCCTGACAGGCCCTTCAATTAGTGAGAAGGAAAGACGCAACCAGGAAGCCACCAAGAGAAAGAGGCATTTT
CTGAAAAGATTCCCCTTTTGGAGAGCCCTACAAGGTATTTACTGAACACTAGACATTGAAGTCTGATTTATCACAATG
TTGAACCCTATGATGAAACAATTCAAGTATAATTGAGTTCGAAACTCGAGCCATGGTGGCCTCAGAGGCCAGCTTGGGGT
AGTTTTACGACACCTTAAATGGAANAAAAAANCCTTGAACCAGTCTGAAAGCTTGAGAATGAACCAGGATCCAAACTC
CAAAAAGGGCAAATCCAGGGGAGAATTACTTCAAGTGCCAAAGCTGGGCCTAATTTCACTTCCACCCACTCAGTAGGG
GTAAACTCCATCCANAAAAACCCNCCCCCCCCACCCTAAGAACAACCTTATTCCAAAAGC

12.2.23. pSBbi-GB-PTEN_C124S

3' sequencing primer: CACTGAACATTGGAATAGTTTCAAAC

AATCCAGATGATTCTTTACAGGTAGCTATAATAATACACATAGCGCCTCTGACTGGGAATAGTTACTCCCTTTTGTCTC
TGGTCTTACTTCCCCATAGAAATCTAGGGCCTCTTGTGCCTTTAAAAATTTGCCCCGATGTAATAAATATGCACATATC
ATTACACCAGTTCGTCCTTTGCCGGCTTTAGAGTGAATTGCTGCAACATGATTGTCATCTTCACTTAGCCATTGGTCAAG
ATCTTCACAAAAGGGTTTGATAAGTTCTAGCTGTGGTGGGTATGGTCTTCAAAGGATATTGTGCAACTCTGCAATTAA
ATTTGGCGGTGTCTAATGCTTTTCAGCACAAAGATTGATATCTTGAATGGTTTTATGCTTTGAATCCAAAAACCTT
ACTACATCATCAATATTGTTCTGTATACGCCTTCAAGTCTTCTGCAGGAAATCCCATAGCAATAATGTTTGGATAAAT
ATAGGTCAAGTCTAAGTCAATCCATCCTCTTGATATCTCCTTTTGTCTGCTAACGATCTCTTTGATGATGGCTGTCA
TAAGCTTGGCCTGACAGGCCCTTCAATTAGTGAGAAGGAAAGACGCAACCAGGAAGCCACCAAGAGAAAGAGGCATTTT
CTGAAAAGATTCCCCTTTTGGAGAGCCCTACAAGGTATTTACTGAACACTAGACATTGAAGTCTGATTTATCACAATG
TTGAACCCTATGATGAAACAATTCAAGTATAATTGAGTTCGAAACTCGAGCCATGGTGGCCTCAGAGGCCAGCTTGGGGT

AGTTTTACGACACCTTAAATGGAAGAAAAAACTTTGAACCACTGTCTGAGGCTTGAGAATGAACCAAGATCCAACTC
AAAAAGGGGCAAATTCAGGAGAATTACATCNGGTGCCAAGCTGGGCCTAACTTTAGTCTCCNCCCACTCAGTATGGG
GGAACTCCTTCGCATAAAACCCCTCCCCCANCTAAAGAACGACGTTAATCCCAAAGCTCCAAG

12.2.24. pSBbi-GB-PTEN_G129E

3' sequencing primer: CACTGAACATTGGAATAGTTTCAAAC

TATCCAGATGATTCTTTACAGGTAGCTATAATAATACACATAGCGCCTCTGACTGGGAATAGTTACTCCCTTTTGTCTC
TGGTCCTTACTTCCCATAGAAATCTAGGGCCTCTTGTGCCTTTAAAAATTTGCCCGATGTAATAAATATGCACATATC
ATTACACCAGTTCGTTCTTGGCGGCTTACAGTGAATTGCTGCAACATGATTGTCATCTTCACTTAGCCATTGGTCAAG
ATCTTCACAAAAGGGTTTGATAAGTTCTAGCTGTGGTGGGTATAGGTCTTCAAAAGGATATTGTGCAACTCTGCAATTAA
ATTTGGCGGTGTCTAATGTCTTTAGCACAAGATTGTATATCTTGAATGGTTTTATGCTTTGAATCCAAAAACCTT
ACTACATCATCAATATTGTTCTGTATACGCCTTCAAGTCTTCTGCAGGAAATCCCATAGCAATAATGTTTGGATAAAT
ATAGGTCAAGTCTAAGTGAATCCATCCTCTTGATATCTCCTTTTGTCTGCTAACGATCTCTTTGATGATGGCTGTCA
TAAGCTTGGCCTGACAGGCCTTGAATTAGTGAGAAGGAAAGACGCAACCAGGAAGCCCAAGAGAAAGAGGCATTTTC
CTGAAAAGATTCCCCTTTTGGAGAGCCCTACAAGGTATTTACTGAACACTAGACATTGAAGTCTGATTTATCACAATG
TTGAACCCTATGATGAAACAATTCAGTATAATTGAGTTCGAACTCGAGCCATGGTGGCCTCAGAGGCCAGCTTGGGGGT
AGTTTTACGACACCTTAAATGGAAGAAAAAACTTTGAACCACTGTCTGAGGCTTGAGAATGAACCAAGATCCAACTC
AAAAAGGGCAAATTCAGGAGAATTACATCAAGTGCCAAGCTGGCCTAACTTCACTCCACCCACTCAGTATGGGGAA
ACTCCATCGCATAAAACCCCTCCCCCAACCTAAAGACGACGTACTCCAAAAGCTCGAGAACTAATCGAGGTGCCGGNAC
GGCGCCCGGTACTCC

12.2.25. pSBbi-GB-PTEN_H93A

3' sequencing primer: CACTGAACATTGGAATAGTTTCAAAC

GATGATTCTTTACAGGTAGCTATAATAATACACATAGCGCCTCTGACTGGGAATAGTTACTCCCTTTTGTCTCTGGTCC
TTACTTCCCATAGAAATCTAGGGCCTCTTGTGCCTTTAAAAATTTGCCCGATGTAATAAATATGCACATATCATTACA
CCAGTTCGTCCCTTTCAGCTTTACAGTGAATTGCTGCAACATGATTGTCATCTTCACTTAGCCATTGGTCAAGATCTTC
ACAAAAGGGTTTGATAAGTTCTAGCTGTGGTGGATTAGCATCTTCAAAAGGATATTGTGCAACTCTGCAATTAATTTGG
CGGTGTCTAATGTCTTTAGCACAAGATTGTATATCTTGAATGGTTTTATGCTTTGAATCCAAAAACCTTACTACA
TCATCAATATTGTTCTGTATACGCCTTCAAGTCTTCTGCAGGAAATCCCATAGCAATAATGTTTGGATAAATATAGGT
CAAGTCTAAGTCNNAATCCATCCTCTTGAAATCTCCCTTTTGTCTGCTAACGATCTCCTTTGATGAAGGGCTGTCTATA
AACCTTGGCCCTGACAGGGCCTTCAAATATTGGAAANGGAAAAACCCACCCCGGAAAGCCCCCCCCAAAAAANNAT
NGGCATTTTCCCGGAAAAAATCCCTTTTGGAAAAACCCCTANCAGGGGTATTTTCCGGGAACCCCTAAAAANNAT
TTGAAAATCCCGGATTTTTTCCCAAGGGGTGAAACCCCTTTGGAGGGGAAACAATT

