Targeting FOXO3a and Sirtuins in breast cancer

Thesis submitted by

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To

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DECLARATION OF ORIGINALITY

Unless otherwise stated in text, this thesis is the result of my own work.

ABSTRACT

SIRT proteins play an important role in the survival and drug resistance of tumour cells, especially during chemotherapy. In this study, we investigated the potency, specificity and cellular targets of three recently identified SIRT inhibitors, Sirtinol, Salermide and EX527. Our results identify the specificity and cellular targets of these novel inhibitors, and suggest that SIRT inhibitors require combined targeting of both SIRT1 and SIRT2 to induce p53 acetylation and cell death in breast cancer cells. Assessing the role of sirtuins in chemoresistance, cisplatin resistant cell lines were developed and characterized. Cisplatin resistant cells (CisR) were found to have higher levels of SIRT1, repressing the activation of FOXO3a. MCF-7 cells overexpressing SIRT1 were also shown to be protected against cisplatin treatment, highlighting its role in resistance.

Sirtuins have many cellular targets, including alpha-tubulin, PARP, ku70 and FOXO3a. Because of the similarities between the regulation of p53 and FOXO3a, we decided to elucidate the effect of sirtinol treatment on FOXO3a. Sirtinol treatment was shown to stabilize FOXO3a, similar to its effect on p53, but at lower concentrations. We have shown previously that FOXO3a is an important downstream mediator of the cytotoxicity of receptor tyrosine kinase targeted therapies. To examine the conjecture whether sirtuin inhibitors could increase the proapoptotic potency of lapatinib through stabilization of FOXO3a, lapatinib was treated alone and in combination with sirtinol and EX527. Sirtinol was found to synergise with lapatinib treatment (in cells containing mutant p53) and this was dependent on the presence of FOXO proteins, highlighting the use of sirtuin inhibitors in increasing the efficacy of therapies that indirectly target FOXO3a.

PUBLICATIONS

Peck B, Chen CY, Ho KK, di Fruscia P, Myatt SS, RC Coombes, Fuchter M, Chwan-Deng H, Lam. SIRT inhibitors induce cell death and p53 acetylation through targeting both SIRT1 and SIRT2. *Mol Cancer Ther* 2010; 9(4): 844-855

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Francis RE, Myatt SS, Krol J, Hartman J, **Peck B,** McGovern UB, Wang J, Guest SK *et al.* FoxM1 is a downstream target and marker of HER2 overexpression in breast cancer. *Int J Oncol* 2009; 25(1):57-68

In memory of my late mother

Brenda Joyce McNicholas

1956 - 2006

For all the things you'll never get to do, I dedicate this piece of work to you

Keep smiling that beautiful smile

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What will I remember most 'fondly' from the PhD – hmmm that would probably have to be the internal bleeding (again – true story…).

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ABBEVIATIONS

AceCS2	Acetylcoenzyme 2
APS	Ammonium persulphate
ARMS	Alveolar rhabdomyosacromas
AROS	Active regulator of SIRT1
Atg7	Autophagy regulating protein 7
ATP	Adenosine Triphophate
BH	Bcl-2 homology
BSA	Bovine Serum Albumin
CAD	Caspase-activated deoyribonuclease
CAT	Central kinase catalytic domain
CBP	CREB binding protein
CDC25b	Cell division cycle 25b
CDK	Cyclin-dependent kinase
CDKI	Cyclin-dependent kinase inhibitor
CML	Chronic myelogenous leukemia
CPS1	Carbamoyl phosphate synthase 1
CR	Caloric restriction
CRM1	Chromosomal region maintenance 1
CXCR4	Chemokine receptor 4
c-MYC	C-myelocytomatosis
DCIS	Ductal carcinoma *in situ*
DBD	DNA-binding domain
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoyribonuclease
DTT	Dithiothreitol
dNTP	Nucleotides
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
ECL	Enhanced Chemi-Luminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
EPC	Endothelial progenitor cells
ER	Estrogen receptor
ERE	Estrogen response elements
ERK	Extracellular signal-regulated kinase (MAPK)
FACS	Fluorescence activated cell sorting (flow cytometry)
FasL	Fas Ligand
FasR	Fas Receptor
FCS	Foetal calf serum

FGFR	Fibroblast growth factor receptor
FOXC	Forkhead box, sub group C, transcription factor
FOXM	Forkhead box, sub group M, transcription factor
FOXO	Forkhead box, sub group O, transcription factor
Gadd45	Growth arrest and DNA-damage 45
GDP	Guanosine diphosphate
GDH	Glutamate dehydrogenase
GNAT	Gen5-related N-acetyl transferase
GTP	Guanosine-5'-triphosphate
h	Hour
H1	Histone 1
H2	Histone 2
H3	Histone 3
H3K9	Histone 3 lysine 9
H3K14	Histone 3 lysine 14
H4	Histone 4
H4K16	Histone 4 lysine 16
HAT	Histone aceyltransferase
HAUSP	Herpesvirus-associated ubiquitin-specific protease
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitors
HER2	Human epidermal growth factor receptor 2
HER3	Human epidermal growth factor receptor 3
HID	HDAC-interacting domain
HRP	Horseradish peroxidase
IARC	International association for research on cancer
ICAD	Inhibitor of caspase-activated DNase
IGF-I	insulin growth factor 1
ING2	Inhibitor of growth protein 2
LAC	Lung adenocarcinoma
m	Milli
M	Molar
MAC	Mitochondrial apoptosis-induced channel
MAPK	Mitogen activated protein kinase
mdm2	Murine double minute 2
Min	Minutes
MAC	Mitochondrial apoptosis-induced channel
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MnSOD	Manganese superoxide dismutase
MOMP	Mitochondrial outer membrane permeabilisation
MOZ	Monocytic leukaemia zinc finger protein
mTOR	Mammalian target of rapamycin
n	Nano
NAD+	Nicotinamide adenine dinucleotide
NaF	Sodium fluoride

Na3VO4	Sodium orthovanadate
NcoR	Nuclear receptor corepressor
NES	Nuclear export sequence
NLS	Nuclear localisation signal
NSC	Non-specific control
NSCLC	Non-small cell lung cancer
PAH	Paired amphipathic alpha-helix domain
PARP	Poly ADP ribose polymerase
PBS	Phosphate buffer saline
PCD	Programmed cell death
PCR	Polymerase chain reaction
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol 3,4,5-triphosphate
PKB (AKT)	Protein kinase B
PLK1	Polo-like kinase 1
PML	Promyelocytic leukemia
Pol I	RNA polymerase I
PtdIns	Phosphatidylinositols
PTEN	Phosphatase and tensin homologue detected on chromosome ten
ROCK1	Rho-associated coiled-coiled containing protein kinase 1
ROS	Reactive oxidative species
RPM	Revolutions per minute
RT	Room temperature
s	Seconds
SAHA	Suberoylanilide hydroxamic acid
SDS	Sodium dodecyl sulphate
SENP1	Sentrin-specific protease 1
SERM	Selective ER modulator
SGK	Serum and glucocorticoid inducible kinase
SHIP	SH2-containing inositol polyphosphate-5-phosphatase)
Sir2	Silent information regulator 2
SIRT1	Silent information regulator two 1
Smac	Second mitochondria-derived activator of caspase
SMRT	Silencing mediator for retinoid and thyroid hormone receptor
PDGFα	Platelet-derived growth factor alpha
TAF68	TATA box-binding protein associated factor 68
TBS	Tris buffered saline
TBST	Tris buffered saline-Tween 20
TCA	Tricholoroacetic acid
TEMED	N,N,N,'N-tetramethylethylenediamine

TIP60	TAT-interactive protein-60
TNF	Tumour necrosis factor
Tp53	Tumour protein 53
TRAIL	Tumour necrosis factor –related apoptosis inducing ligand
Tris	Tris (hydroxymethyl)aminomethane
TSA	Tricostatin A
Tween 20	Polyoxyethylene-sorbitan monolaurate
µ	Micro
UPS	Ubiquitin-proteasome system
UV	Ultra-voilet
VEGFR2	Vascular endothelial growth factor receptor 2
v/v	volume:volume ratio
w/v	weight:volume ratio

CHAPTER 1

INTRODUCTION

1.1 CANCER

In 2002 the International Agency for Research on Cancer (IARC) published figures relating to the incidences of twenty six different types of cancers globally. They showed that in 2002, 10.9 million people were diagnosed with cancer, there were 24.6 million people alive with cancer (within three years of diagnosis) and there were 6.7 million deaths worldwide. Lung cancer and breast cancer are the two most prevalent cancers in the world today, with 1.35 and 1.15 million cases respectively, although lung cancer has a higher mortality to incidence ratio compared to breast cancer due to the relatively more favourable prognosis on detection of the disease [1]. Cancer is the second largest cause of mortality in the western world after cardiovascular disease. In Europe, 3.2 million cases of cancer were diagnosed in 2008 and an estimated 1.72 million people died from cancer, 212,000 of these being breast cancer patients. Although lung cancer was the most common cause of death from cancer in Europe, in women, the most common was breast cancer, 421,000 deaths (28.2% of cancer cases) and the leading cause of death in women (129,000 deaths, 17% of total deaths) [2]. In the UK, there were 45,822 cases of breast cancer in 2006, over 99% of these being women. It is the most common cancer among women in the UK, accounting for 31% of all diagnosed cancer cases (Statistical information team, Cancer Research UK. Breast Cancer. 2009).

1.1.1 Breast cancer

Breast cancer can develop sporadically throughout a woman's lifetime; the likelihood of developing breast cancer can be increased by factors such as obesity, alcohol and exposure to radiation [3]. But an increased risk of developing cancer can also be hereditary, 10% of all breast cancers are thought to have a strong hereditary link due to a family history of breast cancer [4]. Hereditary cancers are predominantly caused by the inheritance of genes that harbour mutations that predispose an individual to developing cancer. In breast cancer

the two most studied genes that increase the risk of developing cancer are breast cancer type 1 susceptibility protein (BRCA1) and breast cancer type 2 susceptibility protein (BRCA2) [5, 6]. BRCA1 and -2 are located on chromosomes 17q21 and 13q12.3 respectively, and both function as tumour suppressors, regulating responses to DNA damage and repair, maintaining genomic integrity. Mutations within BRCA1 and -2 produce truncated proteins that lack appropriate function, increasing the likelihood of accumulating mutations due to DNA damage and promoting tumourigenesis [7]. Both BRCA1 and -2 are important regulators of the estrogen receptor α (ERα), loss of fully functional BRCA1 and -2 can lead to its upregulation. Although the effects of loss of BRCA1 and -2 are multifaceted, affecting multiple pathways that regulate controlled cell growth.

ERα has been shown to be important in breast, colon, ovarian and endometrial cancers [8]. Moreover, 70% of breast cancer cases display an increase in ERα expression – termed ERα positive tumours - and a dependence on ERα-driven proliferation. ERα is activated by the binding of oestrogen – predominantly 17β estradiol – which is produced by the ovaries and the placenta. Once activated ERα functions as a transcription factor, translocating from the extracellular membrane to the nucleus, binding to promoter regions of genes that contain estrogen response elements (EREs), promoting the transcription of genes important in proliferation, differentiation and survival [9]. Estrogen is also thought to contribute to the accumulation of genotoxic stress by increasing potentially damaging factors such as oxidative stress, which can contribute to tumour formation.

The expression of ERα provides a therapeutic opportunity to selectively inhibit tumour growth with anti-estrogen therapies, such as tamoxifen, ICI 182,720 (also known as Faslodex) and aromatase inhibitors, which act as selective ER modulators (SERMS), either antagonizing ER activity or targeting it for proteasomal degradation, inhibiting ERα-dependent proliferation and survival [10]. Unfortunately, prolonged exposure to SERMs can result in tumours that

develop ER-independent growth and resistance to SERM therapy. Recent clinical trials have shown that therapies that target mechanisms that promote estrogen-independent growth can induce apoptosis in breast cancer cell lines and breast tumours. Moreover, targeted therapies such as lapatinib – which will be discussed in more detail later – are able to resensitise tumours to anti-estrogen agents and increase their efficacy [11].

1.2 HALLMARKS OF CANCER

Cancer is a heterogeneous disease, where multiple genetic alterations and dysregulated processes lead to the transformation of normal cells into cancer cells. The seminal paper from Hanahan and Weinburg outlined the 6 essential alternations that cancers have to undergo to transform from normal to cancer cells [12]. These steps comprised of self-sufficiency in growth signals, insensitivity to growth inhibitory signals, limitless replicative potential, evasion of programmed cell death, such as apoptosis, sustained angiogenesis, tissue invasion and metastasis [12].

1.2.1 Oncogenes and tumour suppressors

The genetic alterations observed in the transformation of normal cells to cancer cells occur predominantly within two types of genes, termed oncogenes and tumour suppressors.

1.2.2 Oncogenes

Oncogenes code for proteins that, when inappropriately modified or activated, lead to the transformation of normal cells to undergo tumour development and cancer progression. Most normal genes (proto-oncogenes) that become oncogenes encode proteins that are important in cell cycle progression, proliferation and survival. Oncogenes drive proliferation and survival through their

increased activity or expression, for example, the human epidermal growth factor receptor 2 (HER2) and the fibroblast growth factor receptor (FGFR) are over expressed in 15-20% and 10% of breast tumours respectively, due to their chromosomal amplification [13, 14]. The increased expression of these extracellular receptors results in the increased activation of pro-survival signalling pathways, promoting tumourigenesis.

Oncogenes can also be brought about via chromosomal translocations, this with be discussed in more detail later.

1.2.3 Tumour suppressors

Tumour suppressors are genes that when active regulate normal cellular processes such as proliferation, but are frequently lost, mutated and/or inactivated in cancers, leading to tumour progression.

The protein p53 has been lavished with many titles, referred to as the 'guardian of the genome' [15] and the 'policeman of the oncogenes' [16] and is undoubtedly the most well studied tumour suppressor in cancer cell biology to date, mostly due to the frequency of mutations found in human cancers - ~50% - but also because of the multiple cellular processes that p53 is involved in regulating [17]. The primary function of p53 is as a transcription factor, but it is also known to exert its effect on the cellular phenotype via transcription-independent mechanisms such as mitochondrial membrane disruption, but these studies are still in their infancy and the exact molecular mechanisms need to be more clearly defined [18].