12.2.26. pSBbi-GB-PTEN_V403A

3' sequencing primer: CACTGAACATTGGAATAGTTTCAAAC

AATCCAGATGATTCTTNACAGGTAGCTATAATAATACACATAGCGCCTCTGACTGGGAATAGTTACTCCCTTTTGTCTC
TGGTCCTTACTTCCCATAGAAATCTAGGGCCTCTTGTGCCTTTAAAAATTTGCCCGATGTAATAAATATGCACATATC
ATTACACCAGTTCGTCCCTTTCAGCTTTACAGTGAATTGCTGCAACATGATTGTCATCTTCACTTAGCCATTGGTCAAG
ATCTTCACAAAAGGGTTTGATAAGTTCTAGCTGTGGTGGGTATAGGTCTTCAAAAGGATATTGTGCAACTCTGCAATTAA
ATTTGGCGGTGTCTAATGTCTTTAGCACAAGATTGTATATCTTGAATGGTTTTATGCTTTGAATCCAAAAACCTT
ACTACATCATCAATATTGTTCTGTATACGCCTTCAAGTCTTCTGCAGGAAATCCCATAGCAATAATGTTTGGATAAAT
ATAGGTCAAGTCTAAGTGAATCCATCCTCTTGATATCTCCTTTTGTCTGCTAACGATCTCTTTGATGATGGCTGTCA
TAAGCTTGGCCTGACAGGCCTTGAATTAGTGAGAAGGAAAGACGCAACCAGGAAGCCCAAGAGAAAGAGGCATTTTC
CTGAAAAGATTCCCCTTTTGGAGAGCCCTACAAGGTATTTACTGAACACTAGACATTGAAGTCTGATTTATCACAATG
TTGAACCCTATGATGAAACAATTCAGTATAATTGAGTTCGAACTCGAGCCATGGTGGCCTCAGAGGCCAGCTTGGGGGT
AGTTTTACGACACCTTAAATGGAAGAAAAAANCCTTGAACCACTGTCTGAGGCTTGAGAATGAACCAAGATCCAACTC
AAAAAGGGCAAATTCAGGAGAATTACATCAAGTGCCAAGCTGGCCTAACTTCACTCCACCCACTCAGTATGGGGAA
ACTCCATCGCATAAAACCCCTCCCCCAACCTAAAGACGACGTACTCCAAAAGCTCGAGAACTAATCGAGGTGCCGGAC
GGCGCCCGGTACTCCGTGGAGTCACATGAAGCGACGGCTGAGGACGGAAAGGCCCTTTTCTTTGTGTGGGTGACTCACC
CGCCGCTCTCCNAAGCGCCGCTCCTCCATTTTGAAGTCCCTGCAGCAGGGCCGG

12.2.27. pSBbi-GB-PTEN_K13E&K289E

5' sequencing primer: GTTTGAAACTATTCCAATGTTTCAGTG

AGGTGAAGATTATTCCTCCAATTCAGGACCCACACGACGGGAAGACAAGTTCATGTACTTTGAGTTCCTCAGCCGTTAC
CTGTGTGTGGTGATACAAAGTAGAGTTCTTCCACAAACAGAACAGATGCTAAAAAGGACAAAATGTTTCACTTTTGG
GTAAATCAATTTCTTATACAGGACAGAGGAAACCTCAGAAAAAGTAGAAAAAGTCTATGTGATCAAGAAATCGA
TAGCATTTGCAGTATAGAGCGTGAGATAATGACAAGGAATATCTAGTACTTACTTTAACAAAAAATGATCTTGACAAAG
CAATAAAGACAAAGCCAACCGATACCTTTCTCCAAATTTAAGGTGAAGCTGTACTTCACAAAAACAGTAGAGGAGCCG

TCAAATCCAGAGGCTAGCAGTTCAACTTCTGTAACACCAGATGTTAGTGACAATGAACCTGATCATTATAGATATTCTGA
 CACCACTGACTCTGATCCAGAGAATGAACCTTTTGTAGTAAGATCAGCATACACAAATTACAAAAGTCTGACTCGAGCATG
 CATCTAGAGCTCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTCCCCCTCCCCGTCCTTCC
 TTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCTTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCA
 TTCTATTCTGGGGGTTGGGGTGGGGGCAGGACAGCAAGGGGGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGGAT
 GCGGTGGGCTCTATGGCTTCTGAGGCGGAAAGAACCAGCTGGGCCTACTAGATAGCGGACCCCTTACCGAAACATCGCCG
 CATTCTGCAGAGGAGTCGAACCTTAGTGTATGTAACTTCTGACCCACTGGAATTGTGATACAGTGAATTATAAGTGAAT
 AATCTGTCTGTAAACAATTGTTGGAAAAATGACTTGTGTCTATGCACAAAGTAGATGTCCTAACTGACTTGCCAAAATAT
 TGTGTTGTTAAACAAGAAATTTGTGGAGTAGTTGAAAAACGAGTTTAACTGACTCCAACCTTAAGTGTATGTAACTTCCGAC
 TTCAACTGTATAAGGGATCCAATTTTAACAAAATATTAACGCTTACAATTTCTGA

3' sequencing primer: CACTGAACATTGGAATAGTTTCAAAC

GTCTNTATCCAGATGATTCTTTACAGGTAGCTATAATAATACACATAGCGCCTCTGACTGGGAATAGTTACTCCCTTTT
 GTCTCTGGTCCTTACTTCCCCATAGAAATCTAGGGCCTCTGTGCCTTTAAAAATTTGCCCGATGTAATAAATATGCAC
 ATATCATTACACCAAGTTCCGTCCCTTTCCAGCTTTACAGTGAATTGCTGCAACATGATTGTCATCTTCACTTAGCCATTG
 TCAAGATCTTCAAAAAGGTTTGATAAGTTCTAGCTGTGGTGGGTTATGGTCTTCAAAAGGATATTGTGCAACCCGGG
 ATTAATTTGGCGGTGTCAATGTCTTTCAGCACAAAGATTGTATATCTTGTAAATGGTTTTATGCTTTGAATCCAAAA
 ACCTTACTACATCATCAATATTGTTCTGTATACGCCTTCAAGTCTTCTGCAAGGAAATCCCATAGCAATAATGTTTGGA
 TAAATATAGGTCAAGTCTAAGTCGAATCCATCCTTGTATATCTCCTTTTGTCTGCTAACGATCTCTTTGATGATGGC
 TGTCAATAAGCTTGGCCTGACAGGCCTTCAATTAGTGAAGGAAAGAGCAACCAAGGAGCCCAAGGAGAAAGAGGC
 ATTTCTGAAAAGATTCCCCTTTTGGAGAGCCCTACAAGGTATTTACTGAACACTAGACATTGAAGTCTGATTTATCA
 CAATGTTGAACCCCTATGATGAAACAATTGAGTATAATTGAGTTCGAAACTCGAGCCATGGTGGCCTCAGAGGCCAGCTTG
 GGGGTAGTTTTACGACACCTTAAATGGAAGAAAAAACTTTGAACCACTGTCTGAGGCTTGAGAATGAACCAAGATCCA
 AACTCAAAAAGGGGCAAAATCCAAGGAGAATTACATCAAGTGCAGGCTGACCTAACCTTCACTTCCACCCACTCAGTATG
 GGGAACTCCATCGCATAAAACCCCTCCCCCAACCTAAAGACGACGTACTCCAAAAGCTCGAGAACTAATCGAGGTGCC
 TGGACGGCGCCCGTACTCCGTGGAGTCACATGAAGCGACGGCTGAGGACGGAAGGCCCTTTCTTTGGNTGGGTGAC
 TCACCCGCGCGCTCTCCCGAGCGCGCGCTCCTCCATTTGAGCTCC

12.2.28. pGEM-T-NLK_WT

5' sequencing primer: T7P (TAATACGACTCACTATAGGG)

GCCGCGGNATTNGATTCCGATGGCGGCTTACAATGGCGGTACATCTGCAGCAGCAACAGGTACCAACCACCACCATCACC
 ACCACCTTCCACACCTCCCTCCTCCTCACCTGCACCACCACCACCACCTCAACACCATCTTCATCCGGGGTCCGGTGGC
 GCTGTACACCCTGTACAGCAGCACACCTCTTCGGCAGCTGCGGCAGCCGACGAGCGGCTGCAGCTGCAGCCATGTTAA
 CCCTGGGCAACAACAGCCATATTTCCCATCACCGGCACCGGGGACAGGCTCCTGGACCAGCTGCAGCAGCCCGAGCTCAG
 TACAGGCTGCCGAGCTGCTACAGTTAAGGCGCACCATCATCAGCACTCGCATCATCCACAGCAGCAGCTGGATATTGAG
 CCGGATAGACCTATTGGATATGGAGCCTTTGGTGTGTCTGGTCACTAACAGATCCAAGAGATGGAAGAGAGTAGCGCT
 CAAAAAGATGCCAACGCTTCCAGAATCTGGTCTCTTGCAAAAGGGTCTTCCGGGAATTGAAGATGTTGTTTTTTTA
 AGCATGATAATGTACTCTCTGCCCTTGACATACTCCAACCTCCACACATTGACTATTTTGAAGAAATATATGTTGTCACA
 GAATTGATGCAGAGTGACCTACATAAAATTATCGTCTCTCCTCAACCACTCAGCTCAGATCATGTCAAAGTTTTTCTTTA
 TCAGATTTTGGCAGGTTTGAAATATCTCCATTGAGCTGGCATTTTACATCGAGACATTAAGCCAGGGAATCTCCTTGTGA
 ACAGCAACTGTGTTCTAAAGATTTGTGATTTTGGATTGGCCAGAGTGAAGAATTAGATGAATCCCGTCATATGACTCAG
 GAAGTTGTACTATTATCGGGCTCCAGAAATCCTGATGGGACCGGTCATTACAGCAATGCTATTGACATCTGGTC
 TGTGGGATGTATCTTTGAGAAGTACTAGGACGAAGAATATTGTTTACGGCACAGAGTCCCATTCAGCAGTTGGATTTGA
 TCACGGATCTGTTGGGCACACCATCACTGGAAGCAATGAGGACAGCTTGTGAAGGCGCTAANGCACATATACTCAAGGGG
 TCCTCATAAACAGCCATCTCTCCTGTACTCTATACCCTGGTCTAGCCAGGCTAACCATGAACTTGTTTCATCTCCTTTN
 GCAGGATGTTGG

3' sequencing primer: SP6 (ATTTAGGTGACACTATAG)

GCTCTCCATATGGTCGACCTGCAGGCGGCCGCACTAGTGATTAAGCTTATCACTCCCACACCAGAGGAGATGGGGGCATC
 TCAGATGGCTGAGCAACAGTGGAATAATAAAGCTCTTAAAGCAGCAGACTGAGGGTTGATGCAGAGAGGCACTCTGTT
 TCCTTTCTGCTGTTCCAAAATGAAGTATGAATAATTTCTTTAACCTGTGCGACAGAACTGAGGTTCTTCTCGAAAGTGT
 CATCAAATTTGGGATTGGTGACAGGCTCAAAGTCACTGGTATAAACTCTTCCAGTGAGGTTGGAAGCAACATTTACAC
 ATACATGTGTGATATCGTAGTCGCCCTTCATCTAGGTAGGGTGGGCTAAGGCATCCTTAGCGGATATTCTTTGGATGG
 ATCAAAGACCAACATCCTGCAAAGGAGATGAACAGCTTCATGTGTAGCCTGGCTAGACAGGGTATAGAGTACAGGAAGAG
 ATGGCTGTTTATGAGGACCCCTGAGTATATGTGCCTTAGCGCCTTCAAGCTGTCCTCATTGCTTCCAGTGATGGTGTG
 CCCAACAGATCCGTGATCAAATCCAAGTCTGAATGGGACTCTGTGCCTGAAACAATATTCTTCGTCCTAGTAGTTCTGC
 AAAGATACATCCACAGACCAGATGTCAATAGCATTGCTGTAAATGACGGCTGCCATCAGGATTTCTGGAGCCCGATAAT
 ACTGAGTAAACAATCTCTGAGTCATATGACGGGATTCATTAATTTCCACTCTGGCCAATCCAAAATCAAAAATCTTT
 AGAACACAGTTGCTGTTTCAAAAGGAGATTCCCTGGCTTAATGTCTCGATGTAAATGCCAGCTGAATGGAGATATTTCAA
 ACCTCGCAAAATCTGATAAAGAAAACTTTGACATGATCTGAGCTGAGTGGTTGAGGAGAGACGATAATTTTATGAGGT
 CACTCTGCATCAATTCTGTGACAACATATATTTCTTCAAAATAGTCAATGTGTGGAGGTTGGAGTATGTCAAGGGCAGAG
 AGTACATTATCATGCTTAAAAAANCACAACATCTTCAATTCGNAANACCNNTTTTGAAGAANCCAGATTCTGGAAGAC
 GTTGGGCATCTTTTNGAACGCTACTCTCTTCCATCTCTTGGNATCTGTTACTGGACCA

12.2.29. pGEM-T-NLK_T219G

5' sequencing primer: T7P (TAATACGACTCACTATAGGG)

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CCGGCCGCCNNGCCGCGGGATTAGCTTATCACTCCCACACCAGAGGAGATGGGGGCATCTCAGATGGCTGAGCAACAGTG
GAACTAATAAAGCTCTTAAAGCAGCAGACTGAGGGTTGATGCAGAGAGGCACTCTGTTTCCTTTCTGCTGTTCCAAAAT
GAACTGATGAATAATTTCTTTAACCTGTCCGGACAGAACTGAGGTTCTTCTCGAAAGTGTCATCAAATTTGGGATTGGTGA
CAGGCTCAAAGTCACTGGTATAAACTCTTCCAGTGGAGGTGAAAAGCAACATTTACACATACATGTGTGATATCGTAGT
CGCCCTTCATCTAGGTAGGGGTGGGCTAAGGCATCCTTAGCGGATATTCTTTGGATGGATCAAAGACCAACATCCTGCA
AAGGAGATGAACAGCTTCATGTGTAGCCTGGCTAGACAGGGTATAGAGTACAGGAAGAGATGGCTGTTTATGAGGACCCC
TGAGTATATGTGCCTTAGCGCCTTCAACAGCTGTCTCATTGCTTCCAGTGATGGTGTGCCCCAACAGATCCGTGATCAAA
TCCAAGTGTGAATGGGACTCTGTGCCTGAAACAATATTCTTCGTCTAGTAGTTCTGCAAAGATACATCCACAGACCA
GATGTCAATAGCATTGCTGTAATGACGGCTGCCATCAGGATTTCTGGAGCCCCGATAATACTGAGTAACAACCTTCTGAG
TCATATGACGGGATTCATCTAATCTTCCACTCTGGCCAATCCAAAATCACAATCTTTAGAACACAGTTGCTGTTTACA
AGGAGATTCCCTGGCTTAATGTCTCGATTGTAATGCCAGCTGAATGGAGATATTCAAACCTCGCAAAATCTGATAAAG
AAAAACTTTGACATGATCTGAGCTGAGTGGTTGAGGAGAGACGATAATTTTATGAGGTCACTCTGCATGAGCTCTCCGA
CAACATATATTTCTTCAAAATAGTCAATGTGTGGAGGTTGGAGTATGTCAAGGGCAGAGAGTACATTATCATGCTTAAAA
AANCACAACATCTTCAATTCGCCGGAAGACCCCTTTTGCAAGAGACCAGATTCTGGAAGACGTTGGGCATCTTTTGAACGC
TACTCTCTTCCATCTCTTGGATCTGTTACTGACCAGACAACACCAAGGCTCCATATCCAATAGGTCTATCCGGNTCAA
TATCCAGCTGCTG
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3' sequencing primer: SP6 (ATTTAGGTGACACTATAG)