As a transcription factor p53 is upregulated in response to cellular stresses, such as DNA damage, UV radiation, stress and oncogenic processes [19]. p53 dictates the cellular phenotype by inducing cell cycle arrest (for example, the cyclin-dependent kinase inhibitor (CDKN1) p21 [20]), senescence (the

plasminogen activator inhibitor 1 – PAI-1 [21]), autophagy (the damage-regulated autophagy modulator – DRAM [22]), DNA repair (BTG2 [23]) and programmed cell death, apoptosis (the BH3-containing proteins Bid, Noxa and Puma [24, 25]).

Loss of or impaired p53 function is an integral step in cancer development. Li-Fraumeni syndrome sufferers predominantly have one functional copy of p53 (~80% of patients), which dramatically increases their susceptibility to develop carcinomas of the breast, brain and adrenal glands, as well as other types of malignancies [26].

The increase in cellular expression of p53 is associated with its activation, because of this many mechanisms in tumourigenesis have developed to restrict or halt this upregulation/stabilisation. The E3 ubiquitin ligase murine double minute 2 (mdm2) (along with the other family member, mdm4 [27]), negatively regulates the cellular expression of p53, by blocking its transactivation domain and facilitating its ubiquitination and subsequent degradation by the proteasome [28, 29]. In normal cells, DNA damage results in the disruption of the interaction of p53 with mdm2, resulting in the activation and increased cellular expression of p53. p53 induces the transcription of genes involved with DNA-damage repair, but also the transcription of mdm2, which deactivates p53. This autoregulatory loop ensures that p53 is not inappropriately activated [30].

Mdm2 is upregulated in 7% of human tumours and was found to be predominantly associated with WT p53 (88%), suggesting that the oncogenic role of mdm2 was to suppress normal p53 function [31]. Consequently, because p53 could still function normally – if it were not repressed - chemical inhibitors have already been developed, such as the Nutlins, which bind to the p53-binding pocket within mdm2 inhibiting its ligase activity, resulting in an increase in the cellular accumulation and activation of p53 [32]. Nutlin compounds (notably Nutlin-3) were shown to induce cell cycle arrest and apoptosis in cancer cell lines [32] and exhibit tumour growth inhibition and shrinkage in nude mice bearing

tumour xenographs [33] highlighting that therapeutically targeting mdm2 could provide benefit in tumours that contained inactive WT p53.

p53 is frequently mutated in cancer and is subsequently termed tumour protein 53 (tp53). Mutations predominantly occur within two regions, the DNA-binding domain (DBD) (95%) and the N- and C-terminal regions (5%) [34]. Three-quarters of the mutations within the DBD are missense mutations, which generally produce a full length protein, but their effects either impact on DNA-binding or overall protein structure [35]. Maintaining functional p53 expression is key to controlling cell fate, this is exemplified by the discovery of point mutations within p53 – within the DNA-binding domain - that results in its inactivation and dysregulation. The core domain of p53 is unstable and has a short half-life of 9 minutes. Distinct point mutations within p53 destabilize the protein further reducing DNA-binding efficiency, cellular expression and ability to activate its downstream targets [35]. This loss of function impedes p53s role in maintaining genomic integrity, leading to the accumulation of DNA damage, tumourigenesis, promoting cancer development.

The activation of the specific subsets of targets of p53 is largely dependent upon its posttranslational status and also its association/interaction with known cofactors/co-regulators [36], for example, the posttranslational modification of lysine residues by acetylation dramatically affects the ability of p53 to activate downstream targets involved with inducing a cell cycle arrest and apoptosis. Interestingly, the acetylation of eight key lysine residues within p53 was shown not to effect DNA-binding ability, but did promote the dissociation of mdm2 from the p53 complex, suggesting that acetylation promoted the stabilisation of p53, promoting the full transcriptional potential of p53 [19]. Residues known to be acetylated in p53 are lysines 320 and 382. The acetylation of these residues are associated with p53 stabilisation and activation, predominantly activating genes involved with inducing apoptosis [19].

1.3 MAMMALIAN CELL CYCLE

A eukaryotic cell is required to be able to operate appropriately and maintain the function of distinct tissue types. If there is damage to a tissue and new cells are required, cells undertake a process called the 'cell cycle' to produce two identical daughter cells via mitosis. The mammalian cell cycle is a highly ordered process and contains four established discreet phases: G1, S, G2 and M phase. The two most prominent phases being S phase, where DNA is replicated and M phase, where cells undergo mitosis to product two daughter cells. Between these phases are gap phases, termed G1 and G2 (G1 follows M phase and G2 follows S phase) which allow the cell to prepare for DNA synthesis and cellular division, respectively. There is a fifth stage of the cell cycle G0, which occurs during G1. Entering into G0 (also known as quiescence) is a reversible process where a cell is not actively dividing [37]. This defines G1 as the phase where the cell decides whether to undergo mitosis or not i.e. enter G0, predominantly in response to extracellular stimuli/growth signals. Once the 'decision' has been made, cells are committed to progressing through the cell cycle; this is termed the restriction point. Once this checkpoint has been passed, even if the favourable pro-cyclic stimuli are removed, cells will undergo mitosis [38].

Transition through the phases of the cell cycle is tightly regulated by heterodimeric complexes which contain proteins termed cyclins, and cyclin-dependent kinases (CDKs) (Figure 1.1) [39]. CDKs are serine/threonine protein kinases and as the name suggests, have to associate with specific cyclins to be catalytically active and define their substrate specificity [39]. CDK expression throughout the cell cycle does not change, whereas the expression of cyclins is tightly regulated and is phase specific [40].

To date, twenty CDK-related proteins have been identified in higher eukaryotes [41], although only five (CDK1, -2, -3 -4 and -6) are known to be important in cell cycle progression [42].

To start from the beginning, mitogenic stimuli promotes the transcription and expression of Cyclins D1, -2 and -3, which bind to and activate CDK4 and -6, starting the progression through the G1 phase and preparation to synthesize DNA [43]. These complexes interact with and partially repress the 'pocket proteins' or retinoblastoma (Rb) family of proteins by phosphorylating them on specific residues. There are three family members, termed Rb/p105, p107 and Rb2/p130 [44]. Rb proteins bind to and inhibit the E2F transcription factors. Cyclin D/CDK4/6 complexes disrupt this interaction allowing the transcription of the E-type cyclins (E1 and -2) which form heterodimeric complexes with CDK2 which bind to and phosphorylate Rb again, resulting in its complete inactivation allowing progression into S phase. Cyclin A is consequently upregulated in S phase and associates with CDK2 to drive progression into and through G2 [45]. As cells approach mitosis, progression is driven by type-A cyclins and their activation of CDK1. Once the nuclear envelope has been broken down and the cellular expression of Cyclin A is reduced, CDK1 associates with cyclin B and drives cells through mitosis and the formation of two daughter cells [37].

1.3.1 Cyclin-dependent kinase (CDK) Inhibitors

As with any cellular process there are fail safe mechanisms to protect cells from inappropriate progression through the cell cycle, CDK-mediated progression can be inhibited by proteins called CDK inhibitors (CDKI), these act at various checkpoints throughout the cell cycle to ensure uncontrolled progression does not occur. There are two families of proteins that act as CDK inhibitors, the INK4 (consisting of INK4A, -B, -C, -D) and the Cip and Kip family [42].

The Cip and Kip family consists of $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$. Family members are predominantly regulated by their cellular expression and phosphorylation. Distinct phosphorylation events on specific residues can promote or reduce their binding to specific CDK-Cyclin complexes, as well as regulate their subcellular

localisation and expression [46]. The CDKI p21 can inhibit CDK1 and -2 but is known to predominantly inhibit CDK2. The interaction of p21 with CDK2 reduces the ability of CDK2 to interact with its substrates resulting in Rb being active and trapping E2F, inhibiting E2F-dependent gene transcription, promoting cell cycle arrest [20]. A major regulator of p21 is the tumour suppressor p53, which upregulates p21 transcription and expression in response to DNA damage [47]. Although p21 is able to promote cell cycle arrest at both G1 and G2, the ability to halt the cell cycle at G1 is especially important when there is the possibility of transcribing DNA that is faulty or mutated due to damage. p21 expression allows the cell time to repair DNA and allow mitosis to continue without propagating errors within the genetic material that could promote the development of a disease state [48].

The expression of the CDKI p27 changes throughout phases of the cell cycle and is predominantly upregulated in response to the lack of growth signals [48]. p27 is a potent inhibitor of the CDK2/Cyclin E complex, thus controlling Rb activity and G1/S cell cycle transition. Both p21 and p27 promote Rb activity through inhibiting CDK/Cyclin complexes. As previously mentioned, the Rb family of proteins are integral to the passage from G1 to S phase. Their inhibition by hyperphoshorylation by cyclin/CDK complexes is integral to the release of the E2F transcription factors. At the G1 checkpoint, active (hypophosphorylated) Rb not only binds to and inhibits the pro-transcriptional potency of E2F but also turns them into transcriptional repressors by interacting with and inhibiting them when they are complexed on promoter regions of pro-proliferative genes, halting gene transcription, resulting in an arrest at the G1 boundary [44].

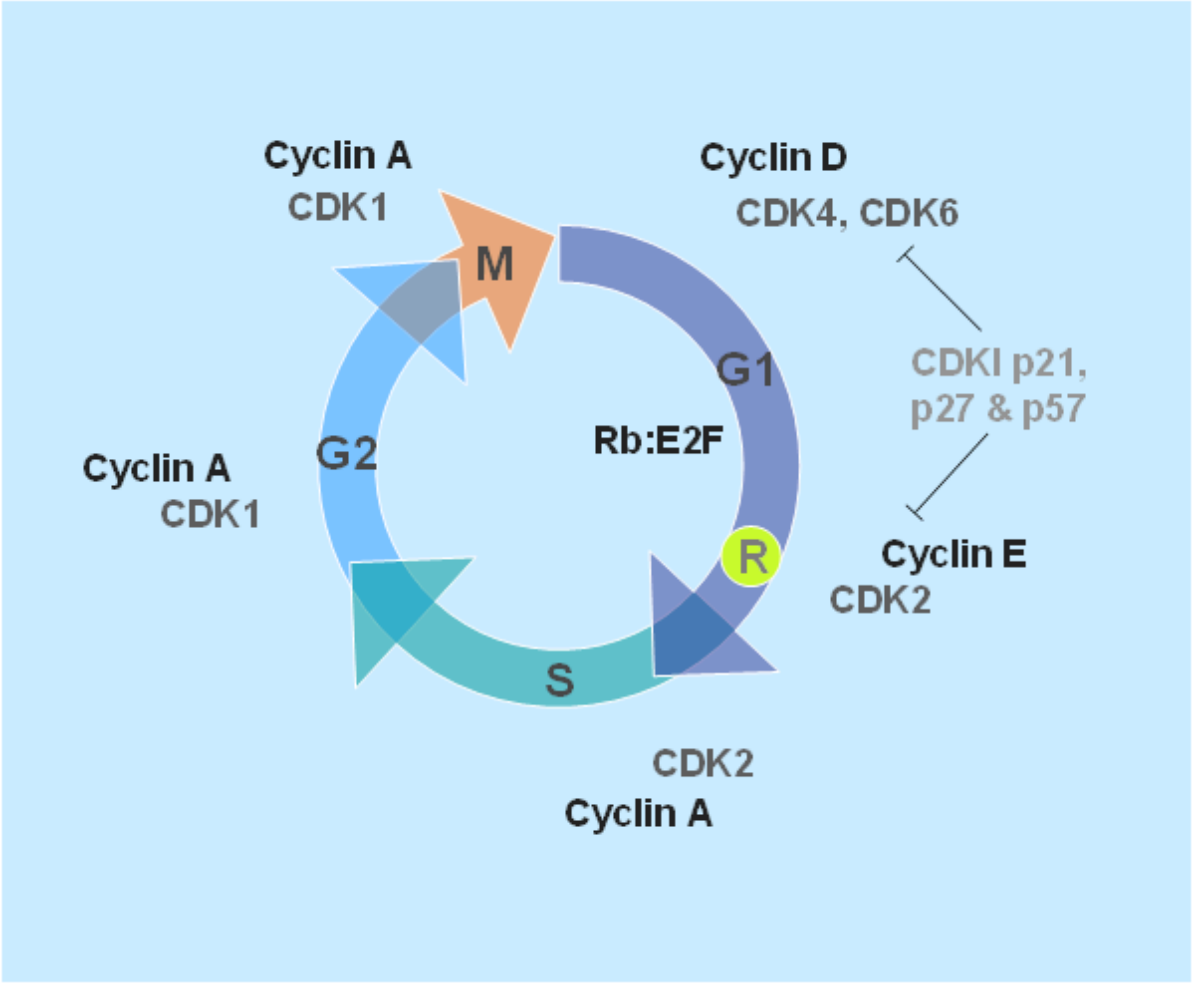


Figure 1.1: The mammalian cell cycle (Modified from Malumbres & Barbacid 2009 and Dehay & Kennedy 2007).

1.4 APOPTOSIS

Cell death is an integral function of multicellular organisms during many stages of development (embryonic), tissue homeostasis and immunity. There are various mechanism of cell death, such as apoptosis, necrosis, mitotic catastrophe and autophagy. Apoptosis is the most relevant to this study due to its dysregulation in cancer [12] and relevance to the research that was undertaken.

Apoptosis is the process of programmed cell death (PCD) and differs from other cell death mechanisms such as necrosis due to its specificity and minimal trauma to surrounding cells and tissues. Necrosis, for example, occurs when cells undergo trauma from an external source, resulting in membrane damage and premature cell death. Necrosis is generally considered to be toxic or detrimental to maintaining healthy tissues [49]. Apoptosis, is a highly ordered process and is particularly important in embryogenesis and development, for example, an embryo will develop both Wolffian and Mullerian ducts, which will become the male and female reproductive organs, respectively. Meaning that one or the other is redundant in each of the sexes, programmed cell death is implemented to removed the structure that will not be required [50]. Moreover, the average human body produces 60 billion new cells a day, and due to the fact that homeostasis must be maintained, roughly the same number of cells must be destroyed [51]. Thus, apoptosis is an indispensible process for higher organisms but whose dysregulation could have lethal consequences.

To date two main apoptotic pathways have been described: the extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathway, but due to the complexities of these pathways it is becoming clear that they may not necessarily be mutually exclusive. Moreover, both pathways are dependent on the caspase – cysteine-aspartic acid protease - signalling cascade [52]. Family members are expressed as catalytically inactive zymogens, requiring cleavage for activation. A

fail-safe mechanism to ensure inappropriate activation of cell death does not occur [53].