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ACACGGGTGGNNNCTCTCCATATGGTGCAGCTGCAGGCGGCCGCACTAGTGATTGAATCCGATGGCGGCTTACAATGGC
GGTACATCTGCAGCAGCAACAGGTCAACCACCACCACCATCACCACCACCTTCCACACCTCCCTCCTCACCTGCACCA
CCACCACCACCCTCAACACCATCTTCATCCGGGGTCCGGCTGCCGCTGTACACCCTGTACAGCAGCACACCTCTTCGGCAG
CTGCGGCAGCCGCGCAGCAGCGGCTGCAGCTGCAGCCATGTTAAACCTTGGGCAACAACAGCCATATTTCCCATCACCGGCA
CCGGGGCAGGCTCCTGGACAGCTGCAGCAGCCCCAGCTCAGGTACAGGCTGCCGAGCTGCTACAGTTAAGGCGCACCA
TCATCAGCACTCGCATCATCCACAGCAGCAGCTGGATATTGAGCCGGATAGACCTATTGGATATGGAGCCTTTGGTGTG
TCTGGTCAGTAACAGATCCAAGAGATGGAAGAGAGTAGCGCTCAAAAAGATGCCCAACGTCTTCCAGAATCTGGTCTCT
TGCAAAAGGGTCTTCCGGGAATTGAAGATGTTGTGTTTTTAAAGCATGATAATGTACTCTCTGCCCTTGACATACTCCA
ACCTCCACACATTGACTATTTGAAGAAATATATGTTGTCCGAGAGCTCATGCAGAGTGACCTACATAAAATATCGTCT
CTCCTCAACCACTCAGCTCAGATCATGTCAAAGTTTTCTTTATCAGATTTTGCGAGGTTTGAAATATCTCCATTACGCT
GGCATTTTACATCGAGACATTAAGCCAGGGAATCTCCTTGTGAACAGCAACTGTGTTCTAAAGATTTGTGATTTTGGATT
GGCCAGAGTGGAAGAATTAGATGAATCCCGTCATATGACTCAGGAAGTTGTTACTCAGTATTATCGGGCTCCAGAAATCC
TGATGGGCAGCCGTCATTACAGCAATGCTATTGACATCTGGTCTGTGGGATGTATCTTTGCAGAACTACTAGGACGAAGA
ATATTGTTTCAGGCACAGAGTCCCATTACAGAGTTGGATTGTATCAGCGATCTGTTGGGCACACCATCACTGGAAACAAT
GAGGACAGCTTGTGAAGGCGCTAAGGCACATATACTCAGGGTCTCATAAACAGCCATCTCTTCTGGAATCTATACCC
TGCTAGCCAGGCTACACATGAA
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12.2.30. pFAST-BAC-GST-NLK_WT

5' sequencing primer: pGEX5' F (GGGCTGGCAAGCCACGTTTGGTG)

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CTCAACGGATCTGGTTCGCGTCCGGGAATCCGATGGCGGCTTACAATGGCGGTACATCTGCAGCAGCAACAGGTCACCAC
CACCACCATCACCACCACCTTCCACACCTCCCTCCTCACCTGCACCACCACCACCCTCAACACCATCTTCATCC
GGGGTCCGGCTGCCGCTGTACACCCTGTACAGCAGCACACCTCTTCGGCAGCTGCGGCAGCCGCGCAGCAGCGGCTGCAGCT
CAGCCATGTTAAACCTTGGGCAACAACAGCCATATTTCCATCACCGGCACCGGGGAGGCTCCTGGACCAGCTGCAGCA
GCCCCAGCTCAGGTACAGGCTGCCGAGCTGTACAGTTAAGGCGCACCATCATCAGCACTCGCATCCACAGCAGCA
GCTGGATATTGAGCCGGATAGACCTATTGGATATGGAGCCTTTGGTGTGCTGGTCAGTAACAGATCCAAGAGATGGAA
AGAGAGTAGCGCTCAAAAAGATGCCCAACGTCTTCCAGAATCTGGTCTCTTGCAAAAGGGTCTTCCGGGAATTGAAGATG
TTGTGTTTTTTAAGCATGATAATGTACTCTCTGCCCTTGACATACTCAAACCTCCACACATTGACTATTTTGAAGAAAT
ATATGTTGTCACAGAATTGATGCAGAGTGACCTACATAAAATATCGTCTCTCCTCAACCACTCAGCTCAGATCATGTCA
AAGTTTTCTTTATCAGATTTTGCGAGGTTTGAAATATCTCCATTACGCTGGCATTTTACATCGAGACATTAAGCCAGGG
AATCTCCTTGTGAACAGCAACTGTGTTCTAAAGATTTGTGATTTTGGATTGGCCAGAGTGGAAGAATTAGATGAATCCCG
TCATATGACTCAGGAAGTTGTTACTCAGTATTATCGGGCTCCAGAAATCCTGATGGGCAGCCGTCATTACAGCAATGCTA
TTGACATCTGGTCTGTGGGATGTATCTTTGCAGAACTACTAGGACGAAGAATATTGTTTCAGGCACAGAGTCCCATTACG
CAGTTGGATTTGATCAGCGATCTGTTGGGCACACCATCACTGGAAGCAATGAGGACAGCTTGTGAAGGCGCTAAGGNNNA
TATACTCAGGGTCTCATAAACAGCCATCTCTTCTGGACTCTAANCCCTGTCTAGCCAGGCTAACAATGAAGCTGGTC
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3' sequencing primer: pFASTBACR (ATCCTCTAGTACTTCTCGAC)

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CTCAGGAGAGAGGGGGCATCTCAGATGGCTGAGCAACAGGTGGAACATAATAAAGCTCTTAAAGCAGCAGACTGAGGGTT
GATGCAGAGAGGCACTCTGTTTCCTTTCTGCTGTTCCAAAATGAAGTATGAATAATTTCTTTAACCTGTCCGACAGAAC
TGAGGTTCTTCTCGAAAGTGTCATCAAATTTGGGATTGGTGACAGGCTCAAAGTCACTGGTATAAACTCTTCCAGTGGAG
GTGGAAGCAACATTTACACATACATGTGTGATATCGTAGTCGCCCTTCATCTAGGTAGGGGTGGGCTNAGGCATCCTT
AGCGNATATTCTTTTGA
```

12.2.31. pFAST-BAC-GST-NLK_T219G

5' sequencing primer: pGEX5' F (GGGCTGGCAAGCCACGTTTGGTG)

TTGGTTCGCGTCCGGGAATCCGATGGCGGCTTACAATGGCGGTACATCTGCAGCAGCAACAGGTCACCACCACCACCAT
CACCACCACCTTCCACACCTCCCTCCTCCTCACCTGCACCACCACCACCACCCTCAACACCATCTTCATCCGGGGTCCGC
TGCCGCTGTACACCCTGTACAGCAGCACACCTCTTCGGCAGCTGCGGCAGCCGAGCAGCGGCTGCAGCTGCAGCCATGT
TAAACCCTGGGCAACAACAGCCATATTTCCCATCACCGGCACCGGGGAGGCTCCTGGACCAGCTGCAGCAGCCCCAGCT
CAGGTACAGGCTGCCGCAGCTGCTACAGTTAAGGCGCACCATCATCAGCACTCGCATCATCCACAGCAGCAGCTGGATAT
TGAGCCGGATAGACCTATTGGATATGGAGCCTTTGGTGTGTCTGGTCAGTAACAGATCCAAGAGATGGAAGAGAGTAG
CGCTCAAAAAGATGCCAACGTCTTCCAGAACTCTGGTCTCTTCAAAAAGGGTCTTCCGGGAATTGAAGATGTTGTGTTTT
TTAAGCATGATAATGTACTCTCTGCCCTTGACATACTCCAACCTCCACACATTGACTATTTTGAAGAAATATATGTTGT
CGGAGAGCTCATGCAGAGTGACCTACATAAAATTATCGTCTCTCCTCAACCACTCAGCTCAGATCATGTCAAAGTTTTTC
TTTATCAGATTTTGGCAGGTTTGAATATCTCCATTGAGTGGCATTTTACATCGAGACATTAAGCCAGGGAATCTCCTT
GTGAACAGCAACTGTCTTAAAGATTGTGATTGGATTGGCCAGAGTGGAAGAATTAGATCAATCCCGTCATATGAC
TCAGGAAGTTGTTACTCAGTATTATCGGGCTCCAGAAATCCTGATGGGCAGCCGTCATTACAGCAATGCTATTGACATCT
GGTCTGTGGGATGTATCTTTGCAGAACTACTAGGACGAAGAATATTGTTTCAGGCACAGAATCCCATTCAGCAGTTGGAT
TTGATCACGGATCTGTTGGGCACACCATCACTGGAAGCAATGAGGACAGCTTGTGAAGGCGCTAAGGCCATATACTCAGG
GTCTCTCATANACAGCCATCTCTTCTGGAATCTAANCCCTGGCTAGCCAGGCT

3' sequencing primer: pFASTBACR (ATCCTCTAGTACTTCTCGAC)

CATCTCAGATGGCTGAGCACAGTGGAACATAAAGCTCTTAAAAGCAGCAGACTGAGGGTTGATGCAGAGAGGCACTCT
GTTTCCTTTCTGCTGTTCCAAAATGAAGTATGAATAATTTCTTAACTGTCCGACAGAACTGAGGTTCTTCTCGAAAG
TGTCATCAAATTTGGGATTGGTGACAGGCTCAAAGTCACTGGTATAAACTCTTCCAGTGGAGGTGGAAGCAACATTTA
CACATACATGTGTATATCGTAGTCGCCCTTCATCTAGGTAGGGGTGGGCTAAGGCATCCTTAGCGGATATTCTTTTGA
TGGATCAAAGACCAACATCCTGCAAAGGAGATGAACAGCTTCATGTGTAGCCTGGCTAGACAGGGTATAGAGTACAGGAA
GAGATGGCTGTTTATGAGGACCCCTGAGTATATGTGCCTTAGCGCCTTCAAGCTGTCTCATTGCTCCAGTGATGGT
GTGCCAACAGATCCGTGATCAAATCCAACCTGCTGAATGGGACTCTGTGCCTGAAACAATATTCTTCGCTCCTAGTAGTTC
TGCAAAGATACATCCCACAGACCAGATGTCAATAGCATTGCTGTAATGACGGCTGCCCATCAGATTTCTGGAGCCCGAT
AATACTGAGTAACAACTTCTGAGTCATATGACGGGATTCATCTAATCTTCCACTCTGGCCAATCCAAAATCACAAATC
TTTGAACACAGTTGCTGTTTACAAGGAGATTCCCTGGCTTAATGTCTCGATGTAATGCCAGCTGAATGGAGATATTT
CAAACCTCGCAAAATCTGATAAAGAAAAAATTTGACATGATCTGAGCTGAGTGGTTGAGGAGAGACGATAATTTTATGTA
GGTCACTCTGCATGAGCTCTCCGACAACATATATTTCTCAAATAGTCAATGTGTGGAGGTTGGAGTATGTCAAGGGCA
GAGAGTACATTATCATGCTTAAAAAANCACAACATCTTCAATTCGCGAAGACCCCTTTTGAAGAGACCAGATTCTGGAA
GACGTTGGGCATCTTTTGAAGCGCTACTCTCTTCCATCTCTTGGATCTGTTACTGACCAGACAACACCAAAGGCTCCAT
ATCCAATAGGTCTATCCGGGTCAATATCCAGCTGCTGCTTG

12.2.32. pcDNA3-FLAG-NLK_WT

First 5' sequencing primer: T7P (TAATACGACTCACTATAGGG)

AGGACGACGACGACAGGAACACAGGACGACGACGACAAGGGATCCACTAGTAACGGCCGCCAGTGTGCTGGAATTCGA
TGGCGGCTTACAATGGCGGTACATCTGCAGCAGCAACAGGTCACCACCACCACCATCACCACCACCTTCCACACCTCCCT
CCTCTCACCTGCACCACCACCACCCTCAACACCATCTTCACTCCGGGTCGGCTGCCGTGTACACCCTGTACAGCA
GCACACCTCTTCGGCAGCTGCGGCAGCCGAGCAGCGGCTGCAGCTGCAGCCATGTTAAACCCTGGGCAACAACAGCCAT
ATTTCCCATCACCGGCACCGGGGAGGCTCCTGGACCAGCTGCAGCAGCCCCAGCTCAGGTACAGGCTGCCGCAGCTGCT
ACAGTTAAGGCGCACCATCATCAGCACTCGCATCATCCACAGCAGCAGCTGGATATTGAGCCGGATAGACCTATTGGATA
TGGAGCCTTTGGTGTGTCTGGTCACTAACAGATCCAAGAGATGGAAGAGAGTAGCGCTCAAAAAGATGCCAACCTCT
TCCAGAATCTGGTCTGTGCAAAAGGGTCTTCCGGGAATTGAAGATGTTGTGTTTTTTAAGCATGATAATGTACTCTCT
GCCCTTGACATACTCCAACCTCCACACATTGACTATTTTGAAGAAATATATGTTGTACAGAATTGATGCAGAGTGACCT
ACATAAAATTTATCGTCTCTCCTCAACCACTCAGCTCAGATCATGTCAAAGTTTTTCTTTATCAGATTTTGCAGGTTTGA
AATATCTCCATTGAGCTGGCATTTTACATCGAGACATTAAGCCAGGGAATCTCCTTGTGAACAGCAACTGTGTTCTAAAG
ATTTGTGATTTTGGATTGGCCAGAATGGAAGAATTAGATGAATCCCGTCATATGACTCAGGAAGTTGTTACTCAGTATTA
TCGGGCTCCAGAAATCCTGATGGGCAGCCGTCATTACAGCAATGCTATTGACATCTGGNCGGGGNAAGTATCTTTGCAG
AACTACTAGGACAAAAANTATTGTTTCAGGCACAANGTCCCATTCAGCATTGGATTGATCCNGGATCTGTTGGGCACA