1.4.1 Extrinsic pathway

The extrinsic pathway requires the activation of death receptors – named due to the presence of an 80 amino acid structure called the 'death domain' in each of the family members - such as Death Receptor 4 (DR4) and the Fas Receptor (FasR) [49]. Expression of the death receptors within the extracellular membrane and the presence of their ligands are key to their activation. The Tumour necrosis factor (TNF)-Related Apoptosis Inducing Ligand (TRAIL) and Fas Ligand (FasL) are ligands for the DR4 and FasR receptor, respectively. Upon ligand binding, conformational changes promote receptor activation, inducing the recruitment of intracellular adaptor proteins to the cytoplasmic death domains. The Fas-associated death domain (FADD) protein binds to the activated receptors (TNF receptors also require the binding of TRADD – TNF Receptor Associated Death Domain), resulting in the recruitment of procaspase-8, forming the death-inducing signalling complex (DISC) [54], promoting the cleavage of procaspase-8 to form the catalytically active caspase-8. Caspase-8 then activates the caspase cascade, cleaving procaspase-3 to form caspase-3.

1.4.2 Intrinsic pathway

The intrinsic pathway is centred around the membrane disruption of the cell powerhouse, the mitochondrion. Both positive and negative stimuli influence the intrinsic apoptotic pathway by driving the expression of proteins that influence the integrity of the mitochondrial membrane, one of the best studied are the Bcl-2 family of proteins.

The Bcl-2 family of proteins consists of more than 30 members, which can promote or inhibit the induction of apoptosis. Family members contain one or

more conserved Bcl-2 Homology (BH) domains termed BH1-4) [55]. Bcl-2 (B cell lymphoma gene 1) was the first family member to be identified and was found to be anti-apoptotic, able to maintain cell survival when pro-survival stimuli was removed, as well as protecting cells from cytotoxic compounds [51], other examples of anti-apoptotic members are BCL-XL and MCL-1. Anti-apoptotic members contain multiple domains but all contain the BH4 domains, whereas pro-apoptotic members can contain either multiple domains, such as Bax and Bak, or only the BH3 domain, such as Bim, Bid, Bad, Noxa and Puma [55].

Family members elicit their function by forming homo- and heterdimers, for example, Bcl-2 binds to and sequesters Bax, inhibiting induction of apoptosis. Upregulation of Bim – or any other BH3-only protein - preferentially binds with Bcl-2, resulting in the release of Bax, promoting apoptosis [51]. Proapoptotic members form mitochondrial apoptosis-induced channels (MACs) within the mitochondrial membrane, which promotes mitochondrial outer membrane permeabilisation (MOMP) [56]. The formation of MACs increase the permeability of the mitochondrial membrane, facilitating the release of Smac (second mitochondria-derived activator of caspase – also known as Diablo) and cytochrome *c* into the cytoplasm. Cytochrome *c* binds to the scaffolding protein Apaf-1 and procaspase-9 forming an apoptosome, promoting the accumulation, cleavage and activation of caspase-9, which in turn cleaves and activates caspase-3 [57].

Both the extrinsic and intrinsic pathways elicit their proapoptotic function through activating initiator caspases (caspase-8 and -9) that activate the effector caspase, caspase-3 (along with the other effector caspases -6 and -7) [53, 58]. Caspase-3 is thought to be the main regulator of apoptosis and is integral for regulating normal development. Caspase-3 knockout mice predominantly die soon after birth, displaying abnormal cell numbers in the brain and other organs [58]. Although in some instances there does appear to be some redundancy between caspase-3 and -7, both have similar substrate specificity and the fact

that mice deficient in both caspases die immediately due to cardiac dysfunction due to improper development [59]. Although they do appear to have discreet roles in apoptosis, caspase-7 is thought to regulate cell viability whereas caspase-3 activation is known to regulate DNA fragmentation [60] and cleave key cellular components, resulting in morphological changes that are associated with apoptosis [57]. But even though this could be the case, caspase-3 and -7 are essential for the induction of apoptosis [60].

As mentioned, caspase-3 promotes DNA fragmentation, supported by the fact that caspase-3 defective mice do not display chromatin condensation and DNA fragmentation [58]. Moverover, DNA fragmentation potential can be reinstated in the MCF7 breast cancer cell line – does not contain caspase-3 – by overexpressing caspase-3 resulting in increased apoptosis [61]. Caspase-3 promotes DNA fragmentation though the activation of the caspase-activated Deoxyribonuclease (CAD) – which facilitates DNA fragmentation through its Deoxyribonuclease (DNase) activity – by cleaving the carboxy-terminal region of the inhibitor of caspase activated DNase (ICAD) which is essential for its activity [62]. DNA fragmentation is defective in ICAD null mice, highlighting its importance in caspase-3-mediated induction of apoptosis [58]. Although mice that lack ICAD expression appear to have no major defects and can still undergo apoptosis, suggesting the chromatin condensation and DNA fragmentation are dispensable in programmed cell death mechanisms [63]. DNA condensation is promoted by the activation of the protein kinase mammalian sterile 20-like protein (MST1) through cleavage of its c-terminal domain and its phosphorylation by caspase-3 which results in its translocation to the nucleus, where it mediates chromatin condensation [64].

Caspase-3 also cleaves the DNA repair protein poly ADP ribose polymerase (PARP) resulting in its inhibition. PARP is important for the repair of damaged DNA, although it achieves this by using the cellular pools of energy (NAD and ATP). Caspase-3 cleavage of PARP promotes the availability of NAD and ATP

that may be important to drive the later stages of apoptosis [59]. Caspase-3 disrupts structural integrity by cleaving and activating the Rho-associated coiled-coiled containing protein kinase 1 (ROCK1) resulting in the phosphorylation of the myosin light chain and contraction of the actin bundle, weakening the cytoskeleton. Moreover, the loss of the cellular scaffold results in the literal 'tearing apart' of the nucleus, promoting apoptosis [65].

1.4.3 Physiology of apoptosis

While undergoing apoptosis cells shrink, resulting in the organelles being more proximal to each other, membrane bledding (irregular budding), collapse of the cytoskeletal framework, condensation of nuclear chromatin, chromosomal DNA fragmentation and the formation of small membrane-bound compartments, called apoptotic bodies [66]. Unlike other forms of cell death, no inflammatory response is associated with cells that have undergone apoptosis. For example, cells that undergo necrosis predominantly release cellular components such as cytochrome *c* into the local environment due to disruption of the cell membrane, promoting an inflammatory response. Due to the organised nature of apoptosis – no membrane disruption and cytoplasm is retained in apoptotic bodies - this does not occur. Moreover, apoptosed cells are phagocytosed by macrophages or surrounding cells, preventing inappropriate inflammation [49].

Apoptosis is a tightly regulated cellular process; dysregulation can lead to multiple disease states, most notably cancer. Suppression of apoptosis is integral to the survival of cancer cells.

1.5 PHOSPHATIDYLINOSITOL 3-KINASE (PI3K) PATHWAY

The phosphatidylinositol 3-kinase (PI3K) signalling pathway consists of the family of enzymes and phospholipid messengers involved with signal transduction

regulating cellular functions such as, cell growth, proliferation, survival and apoptosis.

1.5.1 RAS

Ras is a guanosine nucleotide-binding protein (G protein), which is structurally related to the G_α subunit of heterotrimeric G proteins (large GTPases) but is termed a small GTPase because it consists of a single subunit. Ras, which hydrolyses GTP into GDP and phosphate, binds both GTP and GDP [67]. The binding of GTP results in a conformational change within Ras, adopting the "loaded spring" position due to the additional phosphate that is present on GTP and not GDP resulting in Ras activation. Because of this subtle change, bound GDP is not able to maintain Ras in the same conformation resulting in its deactivation. This change between an on/off state highlights the role of Ras as a cellular switch turning on signal transduction pathways. Ras proteins are also negatively regulated by GTPase activating proteins (GAPs), which promote an increase in Ras GTPase activity resulting in the formation of GDP and its deactivation [68].

Ras activation is known to potentiate extracellular signals through many intracellular signaling cascades, most notably the RAF/MEK/ERK signaling pathway. Active Ras binds to and activates the serine/threonine kinase RAF (c-RAF, BRAF and ARAF) [69], leading to its translocation to the plasma membrane where it phosphorylates and activates the mitogen-activated protein kinase kinases (MAPKKs), MEK1 and MEK2. These in turn phosphorylate and activate the mitogen-activated protein kinases (MAPKs), ERK1 and ERK2 (extracellular signal-regulated kinase 1 and 2). In addition to activating RAF/MEK/ERK signalling cascade Ras also binds directly to and activate the class I PI3K proteins [70].

1.5.2 Class I PI3K proteins

PI3K proteins belong to a large superfamily which consists primarily of three main classes each containing a range of subunits and isoforms, distinct from each other due to their substrate specificity and lipid products [71]. There is also a fourth class but these are more distantly related than the other classes. Although they are indeed serine/threonine kinases, they are not known to target lipids like the other family members [72].

Class I PI3Ks catalyse the phoshorylation at the 3 position hydroxyl group of the inositol ring within lipids, termed phosphatidylinositols (PtdIns) - which are present on the intracellular space of the cell membrane – converting phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-triphosphate (PIP_3). PI3K class I proteins are heterodimeric haloenzymes and comprise of a p110 subunit that contains the catalytic domain and a p85 subunit that contains the regulatory domain. Class I proteins can be further divided into two subgroups, IA and IB. Class IA contain three isoforms of the p110 catalytic subunit, termed p110α, p110β and p110δ, which are coded by three different genes, PIK3CA, PIK3CB and PIK3CD respectively, all structurally similar, containing helical and kinase domains and bindings sites for the regulatory proteins. Class IA also contains three genes that encode the regulatory subunit, PIK3R1, PIK3R2 and PIKR3. Where splice variants of the PIK3R1 encode for the p85α, p55α and p50α proteins, and PIK3R2 and PIK3R3 encode for the p85β and p55γ proteins, respectively [73]. The p85 subunit contains two Src homology 2 (SH2) domains which allow binding to the p110 catalytic subunit. Class IB contains a p110γ catalytic subunit that binds to a different regulatory subunit, termed p101. Unlike class IA, IB PI3Ks are activated by G-protien coupled receptors not receptor tyrosine kinases [72].

Class II PI3Ks are monomeric and do not contain a regulatory subunit. There are three isoforms C2α, C2β and C2γ. Class III PI3Ks are more similar to class I

PI3Ks because they are heterodimers consisting of a catalytic and regulatory subunit. Class I PI3Ks are more extensively studied due to their defined role in the development of cancer, where as Class II and III proteins have not yet been shown to be linked with oncogenesis [74].

The class I PI3K proteins are integral to potentiating extracellular signals from extracellular growth factor and tyrosine kinase receptors into the intracellular environment, regulating transcription factors that activate/inhibit the transcription of genes important in a plethora of cellular process, depending on the signal being received.

When a growth stimuli is received by the cell through an extracellular receptor, it causes the activation and recruitment of the PI3K heterodimer, which primarily consists of p110 (the catalytic subunit) and p85 (the regulatory subunit), to the plasma membrane where it interacts with and phosphorylates its substrate PIP_2, generating PIP_3. PIP_3 generation and accumulation on the plasma membrane attracts proteins containing pleckstrin homology (PH) domains such as the AGC (cAMP-dependent cGMP-dependent and protein kinase C) family members, including the 3'-phosphoinositide–dependent kinase 1 (PDK1)[75]. PIP_3 accumulation also promotes the binding of another AGC family member, the serine/threonine kinase AKT (also known as PKB), to the intracellular space on the plasma membrane, where it is phosphorylated by PDK1 on threonine residue 308, which is within the catalytic site (activation loop) of AKT, leading to its activation and release of AKT into the intracellular space to phosphorylate its downstream targets [76, 77].

AKT is also phosphorylated on serine 473, within the c-terminal hydrophobic motif (HM), by the mammalian target of rapamycin (mTOR) complex 2 [78]. AGC family members are dependent on their phosphorylation to be catalytically active [79, 80]. Dually phosphorylated AKT (308 and 473) is known to have an increased catalytic activity *in vitro* [81, 82] highlighting the importance of both

phosphoryation sites on activity, thus it is rationalised that the phosphorylation of both sites is required to elicit its full activity *in vivo*, but the exact implication of differential phosphorylation on these sites has not been fully elucidated. Jacinto *et al* and Guertin *et al* both showed rather eloquently that differential phosphorylation on serine 473 only affected a subset of AKT downstream targets, most notably the forkhead transcription factors [78, 83, 84].

1.5.3 AKT/PKB

AKT is considered to be one of the most important effectors of the PI3K signalling cascade and has been conserved throughout evolution, from C*aenorhabditis elegans* (*C.Elegans*), *Drosophila* to humans [73]. The maintainance of this signalling axis has lead to a wealth of study of the downstream effector AKT. In mammals there are thee AKT isoforms or family members, termed AKT1 (PKBα), AKT2 (PKBβ) and AKT3 (PKBγ) [73, 85]. Each containing an N-terminal PH domain, a C-terminal region that contains the HM domain and a central kinase catalytic domain (CAT) responsible for the serine/threonine phosphorylating potential of all the AKT isoforms [86].

In humans, AKT1 and AKT2 are expressed in all tissue and cell types, whereas AKT3 expression appears to be more exclusive, predominantly found in the brain. Knockout mice highlighted the different phenotypes that arise from loss of one AKT isoform. AKT1(-/-) mice display placental hypotrophy as well as impaired growth and reduced bodyweight, AKT2(-/-) are hyperinsulemic and hyperglycaemic and AKT3(-/-) have impaired brain development. But all knockout mice are viable and thrive suggesting that compensatory mechanisms exist between the AKT isoforms (reviewed in [87]).

Upon activation (as described above), AKT relocates to the nucleus where it phosphorylates its downstream targets, such as forkhead transcription factors [84], the proapoptotic BH3-containing protein BAD [88], the E3 ubiquitin ligase

MDM2 [89], and many others. All established AKT downstream targets to date have been shown to require the R-X-R-X-X-S/T-B motif (X represents any amino acid, B represents bulky hydrophobic residue) [90, 91].

1.5.4 Phosphatase and Tensin homolog deleted on chromosome 10 (PTEN)

PTEN, a 3'phosphatase stringently controls the cellular levels of PIP_3 by dephosphorylation, converting PIP_3 back to PIP_2, reducing the recruitment of PH containing proteins to the plasma membrane, inhibiting the potentiation of the PI3K signal [77]. Although there are other PIP_3 phosphatases, notably SHIP1 and SHIP2 (SH2-containing inositol polyphosphate-5-phosphatase), knockout mice which lack PTEN develop cancer, whereas SHIP1 knockout mice develop myeloproliferative syndromes - possibly due to the fact that SHIP1 reduces PIP_3 to PIP_2 by removing the phosphate group from the 5-position, not the 3-position of the inositiol ring – establishing PTEN as a true tumour suppressor [73, 92, 93].

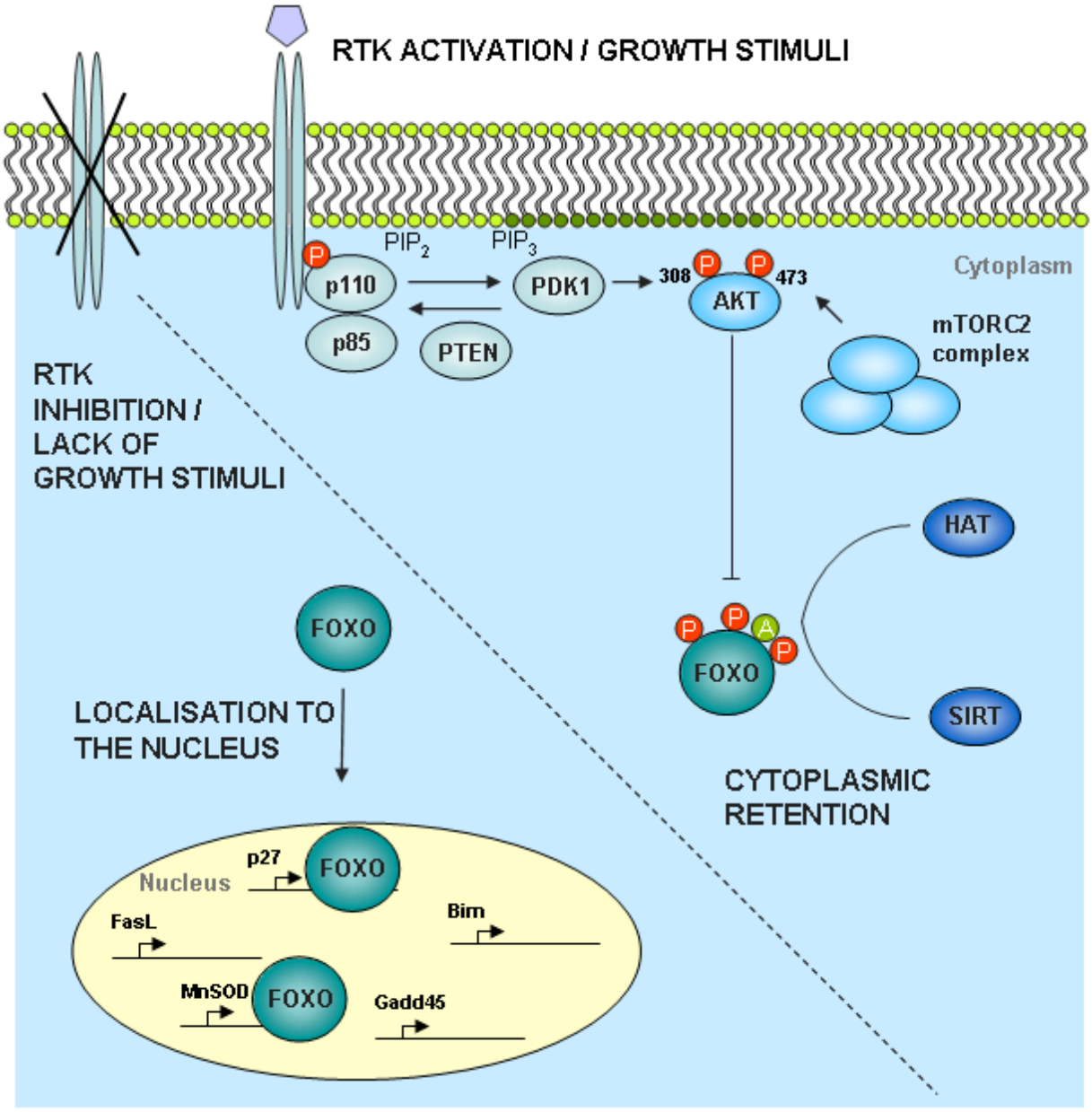


Figure 1.2: The PI3K signaling cascade. A summary of the PI3K cascade and its regulation of the subcellular localisation of the FOXO transcription factors. Post-translational modifications of key signalling components is displayed, phosphorylation (P - red) and acetylation (A - green).

1.5.5 PI3K in cancer

Dysregulation of the intracellular signalling profiles of cells is a prominent feature in the development of cancer. There are many changes that occur to the PI3K in cancer, here I will outline a few that are the most well known and relevant to this study.

Many different extracellular growth factor and tyrosine kinase receptors can activate the PI3K signalling cascade. Due to this redundancy, the PI3K is frequently dysregulated in tumour formation and cancer development.

1.5.5.1 Epidermal growth factor receptor (EGFR, also known as ERBB)

The receptor tyrosine kinase EGFR was one of the first growth factor receptors to be characterised and with the other structurally related ERBB family members HER2 (ERBB2, neu), HER3 (ERBB3) and HER4 (ERBB4) form homodimeric and heterodimeric complexes. Each family member contains an extracellular ligand-binding domain, a trans-membrane region and an intracellular tyrosine kinase domain, although HER3 lacks intrinsic kinase activity due to its lack in ability to bind adenosine triphosphate (ATP) [94-96].

Ligand binding promotes receptor homo/heterodimerisation and phophorylation on lysine 721 (K721) of the EGFR receptor, this in turn promotes ATP binding and activation of the tyrosine kinase domain [95]. Receptor activation results in the recruitment of intracellular proteins important in signal transduction such as proteins involved in the PI3K/AKT signalling cascade, regulating a plethora of cellular processes such as differentiation, proliferation and survival [97, 98].

In cancer, the ERBB family of receptors are activated by a range of mechanisms including activating mutations, overexpression and the autocrine or paracrine production of their activating ligands [96]. The study of 5,232 breast cancer

cases showed 48% to be EGFR positive affirming the importance of EGFR in breast cancer development [99].

In non-small cell lung cancer, anti-EGFR target therapy leads to the development of resistance through mutations in the receptor. The T790M (residue 790 threonine to methionine) mutation increases the binding affinity of the receptor to ATP, as well as sterically hinder the binding of targeted small molecule inhibitors. Moreover, the mutation results in the preferential activation of the PI3K-AKT cascade over that of the RAS-MAPK signaling pathway, suppressing pro-apoptotic mechanisms [100]. Recent publications have shown that the T790M mutation is present in EGFR before the use of targeted therapy [101], highlighting that maintenance of EGFR driven proliferation provides a selective advantage in a heterogeneous tumour environment.

Although EGFR expression does not inversely correlate with patient survival, recent publications are starting to unravel its importance, suggesting that its subcellular localisation is important in breast cancer, showing nuclear accumulation of EGFR is inversely correlated with patient survival suggesting that EGFR has a role in tumourigenesis beyond the activation of intracellular signaling pathways such as the PI3K [102].

1.5.5.2 Human epidermal growth factor receptor 2 (HER2)

Another growth receptor HER2 is implicated in various cancers. The oncogenic tyrosine kinase receptor is overexpressed in 25% of breast cancer cases and is predominantly due to gene amplification [14]. Unfortunately these tumours are more prevalent in premenopausal women and ultimately lead to a poorer clinical outcome. Although its overexpression does present the opportunity to exploit tumour dependence on HER2 driven proliferation by developing HER2 targeted therapies [14].

Transgenic mice overexpressing an active form of the rodent HER2 homologue NEU were shown to rapidly develop multiple mammary tumours [103]. Further studies showed that human HER2 expression in transgenic mice also promotes mammary tumour formation [104].

Recent studies have shown that 20% of breast cancers also express a splice variant of HER2 which lacks a 16 amino acid sequence that codes for the juxtamembrane domain. The truncated form of HER2 is constitutively active due to the fact that it can form homodimers [105]. Furthermore, overexpression of the truncated form of HER2 in mice causes the development of aggressive and invasive mammary tumours [106].

HER2 overexpression is intimately linked with estrogen receptor (ER) endocrine signaling, less than 10% of ER+ tumours have high HER2 expression [107]. Furthermore, increasing HER expression is linked with ER-independent proliferation and resistance to endocrine therapy [108].

1.5.5.3 RAS

Mutations within the RAS family of proteins, most notably, HRAS, KRAS and NRAS - which are structurally similar, containing 85% sequence homology - are found in 25% of all human cancers [109]. Mutations within the different family members is found in different cancer types, for example, HRAS mutations can be found in bladder and renal cancer; KRAS mutations can be found in pancreatic, colorectal and non-small lung cancer; and NRAS mutations can be found acute myelogenous leukemia, melanoma and hepatocellular cancer [110]. Modifications of Ras in cancer are predominantly somatic missense mutations at positions 12, 13 and 61, reducing its GTPase activity and promoting resistance to GAP proteins, resulting in Ras hyperactivation [68]. Although generally lone mutations within Ras do not lead to tumourigenesis, in combination with other processes, Ras overactivation promotes tumour development by activating

prosurvival downstream signaling cascades such as the Raf/MEK/ERK and PI3K signaling pathways [109].

1.5.5.4 PIK3CA

Mutations in the catalytic subunit of class 1A PI3K (PIK3CA) - which encodes the p110α protein - have been found in breast and other tumours [111]. Eighty percent of the mutations found in PIK3CA are located in three main coding regions, two within the helical domain of p110α and one in the kinase domain [112], these mutations result in an increase in activity, highlighting the oncogenic potential of p110α, and its ability to drive PI3K-dependent proliferation and cancer progression. Moreover, mutations within PIK3CA have been found in approximately 30% of ductal carcinomas *in situ* (DCIS) and invasive ductal breast carcinomas (IDC), highlighting the frequency of loss of functional PIK3CA in breast cancer but also that it occurs as a relatively early event, promoting tumour development, rather than being involved in the development of resistance and metastasis [113].

Mutations within PIK3CA are associated with KRAS mutations [77] and HER2 overexpression, suggesting that multiple constituents of PI3K signalling are dysregulated in cancer, highlighting the importance of the PI3K signalling cascade in promoting tumour survival.

1.5.5.5 PTEN

PTEN is essential at controlling PI3K signalling via its regulation of PIP3, inhibiting prosurvival mechanisms such as proliferation and differentiation. Because of its role as a sentinel on the internal surface of the extracellular membrane, guarding the release of serine threonine kinases into the intracellular environment, PTEN was identified in 1997 as a major tumour suppressor [114, 115] and was subsequently found to be frequently mutated in many different

cancers such as lung, prostate, ovarian and breast [116] and to be the main contributing factor to the autosomal dominant syndrome known as Cowdens disease, where 80% of sufferers contain inactivating mutations (up to 70 have been identified) within the PTEN gene [114]. In 2010, 211 sufferers were assessed and 89% were found to be diagnosed with cancer within their lifetime and 81% of female cowden disease suffers developed breast cancer [117]. Seventy five percent of patients (both sexes) also developed benign breast tumours [118]. Its mutation results in a non-functioning PTEN protein resulting in the hyperactivation of the PI3K pathway.

PTEN is deleted in 30-40% of human tumours [119] and is considered one of the most important tumour suppressors known in cancer cell biology to date, matched in frequency by key regulators of cell fate, such as p53 which is mutated in 50% of human cancers [17]. PTEN was found to be mutated in 6% of breast cancer cell lines and xenographs [115]. 48% of breast cancer tissue samples were shown to have lost PTEN expression and this loss correlated with lymph node status (metastasis), loss of ER expression and disease-related death [120].

Loss of PTEN activity results in the hyperactivation of the PI3KAKT signalling axis

1.5.5.6 AKT/PKB

AKT is frequently hyperactivated in cancer through the deregulation of the PI3K signalling cascade. Overexpression of RTKs, loss of function mutations within PTEN and activating mutations within PIK3CA all lead to increased AKT activity which promotes tumour survival and cancer progression.

More recently, somatic mutations within AKT1 were found in 8% of breast cancer patients. The mutation, E17K – within the PH domain – was found to allow binding of AKT to the extracellular membrane without the need of PIP_3

accumulation, promoting the phosphorylation of AKT on S473 (no change in T308 was observed) [121].

Loss of AKT1 expression inhibits the development of Neu-induced tumours in mice, whereas loss of AKT2 accelerated tumour development. Moreover AKT1(-/-)Neu-induced tumours in mice were more invasive than AKT2(-/-)Neu-induced tumours [122] and recently, it has been shown that the dual overexpression of HER2 and AKT2 in mammary epithelial resulted in a markedly increased pulmonary metastasis whereas AKT1 did not [123]. Moreover, the reduction of AKT1 expression using siRNA enhanced migration in cancer cell lines. This finding was reaffirmed using specific short hairpin RNAs (shRNAs) targeting AKT1 and AKT2 specifically. AKT1 silencing enhanced insulin growth factor 1 (IGF-I) and epidermal growth factor (EGF) dependent migration, and AKT2 silencing reduced IGF-I dependent proliferation and survival [85]. Together, these results suggest that the differential expression of the AKT isoforms are linked with different stages of cancer progression and metastasis and that due to redundancy and compensatory mechanisms the distinct inter-regulatory roles of AKT isoforms in cancer need to be more clearly defined, especially with the advent of AKT targeted compounds. Establishing their pre-clinical efficacy will be crucial to achieving patient benefit and increased survival.

1.6 FORKHEAD BOX TRANSCRIPTION FACTORS

Forkhead box proteins are a large family of transcriptional regulators which share an evolutionarily conserved DNA-binding domain (DBD) named the winged helix domain. It consists of three tightly packed α-helical domains at the N-terminal region and three β-sheets and two large loop regions located at the C-terminal end, forming a structure which is similar in appearance to the wings of a butterfly [124, 125].

Since 1989 more than 150 forkhead transcription factors have been identified, including more than 41 in humans [126]. Due to the rapid discovery of forkhead family members Kaestner *et al* outlined a unified nomenclature to minimise confusion, proteins became known as FOXs, named after their 'forkhead box'. Forkhead proteins in chordates were divided into 15 classes or subgroubs, which has since been expanded to 19, each class termed A-S, based on their sequence similarity and structure, not their function [127]. Outside of the DBD, forkhead proteins families vary significantly, in part, leading to their differential roles and regulation in many cellular processes including, metabolism, proliferation, development, migration, invasion, apoptosis and longevity [126, 128]. Of the subgroups, the role and regulation of some are more well defined than others and are established in distinct cellular processes, for example cell cycle proliferation, angiogenesis, cell cycle arrest and apoptosis.