CCATCACTGGAACAATGAAGACACCTTGTGAAGGCCNTAAGGCAATATACTCAGGGGTCTCCAAAAANG

Second 5' sequencing primer: CGGTGGGAGGTCTATATAAGCAGAGCTCTC

TAGNACGGNAATATTCTGGCTATCGAAATTAATACGACTCACTATAGGGAGACCCAAGCTTCACCATGGACTACAAGGTA
CGACGACGACAAGGACTACAAGGACGACGACGACAAGGGATCCACTTANTAACGGCCGCCAGATGTGCTGGAATTCGAT
GGCGGCTTACAATGGCGGTACATCTGCANAGCAACAGGTCACCACCACCACCATCACCACCACCTTCCACACCTCCCTC
CTCTCACCTGCACCACCACCACCACCTCAACACCATCTTCATCCGGGGTCCGGCTGCCGCTGTACACCCTGTACAGNAG
NACACCTCTTCGGCAGCTGCTGCAGCCGCATCAGCGGCTGCAGCTGCAGNCATGTTAAACCCTGGGCAACAACAGCCATA
TTTCCCATCACNGGCNCGGGGAGGCTCCTGGACCAGCTGC

First 3' sequencing primer: SP6 (ATTTAGGTGACACTATAG)

GCTGGCGGCGCNTAGNGATTAGCTTATCACTCCCACACCAGAGGAGATGGGGGCATCTCAGATGGCTGAGCAACAGTGG
AACTAATAAAGCTCTTAAAAGCAGCAGACTGAGGGTTGATGCAGAGAGGCACTCTGTTTCCTTTCTGCTGTTCCAAAATG

AACTGATGAATAATTTCTTTAACCTGTGCGGACAGAACTGAGGTTCTTCTCGAAAGTGTCATCAAATTTGGGATTGGTGAC
 AGGCTCAAAGTCACTGGTATAAACTCTTCCAGTGGAGGTGGAAAAGCAACATTTACACATACATGTGTGATATCGTAGTC
 GCCCTTCATCTAGGTAGGGTGGGCTAAGGCATCTTAGCGGATATTCTTTGGATGGATCAAAGACCAACATCCTGCAA
 AGGAGATGAACAGCTTCCATGTGTAGCCTGGCTAGACAGGGTATAGAGTACAGGAAGAGATGGCTGTTTATGAGGACCCCT
 GAGTATATGTGCCTTAGCGCCTTACAAGCTGTCTCATTGCTTCCAGTGATGGTGTGCCAACAGATCCGTGATCAAAT
 CCAACTGCTGAATGGGACTCTGTGCCTGAAACAATATTCTTCGTCTAGTAGTCTGCAAAGATACATCCACAGACCAG
 ATGTCAATAGCATTGCTGTAATGACGGCTGCCCATCAGGATTTCTGGAGCCCGATAATACTGAGTAACAACCTTCTGAGT
 CATATGACGGGATTCATCTAATTCTTCCACTCTGGCCAAATCCAAAATCACAATCTTTAGAACACAGTTGCTGTTCCAAA
 GGAGATTCCCTGGCTTAATGTCTCGATGTAAAATGCCAGCTGAATGGAGATATTTCAAACCTCGCAAAATCTGATAAAGA
 AAAACTTTGACATGATCTGAGCTGAGTGGTTGAGGAGAGACGATAATTTTATGTAGGTCATCTGCATCAATTCTGTGAC
 AACATATATTTCTTCAAATAGTCAAGGTGTGGAGTTGGAGTATGTCAAGGGCAGAGAGTACATTATCATGCTTAAAAA
 AACACAACATCTTCAATCCCGGAAGACCCTTTTGCAAGAGACCAGATTCTGGAAGACGTTGGGCATCTTTTGGAGCGCT
 ACTCTCTTCCATCTCTGGATCTGTTACTGACCAGACANNCCNAAAGGCTCCATATCCAAANGGTCTATCCGNTCAAT

Second 3' sequencing primer: CTGGCAACTAGAAGGCACAGTCGAGGC

GAATAGNCCNTCTAGATGCATGTCTCGAGCGGCCGCACTAGTGATTAAGCTTATCACTCCACACCACAGGAGATGGGG
 GCATCTCAGATGGCTGAGCAACAGATGGAACATAAAGCTCTTAAAAGCAGCAGACTGAGGGTTGATGCAGAGAGGCAC
 TCTGTTTCTTTCTGCTGTTCCAAAATGAAGTATGAATAATTTCTTTAACCTGTGCGGACAGAACTGAGGTTCTTCTCGA
 AAGTGTCAATAATTTGGGATTGGTGACAGGCTCAAAGTCACTGGTATAAACTCTTCCAGTGGAGGTGGAAAAGCAACAT
 TTACACATACATGTGTGATATCGTAGTCGCCCTTCATCTAGGTAGGGGTGGGCTAAGGCATCCTTAGCGGATATTCTTTT
 GGATGGATCAAAGACCAACATCCTGCAAAGGAGATGAACAGCTTCATGTGTAGCCTGGCTAGACAGGGTATAGAGTACAG
 GAAGAGATGGCTGTTTATGAGGACCCCTGAGTATATGTGCCTTAGCGCCTTACAAGCTGTCTCATTGCTTCCAGTGAT
 GGTGTGCCAACAGATCCGTGATCAAATCAAATGCTGAATGGGACTCTGTGCCTGAAACAATATTCTTCGTCTAGTAG
 TTTCTGAAAGATACATCCACAGACCAGATGTCAATAGCATTTGCTGTAATGACGGCTGCCCATCCTTAGCGGATATTCTTTT
 GATAATACTGAGTAACAACCTTCTGAGTCATATGACGGGATTCATCTAATTCTTCCACTCTGGCCAATCCAAAATCACA
 ATCTTTAGAACACAGTTGCTGTTACAAGGAGATTCCCTGGCTTAATGTCTCGATGTAAAATGCCAGCTGAATGGAGATA
 TTTCAAACCTCGCAAAATCTGATAAAGAAAACTTTGACATGATCTGAGCTGAATGGTTGAGGAGAGACGATAATTTTAT
 GTAAGGTCACTCTGCATCAATTCTGGTGACAACATATATTTCTCAAATAAGCCAATGGGNTGAAGGTTGAATTATGT
 CCAGGNCCAAAAANGTACATT

12.2.33. pcDNA3-FLAG-NLK_T219G

First 5' sequencing primer: T7P (TAATACGACTCACTATAGGG)

CGGACGACGACGACAGGACTACAGGACGACGACGACAAGGGATCCACTAGTAACGGCCGCCAGTGTGCTGGAATTCGGAT
 GGGCGCTTACAATGGCGGTACATCTGCAGCAGCAACAGGTACACCACCACCACCTTCCACACCTCCCTC
 CTCCTCACCTGCACCACCACCACCACCTCAACACCATCTTCATCCGGGGTCCGGCTGCCGCTGTACACCTGTACAGCAG
 CACACCTCTTCGGCAGCTGCGGCAGCCGACGAGCGGCTGCAGCTGCAGCCATGTTAAACCTGGGCAACACAGCCATA
 TTTCCCATCACCGGCACCGGGGACGGCTCCTGGACCAGCTGCAGCAGCCCCAGCTCAGGTACAGGCTGCCGCAGCTGCTA
 CAGTTAAGGCGCACCATCATCAGCACTCGCATCATCCACAGCAGCAGCTGGATATTGAGCCGGATAGACCTATTGGATAT
 GGAGCCTTTGGTGTGTCTGGTCAGTAACAGATCCAAGAGATGGAAGAGAGTAGCGCTCAAAAAGATGCCCCACGTCTT
 CCAGAACTCTGGTCTCTTGCAAAAGGGTCTTCCGGGAATTGAAGATGTTGTGTTTTTTAAGCATGATAATGTACTCTCTG
 CCCTTGACATACTCCAACCTCCACACATTGACTATTTGAAGAAATATATGTTGTGCGGAGAGCTCATGCAGAGTGACCTA
 CATAAAATTATCGTCTCTCCTCAACCACTCAGCTCATGTCAAAGTTTTCTTTATCAGATTTTGCAGGTTTGAA
 ATATCTCCATTGAGCTGGCATTTTACATCGAGACATTAAGCCAGGGAATCTCCTTGTGAACAGCAACTGTGTTCTAAAGA
 TTTGTGATTTTGGATTGGCCAGAGTGGAAGAATTAGATGAATCCCGTCATATGACTCAGGAAGTTGTTACTCAGTATTAT