1.6.1 Forkhead subgroup C (FOXC)

FOXC2 (previously known as Mesenchyme Forkhead 1- MFH1) is known to be a critical regulator of angiogenesis, controlling vascular development, remodelling and migration [129]. FOXC2 is also known to be important in lymphatic development, loss of functional FOXC2 is known to be associated with the inheritable disease Lymphedema-distichiasis, which causes swelling in the lower limbs and irregular eyelash growth [130]. As a transcription factor FOXC2 binds to forkhead-response elements within the promoters of its target genes driving their expression. Established targets of FOXC2 are the chemokine receptor 4 (CXCR4), integrin β3 and the vascular endothelial growth factor receptor 2 (VEGFR2), all which promote endothelial cell migration and angiogenesis [131].

The overexpression of FOXC2 is observed in breast cancer – 85% of invasive breast carcinomas - and has been shown to be a key mediator of epithelial-mesenchymal transition (EMT) [132], which is an essential step in metastasis, allowing cells from the primary tumour to spread and establish themselves in

secondary sites throughout the body [133]. Moreover, it has been shown that inhibiting FOXC2 using small hairpin RNA reduced their metastatic potential, highlighting FOXC2 – and its downstream targets - as a viable target for anti-metastatic targeted therapy [132].

1.6.2 Forkhead subgroup M (FOXM)

FOXM1 is a crucial regulator of cell cycle progression through the transcription of the pro-proliferation genes, cyclin B [134], polo-like kinase-1 (PLK1) [135] and cell division cycle protein 25b (CDC25b) [136], and suppression of CDKIs p21 and p27 through its interaction, promoting their cellular degradation [137, 138].

FOXM1 is only expressed in cells progressing through the cell cycle; it is almost undetectable in quiescent cells. Its expression increases through S phase and continues to increase into G2 and mitosis. FOXM1 is also regulated by its phosphorylation status which positively affects its activity. FOXM1 is known to be phosphorylated by the Raf/MEK/ERK signalling cascade [139] and the CDK2-Cyclin E complex [136], its phosphorylation peaking in G2, reaffirming the role of FOXM1 at modulating the progression of cells into mitosis. Moreover, in development FOXM1-null embryos fail to survive *in utero* past E18.5 due to inability to enter into mitosis [140].

The overexpression of FOXM1 has been identified in many different cancer types; brain, breast, lung, liver and prostate [141] and has been shown to promote tumourigenesis, angiogenesis and metastasis [126]. Recently it has been shown that antibiotic compounds siomycin A and thiostrepton inhibit FOXM1 activity and induces cell death in breast cancer cell lines but not untransformed breast epithelial cells [141].

1.6.3 Forkhead subgroup O (FOXO)

Mammalian FOXO proteins are orthologues of the transcription factor DAF-16 (abnormal DAuer Formation 16) identified in the nematode *Caenorhabditis elegans* (*C.Elegans*), which was found to be an integral component of metabolic insulin signalling and longevity [142]. The FOXO class of proteins consists of four members, FOXO1 (FKHR), FOXO3a (FKHRL1), FOXO4 (AFX) and FOXO6, which are mostly regulated by the PI3K signalling cascade effector protein AKT [84]. FOXO1, -3 and -4 are ubiquitiously expressed although can differ between different tissue/organ types. FOXO1 is expressed more highly in adipose tissues, FOXO3a is expressed highly in the liver, and FOXO4 is highly expressed in skeletal muscle. FOXO6 is predominantly found in the brain [76, 124].

FOXO proteins consist primarily of four domains, the DNA binding domain (DBD) that defines all forkhead proteins, a nuclear localisation signal (NLS) downstream of the DBD, a nuclear export sequence (NES) and a C-terminal transactivation domain (Figure 1.3). FOXO1 and FOXO3 are similar in length (655 and 673 amino acids, respectively) while FOXO4 and FOXO6 are moderately shorter (501 and 559 amino acids, respectively) [143] As well as sequence homology within the DBD, FOXO family members also retain sequence homology that is integral to regulating their subcellular location and consequently, their activity. This will be more clearly defined later.

As transcription factors, FOXO proteins elicit their effect on the cellular phenotype by activating the transcription of their target genes, which differentially effect cell fate in a downstream target-specific manner. FOXO proteins predominantly bind to the consensus motif (also known as forkhead response element) 5'-TTGTTTAC-3' via their DBD (more specifically, through the third α-helix) within the promoter sequences of their downstream targets [142, 144]. Once bound the c-terminal transactivation domain initiates gene transcription. FOXO proteins have also been shown to be keen transcriptional repressors, its

activation halting the expression of the cell cycle progression proteins Cyclin D1 and -2 [145]. Due to the fact that FOXO proteins have a role in a plethora of physiological activities, it is best to outline these subsets of genes within the context of each cellular process.

1.6.3.1 FOXO in oxidative stress

FOXO proteins have also been found to be key sensors of oxidative stress through the regulation of genes involved with detoxification and DNA repair. Reactive oxidative species (ROS) – which includes oxygen free radicals, nitric oxide and hydrogen peroxide - are produced as byproducts from exogenous sources such as, ultra-violet (UV) and ionising radiation [146] or metabolic processes that take place within the mitochondrion [147]. ROS has been shown to be important in some cell signalling pathways and cell cycle progression, but excessive ROS accumulation can be detrimental to the cell and can lead to the induction of apoptosis and cell death. If these cellular processes are perturbed disproportionate ROS accumulation can damage proteins, lipids and DNA leading to cellular transformation, tumourigenesis and cancer [148].

In 1999 *Honda & Honda* showed in *C.Elegans* that the DAF-2/DAF-16 signalling pathway (homologues of mammalian IGFR/FOXO) regulated the expression of SOD-3 – which facilitates the conversion of superoxide into oxygen and hydrogen peroxide [149] - playing a central role in ROS detoxification, having a drastic effect on lifespan [150]. Furthermore, it was shown that this regulation was direct due to the discovery of DAF-16 binding sites within the sod-3 promoter [144]. It was later shown that this pathway was conserved in mammals, FOXO3a being able to upregulate the mRNA and protein expression of manganese superoxide dismutase (MnSOD – homologue of SOD-3) in response to ROS accumulation, promoting cell survival, and that crucially, this mechanism was observed in cancer cells as a mechanism of evading apoptosis [151, 152]. Subsequently, FOXO3a has been found to be upregulated in response to ROS

accumulation and that this coincided with an increase in the expression of its downstream transcriptional targets [153]. Also, it was shown that FOXO4 could also regulate mnSOD expression in response to ROS accumulation and TNFα treatment, reaffirming the role of FOXO family members in regulating cellular stress [146].

FOXO was also found to regulate catalase in response to oxidative stress [154]. Catalase, as the name suggests, catalyses the reaction of breaking down hydrogen peroxide into oxygen and water [155] and was originally identified in *C.Elegans* at being important at regulating longevity via the DAF-16 pathway [154]. It was subsequently shown that hydrogen peroxide treatment in mammalian cells resulted in the activation of FOXO3a and an increase in transcriptional activity. Moreover, FOXO3a bound to the catalase promoter, activating its transcription, but this activation was lost when a FOXO3a inactive mutant was overexpressed [156].

Although mnSOD and catalase are located in different cellular compartments, the mitochondria and peroxisomes respectively, suggesting they have distinct roles in regulating cellular stress [157], they are key transcriptional targets of FOXO proteins and highlight the concerted role of FOXOs in regulating ROS scavengers, driving detoxification and promoting survival. Recently, *Tothova et al* have shown rather excellently that FOXO1,-3 and -4 negative hematopoietic stem cells (HSCs) have marked increases in ROS and a decrease in expression of a subset of genes that regulate ROS accumulation, resulting in dysregulation of the cell cycle and induction of apoptosis, having drastic consequences on self-renewal and differentiation of the HSC pool. Moreover, this phenotype was fully reversible by the treatment of an antioxidant compound, showing that loss of FOXO proteins effect the cellular phenotype predominantly by regulating ROS [158]. Together, these data support the role of FOXO proteins in regulating ROS accumulation and their attempt to maintain normal cell cycle progression through

the transcription of genes important in regulating ROS, dictating the cellular consequences and influencing cell survival.

1.6.3.2 FOXO in DNA repair

FOXO proteins have been shown to protect cells from ROS accumulation by upregulating genes involved with detoxification, but FOXO family members also upregulate the expression of genes known to be important in DNA-damage repair, such as the growth arrest and DNA damage 45 (Gadd45) protein.

Gadd45 acts as a sensor to cellular stress and is known to interact with the cdc25/Cyclin B complex and the CDKI p21, both known to be important in G2-M cell cycle transition. Their interaction with Gadd45 promotes the activation of the G2-M cellular checkpoint, allowing DNA repair mechanisms to be initiated [159]. Gadd45 is also known to interact with the proliferating cell nuclear antigen (PCNA) protein, which is important in DNA-damage repair, maintaining genetic integrity, promoting cell survival [160].

FOXO3a was shown to be activated in response to UV radiation and delay progression of cells through G2-M, increasing the mRNA and protein expression of Gadd45, allowing DNA-repair mechanism to be undertaken [161]. A subsequent study reiterated the importance of FOXO3a in regulating Gadd45 expression and showed that FOXO4 could also promote Gadd45 expression and initiate DNA repair mechanisms [162]. The ability of FOXO proteins to induce DNA-damage repair highlights their role at maintaining genomic integrity, reducing the accumulation of possible oncogenic mutations that could drastically alter cell fate.

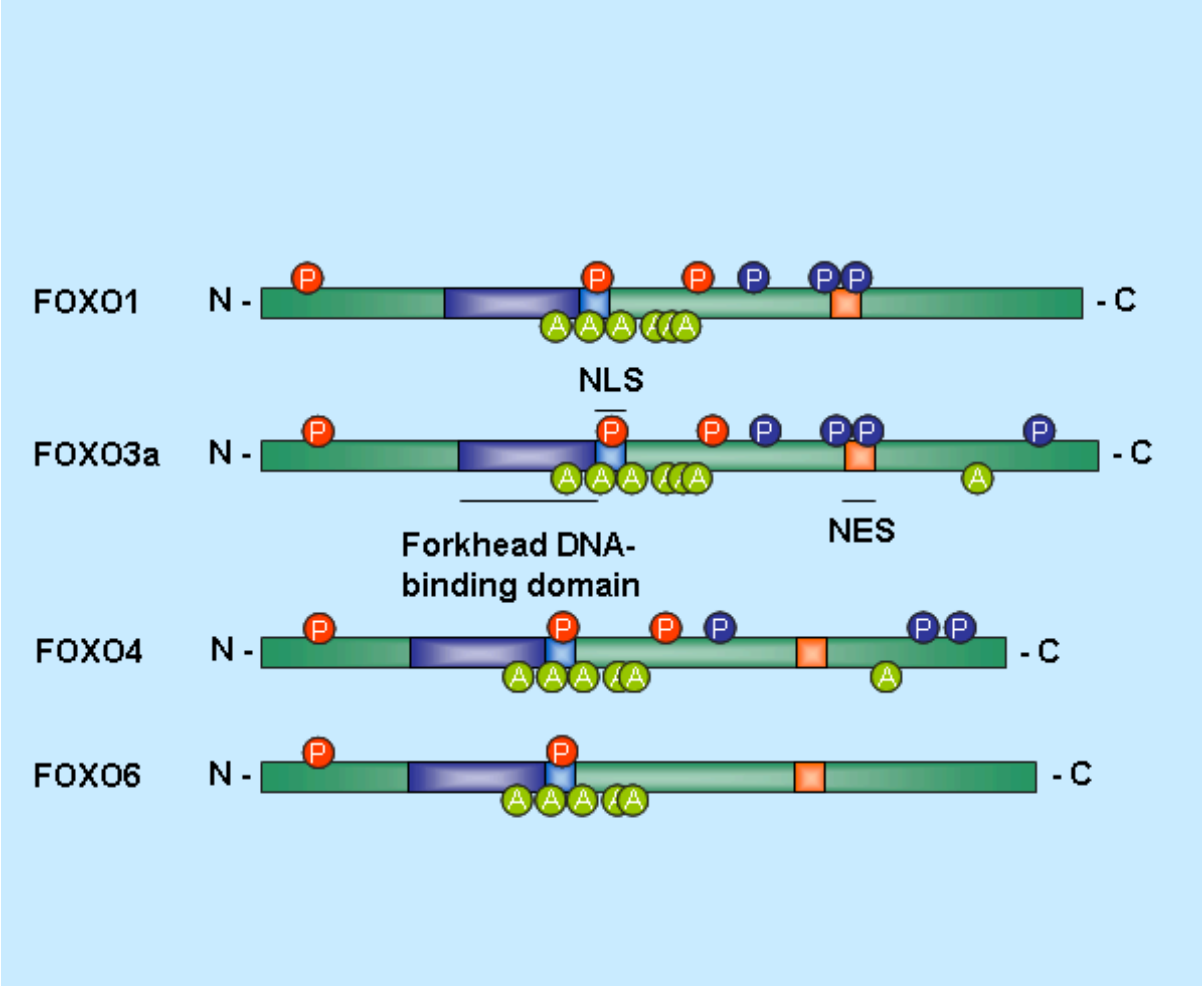


Figure 1.3: Structure and post-translational modification sites of FOXO protein. The basic structure and location of post-translational sites of FOXO family members. The position of the forkhead DNA-binding domain (DBD - purple), nuclear localisation sequence (NLS - blue), nuclear export sequence (NES - orange) are represented diagrammatically. Key post-translational sites such as phosphorylation (P)(red – AKT sites, blue – other) and acetylation (A - green) are also designated. Adapted from Greer and Brunet 2005 [143].

1.6.3.3 FOXO in the cell cycle

FOXO proteins are key regulators of cell cycle inhibition, predominantly responsible for controlling progression through transition of the G1-S phases – but also G2-M - of the cell cycle. FOXO has been shown to regulate both p21 and p27 CDKIs from the Cip/Kip family, as well as INK4 family members [163].