 CGGGCTCCAGAAATCCTGATGGGCAGCCGTCATTACAGCAATGCTATTGACATCTGGTCTGTGGGATGTATCTTTGCAGA
 ACTACTAGGACGAAGAATATTGTTTCAGGCACAGAGTCCCATTACAGCAGTTGGATTNGATCACGGATCTGTTGGGCACAC
 CATCACTGGAANCAATGAGGACAACCTGTGAAAGCC

Second 5' sequencing primer: CGGTGGGAGGTCTATATAAGCAGAGCTCTC

ANTTATACGACTCACTATAGGGGAGACCCAAAGCGTTCCACATGGACTACAAGGACGACGACGACAAGGACTACAAGGACGA
 CGACGACAAGGGATCCACTAGTAACGGCCGCCAGTGTGCTGGAATTCGGATGGCGGCTTACAATGGCGGTACATCTGCAG
 CAGCAACAGGTACACCACCACCACCATCACCACCACCTTCCACACCTCCCTCCTCCTCACCTGCACCACCACCACCACCT
 CAACACCATCTTCATCGGGGTGGCTGCCGCTGTACACCTGTACAGCAGCACACCTCTTCGGCAGCTGCGGCAGCCGC
 AGCAGCGGCTGCAGCTGCAGCCATGTTAAACCTGGGCAACACAGCCATATTTCCCATCACCGGCACCGGGGACGGCTC
 CTGGACCAGCTGCAGCAGCCCCAGCTCAGGTACAGGCTGCCGAGCTGTACAGTTAAGGCGCACCATCATCAGCACTCG
 CATCATCCACAGCAGCAGCTGGATATTGAGCCGGATAGACCTATTGGATATGGAGCCTTTGGTGTGTCTGGTCAGTAAC
 AGATCCAAGAGATGGAAGAGAGTAGCGCTCAAAAAGATGCCAACGTCTTCCAGAATCTGGTCTCTTGCAAAAGGGTCT
 TCCGGGAATTGAAGATGTTGTGTTTTTTAAGCATGATAATGTAACCTCTGCCCCTTGACATACTCAACCTCCACACATTGA

First 3' sequencing primer: SP6 (ATTTAGGTGACACTATAG)

CGCTCGGCGGCGCCTAGTGATTAGCTTATCACTCCACACCAGAGAGATGGGGGCATCTCAGATGGCTGAGCAACAGTG
 GAACTAATAAAGCTCTTAAAAGCAGCAGACTGAGGGTTGATGCAGAGAGGCACTCTGTTTCTTTCTGCTGTTCCAAAAT
 GAACTGATGAATAATTTCTTTAACCTGTGCGGACAGAACTGAGGTTCTTCTCGAAAGTGTCATCAAATTTGGGATTGGTGA
 CAGGCTCAAAGTCACTGGTATAAACTCTTCCAGTGGAGGTGGAAAAGCAACATTTACACATACATGTGTGATATCGTAGT
 CGCCCTCATCTAGGTAGGGGTGGGCTAAGAGCTCCTTAGCGGATATTCTTTGGATGGATCAAAGACCAACATCCTGCA
 AAGGAGATGAACAGCTTCATGTGTAGCCTGGCTAGACAGGGTATAGAGTACAGGAAGAGATGGCTGTTTATGAGGACCC
 TGAGTATATGTGCCTTAGCGCCTTACAAGCTGTCTCATTGCTTCCAGTGATGGTGTGCCAACAGATCCGTGATCAA

TCCAAC T GCTGAATGGGACTCTGTGCCTGAAACAATATTCTTCGTCTAGTAGTTCTGCAAAGATACATCCCACAGACCA
 GATGTCAATAGCATTGCTGTAATGACGGCTGCCCATCAGGATTTCTGGAGCCCCGATAAATACTGAGTAACAACCTTCCTGAG
 TCATATGACGGGATTATCTAATTCTTCCACTTGCCCAATCCAAAATCACAATCTTTAGAACACAGTTGCTGTTTACA
 AGGAGATTCCCTGGCTTAATGTCTCGATGTAAATGCCAGCTGAATGGAGATATTTCAAACCTCGCAAAATCTGATAAAG
 AAAAACTTTGACATGATCTGAGCTGAGTGGTTGAGGAGAGACGATAATTTTATGAGGTCACTCTGCATGAGCTCTCCGA
 CAACATATATTTCTTCAAATAGTCATGGTGTGGAGGTTGGAGTATGTCAAGGGCAGAGAGTACATTATCATGCTTAAAA
 AANCACAACATCTTCAATCCCCGGAAGACCCTTTTGAAGAGACCAGATTCTGGAAGACGTTGGGCATCTTTTGAACGC
 TACTCTCTTCCATCTCTGGATCTGTTACTGACCAA

Second 3' sequencing primer: CTGGCAACTAGAAGGCACAGTTCGAGGC

GGGCCNACCCGTGNGCTCTGNATCTGNNNTTTCGGGGNANAGGATTAAGGCTTACNNTNGCTATCCTCGACTNGANGGG
 NNNNACAAACCATGGCTGNTGNACCGTNGNANCTGNNNAAGNGTCTTTCTNGCCNCANACNGAGGGTTGATGNANANNNG
 CNNTCTGTTTCCATTCTGCTGNTCGNGNAGACCTGNTGAATNATNNNTTANCCNGCNGGACANNACTGAGGNTCTTNTC
 TNNNNTGNCCNCCNTTNGGGATTGGTGACAGGCTCNAAGNCNATGGTATAAACTCTTCAAGTGGAGGTGGAAAAGCANC
 ATTTNCACATACATGTGTGATATCGTANTGCCCTTCATCTANGTAGGGGTGGGCTAAGGCATCCTTAGCGGANATTCTT
 TTGGATGGATCANNGACCAACNTCTGCAAANGAGATGAACAGCTTCATGTGTAGCCTGCCTAGACAGGGTATAGAGTAC
 AGGAAGAGATGGCTGTTTATGAGGACCCCTGAGTATATGTGCCTTAACGCCTTCACAAGCTGTCTCATTGCTCCAGTG
 ATGGTGTGCCCAACAGATCCGTGATCAAATCCANCTGCTGAATGGGACTCTGTGCCTGAAACAATATTCTTCGTCTAGT
 AGTTCTGCNAAGATACATCCCACAGACCAGATGTCNNTANCATTGCTGTACTGACGGNTGNCCATCANGATTTCTGGAGC
 CCGATAAATACTGAGTAACAANTTCTGAGTCATATGACGGGATTCATCTANTTCTTCCACTCTGGGCCAATCCAAAAATC
 ACAAATCTTTCANAACNCNGTTTGTGNTTACAAGGAAGATTCCTCTGGCTTAATGTCTCGATGTTAAATGNCCANC
 CTGAATGGAATAATTTTCAAACCCNCGCAAAAATCTGAATAAAAAAAACTTTNGANATGNNNCTGAACCTTGAAATN

12.2.34. pSBbi-GB-NLK_WT

5' sequencing primer (EF-1 Alpha): TCAAGCCTCAGACAGTGGTTC

ACCCNAGCTGGCCTCTGAGGCCCNATGGCTCGAGTTTCTGAACCTCAATTATACTGAATTGTTTCATCATAGGGTTCAACAT
 TGTGATAAATCAGGACTTCAATGTCTAGTGTTCAAGTAAATACCTTGAGGGCTCTCCAAAAAGGGGAATCTTTTCAGGAA
 ATGCCTCTTTCTCTTGGTGGGCTTCTGTTGCGTCTTTCTTCTCACTAATTCGAAGGCCTGTCAAGGCCAAGCTTCACC
 ATGGACTACAAGGACGACGACGACAAGGACTACAAGGACGACGACGACAAGGGATCCACTAGTAACGGCCGCCAGTGTG
 TGAATTCCGATGGCGGCTTACAATGGCGGTACATCTGCAGCAGCAACAGGTCAACACCACCACCATCACCACCACCTTC
 CACACCTCCCTCCTCCTCACCTGCACCACCACCCACCTTCAACACCATCTTTCATCCGGGTGGGCTGCCGCTGTACAC
 CCTGTACAGCAGCACACCTCTTCGGCAGCTGCGGCAGCCGACGAGCGGCTGCAGCTGCAGCCATGTTAAACCTGGGCA
 ACAACAGCCATATTTCCCATCACCGGCACCCGGGGCAGGCTCCTGGACCAGCTGCAGCAGCCCCAGCTCAGGTACAGGCTG
 CCGCAGCTGCTACAGTTAAGGCGCACCATCATCAGCACTCGCATCATCCACAGCAGCAGCTGGATATTGAGCCGGATAGA