FOXO family members FOXO1, -3 and -4 (predominantly FOXO3a) were show to bind to the p21 promotor in a Smad-dependent manner, promoting its transcrption in response to the cytostatic transforming growth factor β (TGF-β) cytokine signal. Furthermore, when FOXO activity was inhibited using targeted short interfering RNA or the overexpression of a functionally inactive FOXO mutant, p21 expression was lost following TGF-β treatment while other downstream targets remained unchanged [164].

The overexpression of FOXO3a and FOXO4 were shown to induce the expression of p27, disrupting the cyclin E-cdk2 complex causing a cell cycle arrest at the G1 cellular check point. Furthermore, this arresting potential was significantly decreased in p27-/- cells, compared to WT cells, due to the fact that loss of cell cycle control is a common feature of cancer development, the hyperactivation of the PI3K/AKT axis was linked to inhibition of FOXO family members and consequently uncontrolled cell growth [165]. The overexpression of a constituitively active FOXO4 was also able to positively regulate the expression of p27 by promoting its stabilization [166]. Moreover, the pro-oncogene c-myelocytomaosis (c-MYC) was shown to repress FOXO3a activity leading to loss of p27 expression, an event which is observed in B-cell lymphomagenesis [167]. The regulation of p27 by FOXO3a was also shown to be important in the induction of apoptosis in both B and T cells through loss of Interleukin 3 (IL-3) and Interleukin 2 (IL-2) mediated survival, respectively [168, 169].

FOXO proteins were also shown to regulate cell cycle inhibition by driving the transcription of the Rb family member, p130. FOXO3a and FOXO4 were shown to bind to the p130 promoter and increase its cellular expression, as well as influence its phosphorylation status and interaction with E2F transcription factors. FOXOs activation of p130 promoted the exit from the cell cycle and entering into a reversible quiescent state and is thought to complement p27 upregulation (p27 inducing a cell cycle arrest and p130 promoting exit from the cell cycle) [170].

As well as upregulating proteins involved with cell cycle inhibition FOXO proteins are also known to repress proteins involved with promoting cell cycle progression. FOXO proteins are known to decrease CDK/Cyclin D complex activity by upregulating p27 expression, but it has also been shown that they are able to decrease the cellular expression of Cyclin D1 by inhibiting promoter activity in a p27 independent manner. Overexpression of Cyclin D1 was also sufficient to overcome the FOXO induced cell cycle arrest reasserting the transcriptional control that FOXO proteins have over Cyclin D1 expression [171].

1.6.3.4 FOXO in cell death and apoptosis

1.6.3.4.1 Extrinsic apoptosis

FOXO family members are also known to regulate a distinct subset of genes that facilitate the induction of programmed cell death, apoptosis. FOXO3a is known to regulate the expression of components of the extrinsic apoptotic pathway. The activating ligand, tumour necrosis factor (TNF) cytokine family member, FasL (Fas ligand) was one of the first identified targets of the AKt/FOXO signalling pathway [84]. FasL binds to the Fas receptor, activating the caspase-8 dependent proapoptotic pathway [172]. FOXO3a was also found to positively drive the direct transcription of TRAIL, also a member of the TNF super family. Once cleaved, TRAIL forms homo-trimeric structures which activate apoptosis

through binding to the death receptor 5 (DR5) and DR4, activating a caspase-8-dependent proapoptotic mechanism [173, 174].

1.6.3.4.2 Intrinsic apoptosis

In addition to regulating proapoptotic extracellular receptors, FOXO proteins also regulate the expression of genes involved with destabilizing the mitochondrial membrane, namely the Bcl-2 family members - BH3-only proteins - Puma [175] and Bim [176], promoting the intrinsic activation of apoptosis.

The proapoptotic protein Bim was initially discovered to be a downstream target of FOXO3a in the mouse pro-B cell line Ba/F3, after FOXO3a activation due to cytokine deprivation (IL-3). Moreover, Bim -/- leukocytes were shown to be more resistant to cytokine withdrawal and apoptosis, highlighting the importance of Bim at initiating apoptosis [176, 177]. IL-2 deprivation in the murine T cell line CTLL-2 also led to the induction of Bim and p27. Interestingly, the induction of Bim was preceded by an increase in p27, which coincided with the induction of a cell cycle arrest, then apoptosis, suggesting that the differing downstream targets of FOXO3a cooperated to elicit their effect on the cellular phenotype [169]. Subsequent studies also showed FOXO3a-dependent induction of Bim in neuronal cell lines [178], endothelial progenitor cells (EPC) [179] and osteosacroma cells [180]. The latter study being of particular interest due to the fact that it showed the UV-induced apoptosis was independent of functional p53, highlighting FOXO proteins as keen inducers of apoptosis in response to DNA damage [180].

Further studies into the regulation of Bim by FOXO3a showed that it was possible for the pro-apoptotic function of FOXO proteins to be exploited therapeutically. Chronic myelogenous leukemia (CML) cell lines – which are driven by the Bcr-Abl chimeric oncogene [181] – and cell lines over expressing Bcr-Abl, upon treatment of the Bcr-Abl inhibitor STI571, caused the activation of

FOXO3a and increased FOXO3a-dependent Bim expression resulting in the induction of apoptosis. Moreover, the induction of Bim expression by STI571 treatment was lost when the cellular pool of FOXO3a was reduced using siRNA, the cells maintaining a cell cycle arrest rather than undergoing apoptosis. Furthermore, Bim silencing with siRNA also abrogated the induction of apoptosis in STI571 treated cells [182]. Subsequent studies in the breast cancer cell line MCF-7 have shown that FOXO3a also drives the expression of Bim in response to the chemotherapeutic DNA-damaging agent paclitaxel, inducing apoptosis [183].

The BH3-only protein PUMA has recently be also shown to be regulated by FOXO3a in p53-dependent and independent mechanisms. MEFs which were negative for PUMA and BAX were used to show that the proapoptotic potency of FOXO3a was dependent on their cellular expression [184]. Moreover, it was shown that FOXO3a induced apoptosis in T-cells following IL-2 withdrawal through the transcriptional activation of Bim and PUMA. Although it was hypothesised that loss of Bim had a more drastic effect on cell fate due to both transcriptional and post-translational regulation, whereas PUMA was only regulated transcriptionally [175].

Bim and PUMA induce apoptosis by binding to Bcl-2, facilitating the release of Bax, which reduces the integrity of the mitochondrial outer membrane, facilitating the release of factors that activate the caspase cascade, inducing apoptosis [185]. These findings highlight the importance of FOXO3a-mediated induction of apoptosis and the central role FOXOs pro-apoptotic targets play in dictating cell survival.

1.6.4 Regulation of FOXO proteins

As discussed previously the FOXO transcription factors have a diverse range of transcriptional targets involved in different cellular processes such as, cell cycle

regulation (e.g., p21, *p27*, *p130(RB2)*, *cyclin D*), apoptosis (e.g., *Bim, Fas-ligand* and *TRAIL*) and oxidative stress (e.g., *mnSOD and Catalase*) [84, 151, 156, 165, 170, 171, 173, 176]. To achieve successful regulation of this repertoire of target genes, the activity of FOXO transcription factors is regulated by a range of posttranslational processes including phosphorylation, ubiquitination, acetylation, which allows the activation of specific subsets of genes that influence cell fate [126].

1.6.4.1 Phosphorylation

Phosphorylation is the chemical modification of serine, threonine and tyrosine (although the latter is the least common) residues by the addition of a phosphate group. The post translational status of proteins is a dynamic process, the modification of residues is regulated by kinases and phosphatases which phosphorylate and dephosphorylate protein substrates, respectively. The phosphorylation status of proteins is predominantly linked heavily with their activity, phosphorylation promoting its activity or reducing it, in a substrate specific manner. FOXO3a is known to be phosphorylated by the oncogenic kinases AKT, IKK and ERK, all three commonly activated in cancer [186].

All FOXO family members contain multiple AKT consensus sequences meaning they are prime cellular targets for phosphorylation by AKT. Moreover, these phosphorylation sites are conserved throughout evolution, from *C.Elegans* to mammals. AKT and other closely related kinases, such as the serum and glucocorticoid inducible kinase (SGK) phosphorylate FOXO3a on Threonine 32, Serine 253 and Serine 315. The phosphorylation of FOXO3a on these residues results in an increased interaction with the chaperone protein 14-3-3, and the rapid export of FOXO3a from the nucleus into the cytoplasm, inhibiting FOXO3a activity [143]. The 14-3-3 family of proteins are actively involved in nuclear/cytoplasmic shuttling processes, transporting proteins from one compartmentalised space to another, they are predominately found within the

cytoplasmic space. 14-3-3 proteins form homo and heterodimeric U-shaped structures and bind to their targets via the central channel. Phosphorylation within the NES sequence of FOXO promotes the interaction with the 14-3-3 complex; this binding decreases the binding affinity of FOXO proteins to DNA and possibly obscures the NLS sequence [187-189]. The nuclear export of the FOXO:14-3-3 complex is facilitated by chromosomal region maintenance 1 (CRM1) and Ran GTPase [152].

The loss or withdrawal of growth stimuli results in the inactivation of the PI3K signalling cascade. Inactive AKT and other AGC family members are unable to phosphorylate FOXO3a and prolong cytoplasmic retention. Thus, unphosphorylated FOXO3a translocates to the nucleus where it activates its downstream targets.

Hu *et al* elucidated the complex nature of the AKT/FOXO axis by showing in breast tumour samples that indeed AKT phosphorylation did correlate with the cytoplamic retention of FOXO3a, but there were also a significant proportion of samples that did not, suggesting the subcellular location of FOXO3a was regulated by other pathways. Investigating this further they showed that FOXO3a cytoplasmic retention correlated with the upregulation of the inflammatory pathway protein IKKβ and showed that it directly interacted with FOXO3a, phosphorylating it on serine 644 promoting its exclusion from the nucleus and degradation by the proteasome. Moreover, this regulation was independent of AKT, suggesting multiple mechanisms such as increased IKK signalling – which also occurs in cancer – is increased to inhibit the tumour suppressor function of FOXO family members [190-192].

The downstream effector of the RAS-MEK pathway ERK, has also been shown to regulate the phosphorylation status of FOXO3a. ERK phosphorylation on Serine 294, Serine 344 and Serine 425 promotes the dissemination of FOXO3a

throughout the cytoplasm where the likelihood of interaction with mdm2 is increased, reducing the cellular expression of FOXO3a [193].

1.6.4.2 Ubiquitination

When located in the cytoplasm, FOXO3a is exposed to mechanisms that regulate the cellular of accumulation of proteins, one such way is ubiquitin-dependent proteasomal degradation.

There are two types of ubiquitination, monoubiquitination, which is reversible and polyubiquitination which is not. Polyubiquitation results in degradation via the proteasome, a process known as the ubiquitin-proteasome system (UPS) [194]. The UPS is essentially a two step process, firstly, the covalent attachment of ubiquitin molecules to a particular cellular substrate – this is facilitated by a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin ligase (E3) - and then the degradation of this protein via the 26S proteasome complex [195]. The 26S proteasome complex can be located in either the nucleus or cytoplasm (proximal to centosomes, endoplasmic recticulum and cytoskeletal structures) and has been shown to be active in both compartments [196]. Entry to the nucleus can be obtained via nuclear pores or during the reassembly of the nuclear envelop after cells have undergone mitosis [197].

The phosphorylation of FOXO1 by AKT results in its nuclear exclusion but also promotes the interaction with the F-box protein Skp2, which as its predominant E3 ubiquitin ligase, facilitates its polyubiquitination. Although to date, this regulation is not believed to be shared by the other family members [198].

Although FOXO3a was known to be regulated by the proteasome [199], it was not until Yang *et al* displayed definitively that FOXO3a was regulated by the E3 ubiquitin ligase mdm2 - that interestingly is also, in part, responsible for

regulating the cellular accumulation of the tumour suppressor p53 [28] - that the process became more apparent. ERK phosphorylation on Serine 294, Serine 388 and Serine 425 promotes the localisation of FOXO3a to the cytoplasm where the interaction with mdm2 is increased. Polyubiquitination by mdm2 results in the degradation by the proteasomal pathway [193].

Van der Horst *et al* showed rather eloquently that FOXO4 – and possibly other family members – is monoubiquitinated in response to oxidative stress, resulting in its translocation to the nucleus and an increase in transcriptional activity and that this was negatively regulated but the herpesvirus-associated ubiquitin-specific protease (HAUSP) [200]. HAUSP had previously been identified as a regulator of p53 ubiquitination, competing with mdm2 at modulating its post-translational status, again reiterating the similarities of FOXO regulation with p53 [201, 202]

1.6.4.3 Cytosolic FOXO

New research is bringing to light the possible transcriptionally-independent role for FOXO1 taking place within the cytoplasm. Zhao *et al* have shown that acetylated cytoplasmic FOXO1 activates the autophagy regulating protein 7 (Atg7), promoting the induction of autophagy, having a dramatic effect on cell fate independent of its transcriptional potential [203]. This study highlights the need for more research into the many ways in which FOXO family members can dictate cell fate, and how these mechanisms could be important in different disease states, such as cancer [204].

1.7 THE DYNAMIC REGULATION OF THE ACETYLATION STATUS OF PROTEINS

Acetylation is the chemical modification of the ε-amino group of lysine residues where an acetyl group from the acetyl co-enzyme is transferred to the lysine

residue on the target protein [205]. Acetylation can occur on a range of proteins with diverse cellular functions, most notably histones, due to the fact that they were the first cellular components found to be acetylated in the late 1960s [206]. Because of this fact, proteins that control the acetylation status of histone and non-histone proteins are called histone deacetylases (HDACs) and histone acetyltransferases (HATs). The acetylation of histone and non-histone proteins is controlled by the dynamic interaction of HDACs and HATs in a substrate specific manner.

Highly conserved throughout evolution, histone proteins package and arrange DNA into nucleosomes, giving them protection, structure and order. Nucleosomes consist of 146 base pairs of genomic DNA wrapped around a histone octamer (consisiting of two pairs of H2A, H2B, H3 and H4). The amino-terminal and carboxyl-terminal ends of histones project out of the main 'bead-like' structure of the nucleosome earning them the name 'histone tails' [207].

Nucleosomes coupled with DNA are the main constituents of chromatin, which make up chromosomes. The modification of the acetylation status of these histones can alter chromatin structure which in turn leads to alterations in gene expression [208]. Acetylation neutralizes the charge of the histone tails, resulting in the decreased ability of the histone to interact with DNA, meaning the tight DNA-nucleosome structure is relaxed forming euchromatin, allowing the access of transcription factors and other associated proteins that can initiate gene transcription. When histones are not acetylated their affinity to interact with DNA is not reduced meaning they can form tightly packed heterochomatin, which limits the ability of factors to bind and interact with the DNA, leading to silencing of genes [209, 210].

1.7.1 Histone Acetyltransferases (HATs)

There are several different HAT protein families, based on conserved structures within each of the members. The three most established families are Gen5-related N-acetyl transferase (GNAT), p300/CBP (CREB binding protein) and MYST, which is named after founding members monocytic leukaemia zinc finger protein (MOZ), YBF2/SAS3, SAS2 and TAT-interactive protein-60 (TIP60) [209, 211]

HATs are known to regulate the acetylation status of both histone and non-histone proteins, for example, p300/CBP regulates the acetylation status of p53 and FOXO family members [212], influencing their cellular accumulation and activity.

1.7.2 Histone Deacetylase (HDAC) class I and II

There are four classes of HDACs, class I, II and IV are zinc-dependent and known as the 'classical' family. Class III being the relatively new family of HDACs, which are termed Sirtuins and are NAD+ (nicotinamide adenine dinucleotide-positive) dependent [206].

Class I members are evolutionarily homologous to the yeast protein Rpd3 and comprises of HDAC1, -2, -3 and -8, all containing a high degree of sequence homology to Rpd3 (HDAC1 and –2 being the most closely related and structurally similar). Class I HDACs are ubiquitously expressed in all cells [213] and are predominantly found in the nucleus [214]. Class I HDACs predominantly elicit their effect on their substrates and the cellular phenotype through regulating gene transcription as members of large mutliprotein complexes, for example, the Sin3 complex can act together to facilitate the function of the individual components that on their own cannot interact with DNA and inhibit gene transcription. Sin3 does not contain a DNA interacting domain, requiring other

proteins to perform this function [215]. One protein that can fulfil this role is the transcription factor mitotic arrest deficient-like 1 (Mad1), which was the first transcription factor found to interact with Sin3. Sin3 binds to Mad1 using its paired amphipathic alpha-helic domain (PAH) two (PAH2) [216]. HDAC1 and -2 have been shown to directly interact with Sin3 through a HDAC interacting domain (HID) and one is required to repress transcription in a Mad1-dependent manner. Although HDAC1 and -2 cannot interact with Mad1 directly, requiring Sin3 to facilitate complex formation [217]. The aggregation of proteins into complexes also aids defining substrate specificity, for example, the inhibitor of growth protein 2 (ING2) binds to the mSin3a-HDAC1 complex in response to DNA damage and interacts with a discreet subset of genes involved with promoting proliferation. The binding of mSin3a-HDAC1 represses gene transcription halting cell proliferation allowing the cells to mediate DNA repair mechanisms [211, 218].

HDAC3 is a major component of the nuclear receptor corepressor (NCoR) and the silencing mediator for retinoid and thyroid hormone receptor (SMRT) transcriptional repressor complexes [219].

Class II HDACs are evolutionarily derived from the yeast protein, Hda1 and are subdivided into two subclasses, IIa and IIb. Class IIa containing HDAC4, -5, -7 and -9, class IIb containing HDAC6 and -10 [206, 220]. Although derived from Hda1, class II HDACs only share homology within the coding of the catalytic domains but vary significantly in other structures and their overall functions [219] (Not unlike the forkhead family of proteins). Class II HDACs are predominantly located in the cytoplasm but are thought to translocate to the nucleus when required for transcriptional repression [220].

The fourth class has only one member, HDAC11 and is distinct from classes I and II due to the fact that it contains structural features similar to both Rpd3 and Hda1 [221].

1.7.3 Histone Deacetylase (HDAC) class III, also termed Sirtuins

Sirtuins are the most recently discovered HDAC class and are different to other families due to the fact they require NAD+ and are resistant to compounds that target and inhibit class I and II HDACs. Moreover, HDACs are known not to solely regulate the acetylation status of histones but also regulate the acetylation status of non-histone proteins. Sirtuins are a perfect example of this, influencing the posttranslational status and activity of proteins key to maintaining cell viability, such as p53 [222], FOXO family members [223] and the DNA-repair protein Ku70 [224].

There are seven human orthologues of silent information regulator 2 (sir2), termed SIRT1-7, which possess primarily histone deacetylase (SIRT1, -2, -3, -5) or mono-ribosyltransferase activity (SIRT4 and SIRT6) [225, 226], the enzymatic ability of SIRT7 is yet to be fully determined (Figure 1.4) .

Sirtuins are ubiquitously expressed although they occupy distinct subcellular localisations and consequently interact with different intracellular targets – suggesting that their main role is mediating the acetylation status of non-histone proteins, otherwise they would all be located in the nucleus - for example, SIRT1 is primarily located in the nucleus – but can also be found in the cytoplasm - whereas SIRT2 is predominantly found in the cytoplasm [227, 228]. SIRT3, -4 and -5 are located in the mitochondrion [229], although recently SIRT3 has also be found in the nucleus [230]. SIRT6 and -7 are also located in the nucleus, although they are predominantly found in distinct regions, SIRT6 is associated with heterochromatin, whereas SIRT7 is found in the nucleoli [231].

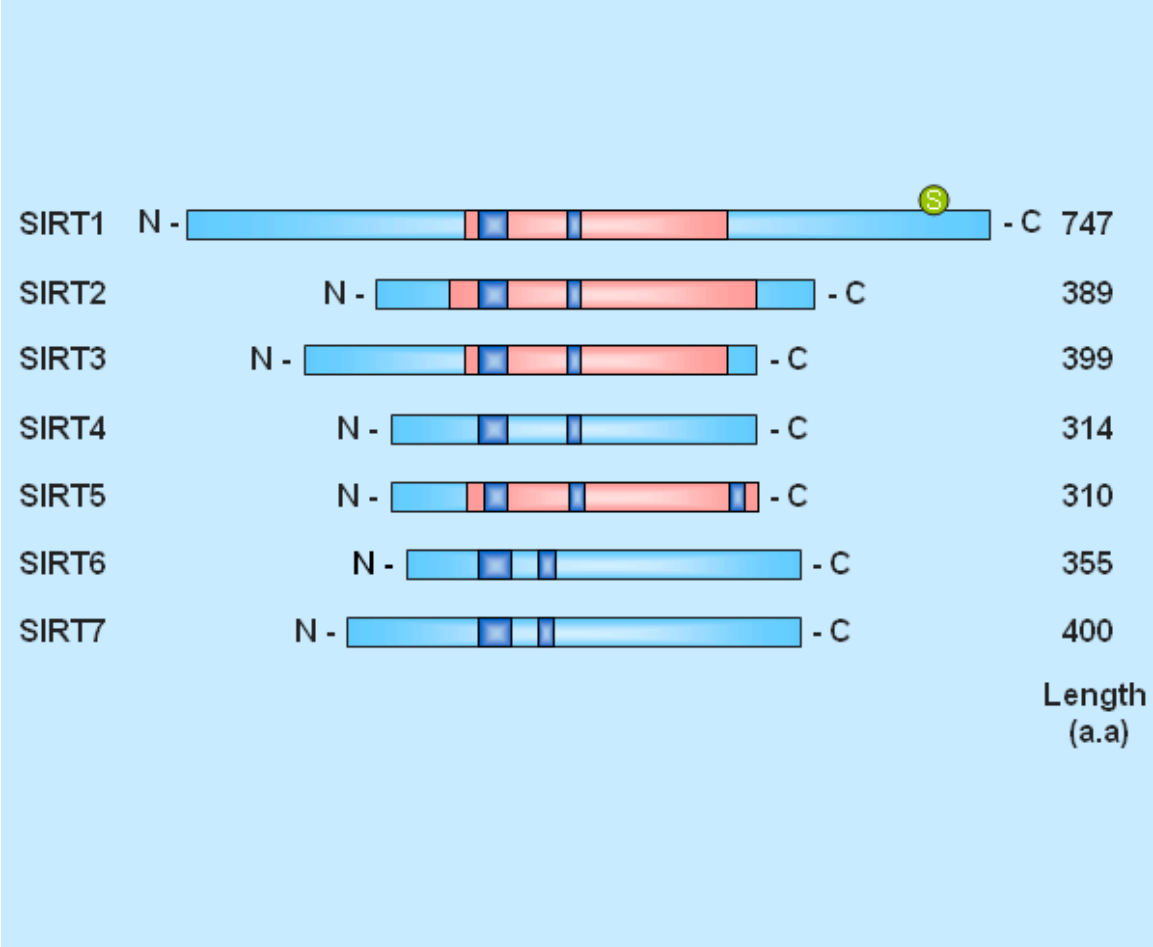


Figure 1.4: The structure of mammalian sirtuins. The basic structure of Sirtuin proteins, displaying the NAD+ binding site (dark blue), HDAC catalytic domain (pink) and sumoylation site (green). Only SIRT1, -2, -3 and -5 are known to possess histone deacetylase activity. Adapted from Yamamoto *et al* 2007 [232].

1.7.3.1 SIRT1

SIRT1 is the most studied sirtuin family member, it being the most homologous to Sir2, the first sirtuin to be discovered in *Saccharomyces cerevisiae* (*S.cerevisiae*). Sir2 was found to promote longevity, increasing lifespan by 30% when an extra copy was integrated into the genome. Moreover, deletion of Sir2 resulted in a drastic shortening of lifespan [233]. Further interest was sparked when Sir2.1 - sharing 31% sequence similarity in the 'conserved core' domain [234] - was found to increase lifespan in *C.Elegans* by 50% when its expression was increased due to gene duplication. Moreover, Sir2.1 was able to promote longevity in response to caloric restriction (CR). The increased availability of NAD+ during CR facilitated an increase in Sir2.1 activity, driving survival [234]. Furthermore, transgenic mice overexpressing SIRT1 showed traits associated with caloric restriction, for example, reduced body weight, lower blood cholesterol, increased metabolic rate, lower body fat and improved physicality [235]. Mice deficient in SIRT1 were shown unable to develop these traits under conditions of CR, suggesting that SIRT1 is indispensible for regulating CR in mice [236]. In mammalian cells SIRT1 – along with SIRT2 and -3 [237] - were also found to be activated in response to CR, reducing stress induced apoptosis and promoting longevity, suggesting that sirtuins can cooperate in deciphering cell fate in response to different forms of stress [238].

SIRT1 is known to regulate the acetylation status of histones H2A, H2B, H3 and H4, although it shows preference to regulating the specific lysine residues histone 3 lysine 9 (H3K9), histone 3 lysine 14 (H3K14) and histone 4 lysine 16 (H4K16) . H4K16 is an important posttranslational modification, it has been shown to regulate chromatin structure, acetylated H4K16 disrupting the formation of compact chromatin, a trait not shared by other lysines residues on histones [239]. SIRT1 is a key modulator of H4K16, its overexpression able to decrease H4K16 in response to various cytotoxic agents [240].

SIRT1 is known to be involved in deciphering cell fate through its negative regulation of p53. Acetylation is essential for p53 activation [36] as well as potentiating its activation by increasing the cellular accumulation of p53 by inhibiting its mdm2-dependent degradation by the proteasome. The deacetylation of p53 by SIRT1 suppresses its stabilization and transcriptional activation, inhibiting p53s ability to activate targets involved with cell cycle arrest and apoptosis, such as p21 and BAX [241]. Moreover, acetylation has been shown to be crucial for the transcription-independent activation of BAX by p53, suggesting that the effect of acetylation on p53 is complex, but ultimately the effect on cell fate is the same, p53 acetylation promotes the induction of programmed cell death [242].

1.7.3.2 SIRT2

SIRT2 is a major regulator of cytoskeleton dynamics, predominantly through its deacetylation of α-tubulin and microtubule regulation influencing processes such as cell division, intracellular transport and motility [243]. Validating α-tubulin as an important downstream target of SIRT2, the knockdown of SIRT2 using siRNA in 293T cells resulted in the hyperacetylation of tubulin [243]. Although SIRT2 is predominantly cytoplasmic protein it is also known to regulate the acetylation status of H4K16 and H3K9, although this is thought to only occur during mitosis when the nuclear membrane has disassembled allowing cytoplasmic proteins to interact with chromatin. SIRT2 deactylation encourages progression through the G2-M phase of the cell cycle by promoting chromatin condensation which precedes chromatin segregation in cells undergoing mitosis [244, 245]. Supporting this function of SIRT2, H4K16 acetylation levels decrease when SIRT2 is seen to associate with chromatin in G2-M cell cycle progression. Moreover, SIRT2-/- MEFs displayed an inability to deacetylate H4K16, hyperacetylation was observed in cells undergoing mitosis, delaying the entry into s phase suggesting SIRT2 as an important regulator of cell cycle progression [244, 246].

1.7.3.3 Mitochondrial Sirtuins (SIRT3, -4 and -5)

The role of sirtuins in mitochondrial biology and the consequent influence on cell fate is particularly interesting when you consider that a significant proportion of mitochondrial proteins can be acetylated (at least 20%) [247]. Furthermore, SIRT3-/- null mice (but not SIRT4-/- or SIRT5-/-) display hyperacetylation of mitochondrial proteins which can be reduced by reinstating SIRT3 expression, suggesting that it plays a major role in regulating the posttranslational status of this compartmentalized pool of proteins.

It has been shown that SIRT3 - the most well defined mitochondrial sirtuin - is found in both the mitochondrion and the nucleus. Although recent publications have shown that full length SIRT3 can be found in the nucleus, SIRT3 is translocated to the mitochondrion where it undergoes proteolytic cleavage [248]. Furthermore, all mitochondrial sirtuins contain N-terminal sequences that promote their localization to the mitochondrion where they undergo proteolytic cleavage [249]. Moreover, full length SIRT3 has been found to lack deacetylase activity and can only possess deacetylase activity once it has been cleaved, suggesting that the translocation of SIRT3 to the nucleus must only occur under specific types of physiological stress.

SIRT3 is known to deacetylate the mitochondrial matrix protein acetylcoenzyme A synthase 2 (aceCS2) on residue K642 increasing its enzyme activity [250]. AceCS2 facilitates the formation of acetyl-CoA from acetate, CoA and ATP, which can be used to generate energy through the tricarboxylic acid (TCA) cycle [251]. SIRT3 also associates with components of Complex I of the electron transport chain, promoting its activity and the generation of ATP [252]. These two intracellular targets reiterate the intimate role of SIRT3 at regulating the cellular availability of metabolic energy. SIRT3 expression is also known to positively correlate with the expression of mnSOD [237]. Also, SIRT3 corroborates with

SIRT4 to regulate NAD+ levels in mitochondria following stress, promoting cell survival [253].