 CCTATTGGATATGGAGCCCTTTGGTGTGCTGGTCAAGTAAACAGATCCAAGAGATGGAAAGAGAGTAGCGCTCAAAAAGAT
 GCCCCACGTCTTCCAGAATCTGGTCTCTTGAAGGGTCTTCCGGGAATTGAAGATGTTGTGTTTTTTAAGCATGATA
 ATGTACTCTCTGCCCTTGACATACTCCAACCTCCACACATTGACTATTTGAAGAAATATATGTTGTACAGAATTGATG
 CAAAATGACCTACATAAAATATCGTCTCTCTCAACCACTCAGCTCAGATCATGTCAAAGTTTTCTTTATCAGATTTT
 GCAAGGTTNGAANTATCTCCATTACG

3' sequencing primer (BGHR): TAGAAGGCACAGTTCGAGG

CCAGCTTATCACTCCNACCAGAGGAGATGGGGCATCTCAGATGGCTGAGCAACAGTGGAACATAATAAGCTCTTAAAA
 GCAGCAGACTGAGGGTTGATGCAGAGAGGCACTCTGTTTCTTTCTGCTGTTCCAAAATGAAGTATGAATAATTTCTTT
 AACCTGTGCGACAGAAGTGAAGTTCTTCTGAAAAGTGTCAAAATTTGGGATTGGTGACAGGCTCAAAGTCACTGGTAT
 AAACCTCTTCCAGTGGAGGTGGAAAAGCAACATTTACACATACATGTGTGATATCGTAGTCGCCCTTCATCTAGGTAGGGG
 TGGGCTAAGGCATCCTTAGCGGATATTCTTTGGATGGATCAAAGACCAACATCCTGCAAAGGAGATGAACAGCTTCATG
 TGTAGCCTGGCTAGACAGGGTATAGAGTACAGGAAGAGATGGCTGTTTATGAGGACCCCTGAGTATATGTGCCTTAGCGG
 CTTCAACAGCTGTCTCATTGCTTCCAGTATGGTGTGCCCAACAGATCCGTGATCAAATCCAAGTCTGAATGGGACTC
 TGTGCCTGAAACAATATTCTTCGTCTAGTAGTTCTGCAAAGATACATCCCACAGACCAGATGTCAATAGCATTGCTGTA
 ATGACGGCTGCCCATCAGGATTTCTGGAGCCCGATAATACTGAGTAACAACCTTCTGAGTCATATGACGGGATTCATCTA
 ATTCTTCCACTCTGGCCAAATCCAAAATCACAATCTTTAGAACACAGTTGCTGTTTACAAGGAGATTCCTGGCTTAATG
 TCTCGATGTAAATGCCAGCTGAATGGAGATATTTCAAACCTCGCAAAATCTGATAAAGAAAACTTTGACATGATCTGA
 GCTGAGTGGTTGAGGAGAGACGATAATTTTATGAGGTCACTCTGCATCAATTCTGTGACAACATATATTTCTTCAAATA
 GTCATTGTGTGGAGGTTGGAGTATGTCAAGGGCAGAGAGTACATTATCATGCTTANAAAAACACAACATCTTCAATCCCC
 GGAAGAACCCTTTTGAANGACCAANTTCTGGAAGANNTTGGGCATCTTTTGAAGCGCTACNNTTCTTCTCTTGGAT
 CGGTTACGGACCAANCAACCCNAAGGCTCCTATCC

12.2.35. pSBbi-GB-NLK_T219G

5' sequencing primer (EF-1 Alpha): TCAAGCCTCAGACAGTGGTTC

TACCCNAGCTGGCCTCTGAGGCCACCATGGCTCGAGTTTCTGAACCTCAATTATACTGAATTGTTTCATCATAGGGTTCAACA
 TTGTGATAAATCAGGACTTCAATGTCTAGTGTTCAAGTAAATACCTTGAGGGCTCTCCAAAAAGGGGAATCTTTTCAGGA
 AATGCCTCTTTCTCTTGGTGGGCTTCTGTTGCGTCTTTCTTCTCACTAATTCGAAGGCCTGTCAAGGCCAAGCTTCAC
 CATGGACTACAAGGACGACGACGACAAGGACTACAAGGACGACGACGACAAGGGATCCACTAGTAACGGCCGCCAGTGTG
 CTGGAATTCGATGGCGGCTTACAATGGCGGTACATCTGCAGCAGCAACAGGTCAACACCACCACCATCACCACCACCTT
 CCACACCTCCCTCCTCCTCACCTGCACCACCACCACCCCTTCAACACCATCTTTCATCCGGGTGGGCTGCCGCTGTACA
 CCCTGTACAGCAGCACACCTCTTCGGCAGCTGCGGCAGCCGACGAGCGGCTGCAGCTGCAGCCATGTTAAACCTGGGC

AACAACAGCCATATTTCCCATCACCGGCACCGGGGCAGGCTCCTGGACCAGCTGCAGCAGCCCCAGCTCAGGTACAGGCT
GCCGCAGCTGCTACAGTTAAGGCGCACCATCATCAGCACTCGCATCATCCACAGCAGCAGCTGGATATTGAGCCGGATAG
ACCTATTGGATATGGAGCCTTTGGTGTGTCTGGTCAGTAACAGATCCAAGAGATGGAAAGAGAGTAGCGCTCAAAAAGA
TGCCCAACGTCTTCCAGAATCTGGTCTCTTGCAAAAGGGTCTTCCGGGAATTGAAGATGTTGTGTTTTTTAAGCATGAT
AATGTACTCTCTGCCCTTGACATACTCCAACCTCCACACATTGACTATTTTGAAGAAATATATGTTGTGCGGAGAGCTCAT
GCAGAGTGACCTACATAAAATTATCGTCTCTCCTCAACCACTCAGCTCAGATCATGTCAAAGTTTTCTTTATCAANTTT
TGCGAGGTTTGAAATATCTCCATTCAGCTGGCATTTTACATCGAGACATTAAGCCAGGGAATCCTTGTGAACAGCACT
GTGTTCTAANGATTTGTGATTTTGATTGGCCNNAGNGAANNAATTAANTGATNCCNGTCTA

3' sequencing primer (BGHR): TAGAAGGCACAGTCGAGG

AGCTTATCACTCCCACACCAGAGGAGATGGGGGCATCTCAGATGGCTGAGCAACAGTGGAATAATAAGCTCTTAAAAG
CAGCAGACTGAGGGTTGATGCAGAGAGGCACTCTGTTTCTTTCTGCTGTTCCAAAATGAACTGATGAATAATTTCTTTA
ACCTGTCTGGACAGAAGTGAAGTTCTTCTCGAAAGTGTCAATTTGGGATTGGTGACAGGCTCAAAGTCACTGGTATA
AACTCTTCCAGTGGAGGTGGAAAAGCAACATTTACACATACATGTGTGATATCGTAGTCGCCCTTCATCTAGGTAGGGGT
GGGCTAAGGCATCCTTAGCGGATATTCTTTTGATGGATCAAAGACCAACATCCTGCAAAGGAGATGAACAGCTTCATGT
GTAGCCTGGCTAGACAGGGTATAGAGTACAGGAAGAGATGGCTGTTTATGAGGACCCCTGAGTATATGTGCCTTAGCGCC
TTCACAAGCTGTCTCATTGCTTCCAGTGATGGTGTGCCAACAGATCCGTGATCAAATCCAAGTCTGAATGGGACTCT
GTGCCTGAAACAATATTCTTCGTCTAGTAGTTCTGCAAAGATACATCCACAGACCAGATGTCAATAGCATTGCTGTAA
TGACGGCTGCCCATCAGGATTTCTGGAGCCCGATAATACTGAGTAACAACCTCCTGAGTCATATGACGGGATTCATCTAA
TTCTTCCACTCTGGCCAATCCAAAATCACAAATCTTTAGAACACAGTTGCTGTTTACAAAGGAGATTCCCTGGCTTAATGT
CTCGATGTAAATGCCAGCTGAATGGAGATATTTCAAACCTCGCAAATCTGATAAAGAAAACTTTGACATGATCTGAG
CTGAGTGGTTGAGGAGAGACGATAATTTTATGTAGGTCACTCTGCATGAGCTCTCCGACAACATATATTTCTTCAAATAG
TCATGGTGTGGAGGTTGGAGTATGTCAAGGGCAGAGAGTACATTATCATGCTTAAAAAANCACAACATCTTCAATTCCTCG
GAAGACCCCTTTTGCAAGAGACCAGATTCTGGAAGACGTTGGGCATCTTTTGAGCGCTACTCTTTCCATCANNCTTGGA
TCTGTTACTGACCAGACAACACCAAAGGCTCCATATCCAATAGGTCTATCCGGCTCAT