SIRT4 does not possess HDAC activity but acts as an ADP-ribosyltransferase, depositing ADP-ribose from NAD+ onto its target proteins [254]. One of known cellular targets being glutamate dehydrogenase (GDH), which converts glutamate to α-ketoglutarate and ammonia, promoting the production of ATP and secretion of insulin. SIRT4 negatively regulates the activity of GDH, and SIRT4s regulation of insulin secretion is GDH-dependent [255]. To confirm GDH as an important cellular target of SIRT4, pancreatic islets from SIRT4-/- mice displayed increased insulin secretion and significantly elevated insulin levels in the blood compared to WT mice [249].

SIRT5 does display weak HDAC activity and is able to deacetylate H4 *in vitro* [256] and to date, is known to regulate the acetylation status of two mitochondrial proteins, cytochrome c - which is integral to the induction of apoptosis – and carbamoyl phosphate synthase 1 (CPS1) [257]. The deacetylation of CPS1 increases its activity, promoting the activation of the urea cycle and reduction in accumulation of ammonia. SIRT5-/- mice displayed increased ammonia levels after prolonged fasting, suggesting that SIRT5 is a major regulator of ammonia accumulation [258].

1.7.3.4 SIRT6

SIRT6-knockout mice are susceptible to genotoxic insults, genomic instability and the development of premature aging [259]. Although SIRT6 was initially identified to contain ADP-ribosyltransferase activity [226], it has recently been shown that SIRT6 also possesses histone deacetylase activity and that this may be responsibly for its ability to regulate cell survival [260]. Supported by the fact that SIRT6 is located in the nucleus, it appears to protect cells from senescence and aging by deacetylating H3K9 at the terminal telomeric regions of chromatin,

maintaining telomere structure and reducing the development of abnormal chromosomal aberrations [261]. SIRT6 also interacts with components of the NF-κB complex, where upon binding to the promoters of its target genes modulated the acetylation of H3K9 inhibiting gene transcription. Moreover, SIRT6 null mice were found to contain increased expression of NF-κB regulated genes [258, 261].

1.7.3.5 SIRT7

SIRT7 deficient mice have been developed and shown to have a decreased lifespan due to cardiac dysfunction. Mice lacking SIRT7 have an increase in p53 activity and apoptosis in cardiomyocytes, whereas SIRT7 overexpression increase stress resistance and reduces the induction of apoptosis [262]. SIRT7 also is known to regulate ribosomal RNA transcription by directly binding to RNA polymerase I (Pol I), increasing its activity [263], which is in contrast to SIRT1 which competes with the HAT PCAF to negatively regulates the acetylation status – and activity - of the TATA box-binding protein associated factor 68 (TAF68) which promotes Pol I transcription [264].

1.8 FOXO AND ACETYLATION

The origins of interest in the regulation of FOXO by sirtuins came from the studies in *C.Elegans*, which showed that Sir2.1 was able to promote longevity and survival, and appeared to corroborate with the DAF-16 signalling pathway to achieve this [234]. In 2004 Brunet *et al* and Motta *et al* both showed that SIRT1 directly interacted with FOXO3a. Motta *et al* elucidated the mechanism by which SIRT1 counteracted the HAT p300 in dynamically regulating the acetylation status of FOXO3a (and FOXO4). The deacetylation of FOXO3a by SIRT1 inhibiting its ability to activate the downstream targets p27 and Bim, inhibiting the induction of apoptosis [265]. Brunet *et al* showed rather eloquently that SIRT1 regulated the acetylation of FOXO3a in response to hydrogen peroxide induced oxidative stress, promoting the transcription of genes favouring a cell cycle arrest

and DNA damage repair phenotype, such as p27 and Gadd45 [223]. Although the papers have differing opinions on the regulation of p27 by acetylated FOXO3a, they both concur with the fact that SIRT1s deacetylation of FOXO3a promoted survival and suppressed a FOXO3a-dependent induction of apoptosis. Subsequent publications later that year also showed that SIRT1 deacetylated FOXO4, competing with CBP to control its acetylation status. SIRT1-mediated deacetylation increased the transcriptional activity of FOXO4 [266]. Moreover, mouse Sir2 was found to compete with CBP to control the acetylation of FOXO1, deacetylating FOXO1 on lysines 242, 245 and 262, potentiating its transcriptional activity, promoting the upregulation of p27 and mnSOD [267]. Furthermore, Matsuzaki *et al* showed that the acetylation of these residues decreased DNA-binding affinity of FOXO1, increasing its phosphorylation at a proximal residue - serine 253 – which is controlled by AKT, promoting its nuclear exclusion and inhibition by cytoplasmic retention [268].

Subsequent publications have also shown that SIRT2 and SIRT3 interact directly with FOXO3a regulating its acetylation status and ability to regulate cellular ROS accumulation [269, 270]. A particularly interesting study showed that SIRT3-/- mice displayed cardiac dysfunction and that overexpression of SIRT3 inhibited this in a FOXO3a-dependent manner via the upregulation of genes involved with decreasing ROS accumulation and reducing cellular stress [271]. Although, these studies do require further examination to determine the exact consequence on cell fate of multiple SIRTs interacting with FOXO3a. The regulation of the acetylation status of p53 is also regulated by multiple sirtuins, SIRT1, -2 and -3, suggesting that there is redundancy between family members and that sirtuins play a central role at regulating cell fate [272-274].

When considering the acetylation of FOXO3a most publications centre on the regulation of lysines within the DBD. The general consensus is that the deacetylation of FOXO3a promotes the activation of a subset of genes predominantly involved with survival, promoting the transcription of genes

involved with responding to stress and inhibiting the induction of apoptosis [143]. This suggests that the acetylation of FOXO3a could promote the transcription of genes involved with initiating apoptosis. In support of this hypothesis, Yang *et al* 2009 recently showed that the class I and II HDAC inhibitor depsipeptide caused the acetylation and increased cellular accumulation of FOXO1 in human lung cancer cells. Moreover, depsipeptide treatment resulted in FOXO1-dependent induction of Bim, resulting in the induction of apoptosis [275]. Also, Brunet *et al* did show that inhibition of SIRT1 with the pan inhibitor nicotinamide did reduce the transcription of p27 and mnSOD and an increase in Bim expression. However they did not suggest whether this was due to changes of chromatin structure due to HDAC inhibition, or the direct aceylation of FOXO [223]. In pancreatic β cells the acetylation of FOXO1 inhibits its polyubiquitation and subsequent degradation by the proteasome. Moreover, its acetylation promotes the interaction with promyelocytic leukemia (PML) protein and PML bodies within the nucleus, not promoting its phosphorylation and nuclear exclusion [276]. This is particularly interesting when you consider that this is the same subcellular location where p53 accumulates [274] and that PML bodies are associated with increased cellular stress and apoptosis, particularly Fas and TNF mediated cell death [277].

Smith & Shanley have investigated the posttranslational status of FOXO proteins in response to various regulatory factors using mathematical models and found that acetylation protected FOXO proteins from degradation [278]. Furthermore, although acetylated FOXOs transcriptional activity was lower than the non-acetylated form, this could have been a result of monitoring only a subset of downstream targets [278].

Research into the effect of acetylation on FOXO3a is still in its infancy and requires more investigation to prove a consensus of how the modulation of specific lysine residues affects the role of FOXO proteins in determining cell fate. One way to examine this would be to perform ChIP-seq. Although, an

appropriate cellular system would have to be found where you would not have compensation from other forkhead family members. CHIP-seq would allow the assessment of the activation/repression of all FOXO3a target genes (established and novel), taking into account cofactor binding and promoter architecture. The one caveat would be producing site-specific mutants of acetylation mimic and non-acetylatable FOXO that reflected the acetylation status found in vivo through HAT/HDAC regulation.

1.9 FOXO IN CANCER

Three of the four group members were initially identified in humans because they were found at chromosomal translocations in tumours, suggesting that they may play a role in tumour development [143]. FOXO1 was found to be fused to PAX proteins 3 and 7, in two translocation sites, t(2;13) and t(1:13) respectively, in a number of alveolar rhabdomyosacromas (ARMS) [124]. PAX proteins – named because of the presence of the 'paired box' DNA-binding domain within each family member - are transcription factors that are important in cellular differentiation and tissue development, most notably, skeletal muscle. They promote cell proliferation, migration and survival [279].

The PAX3-FOXO1 chimeric protein contains the potent FOXO transactivation domain and the PAX3 DNA-binding domain, which results in the fusion protein being more transcriptionally active than the normal PAX3 protein. The PAX3-FOXO1 protein is constitutively active and localised solely in the nucleus where it can activate its downstream targets more readily, leading to oncogenic transformation and tumour development [280]. Moreover, the presence of the PAX3-FOXO1 protein results in a loss of expression of endogenous FOXO1, which is a critical mediator of cell cycle control in normal tissues. This loss of expression could be a key event in the development of tumourigenesis [76]. The loss of FOXO1 though chromosomal deletion (13q14) can be found in different prostate cancer types and its loss is known to promote tumourigenesis and the

development of androgen independence [281]. Furthermore, the PAX3-FOXO1 protein promotes transcription of platelet-derived growth factor alpha (PDGFα), which is neither a target of PAX3 or FOXO1, suggesting that the fusion protein could have distinct downstream targets to that of the individual transcription factors. PDGFα activates the PI3K cascade, negatively regulating FOXO1 transcriptional activity, promoting tumour progression [128]. Of course, *in vivo* the development and progression of ARMS is likely to be far more complex, involving a combination of all known and other yet unknown mechanisms that provide the tumour with a select advantage over its environment, but what is clear is FOXO dysregulation can provide this advantage. In metastatic ARMS patients, half the patients display metastasis to the bone barrow and a quarter present with metastasis to the lungs. Ultimately this leads to a poor clinical outcome, only 8% of sufferers having a 4-year survival rating [279, 282].

FOXO proteins function as tumour suppressors in a range of cancers, including lung, prostate and breast cancers [76]. The loss of expression or dyregulation of FOXO proteins can have drastic effects on cell fate. The loss of functional FOXO has been investigated using triple knockout mice - who do not express FOXO1, -3 and -4 – the lack of the three principal FOXO proteins leading to the development of hemangiomas and lymphoproliferative disease [283, 284]. The loss of FOXO3a mRNA and protein expression has been observed in lung adenocarcinoma (LAC) and non-small cell lung cancer (NSCLC). In 24.2% of LAC samples, FOXO3a expression was lost due to homozygous deletion and a further 60.6% displayed a significant loss of FOXO3a expression [285], supporting the hypothesis that FOXO3a functions as a tumour suppressor. Decreased expression of FOXO3a has also been observed in human prostate cancer cells when developing androgen-independent growth [286]. Furthermore, prostate cancer clinical samples displayed a decreased transcriptional expression of FOXO3a in high grade prostate tumours [287]. Interestingly, it was also shown that in low grade tumours, FOXO3a expression was comparable to benign tissue samples but in low grade prostate tumour samples, FOXO3a was

retained in the cytoplasm, suggesting that FOXO3a inactivation is an early step in prostate cancer development, followed by loss of FOXO3a expression in high-grade tumours [287].

Not only does its loss of expression lead to increased tumourigenesis but recent studies have shown that its subcellular location is integral to its role at regulating cell fate. The cytoplasmic retention of FOXO3a has been observed in breast cancer and shown to be due to the hyperactivation of regulatory kinases, such as PI3K/AKT, RAS/MEK/ERK and IKK [186, 190]. Activation of these pathways through loss of functional PTEN, PIK3CA and increased expression of extracellular receptors all lead to the inactivation of FOXO3a and its retention in the cytoplasm. The suppression of FOXO3a activity through overactivation of upstream regulatory pathways has been implicated in breast cancer tumour progression, for example, in tumour cell lines that harbour PTEN mutations, FOXO1 and -3a are retained in the cytoplasm resulting in their inhibition. However, the reintroduction of functional PTEN or overexpression of FOXO family members resulted in a G1 cell cycle arrest and induction of apoptosis, highlighting the importance of PI3K hyperactivation in breast cancer and its suppression of FOXO proteins, promoting tumour progression and survival [165, 288]. Moreover, hyperactivation of the IKK inflammatory pathways has also been shown to inversely correlate with the active/nuclear FOXO3a in breast cancer. A total of 131 breast cancer tumours were examined, 113 (86%) were found to positively express FOXO3a and that it was located in the cytoplasm and that this repression of FOXO3a positively correlated with a poor clinical outcome [190]. The retention of FOXO3a in the cytoplasm inhibits its ability to transcribe its downstream targets – which are predominantly involved with regulating cell cycle progression – resulting in tumour progression and resistance to apoptosis. Combined these data support the hypothesis that suppression of proapoptotic potential of FOXO3a can play a pivotal role in tumour progression and survival. What is clear is, dysregulation of the normal function of FOXO proteins is a

frequent event in cancer and restoring their activity could reinstate proliferative control.

1.9.1 Therapeutic approaches to targeting the PI3K and FOXO family members in cancer

Due to the fact that the activity of FOXO proteins is regulated by signalling pathways that are positioned upstream of the transcription factors and that these pathways are frequently hyperactivated in cancer, compounds that impede this hyperactivation are potent activators of FOXO proteins, for example, PI3K inhibitors have been identified using large-scale screens that specifically assess the ability of compounds to promote the translocation of FOXO3a to the nucleus, inhibiting proliferation in human cancer cell lines [289].

In recent years, a fervour of research into the development of smart therapies or targeted therapies has produced a myriad of specifically targeted compounds and monoclonal antibodies that inhibit various constituents of the PI3K signalling system, ranging from the extracellular receptors to the intracellular signalling machinery. Probably the most widely known and clinically tested therapies are gefitinib, lapatinib and herceptin which target EGFR, EGFR and HER2, and HER2 respectively, resulting in the inhibition of the PI3K cascade, inhibiting tumour growth.

Lapatinib is a potent proapoptotic compound, inducing cell cycle arrest and apoptosis in cell lines or xenographs that are dependent on EGFR or HER2-driven proliferation [290, 291]. We and others have shown that FOXO3a is indirectly activated in response to lapatinib and gefitinib treatment, and that its activation is integral to modulating the induction of apoptosis [98, 292, 293]. Moreover, since HER2 overexpression is predominantly linked with ER-independent growth and resistance to anti-estorogens, such as tamoxifen, lapatinib treatment can resensitises cell lines to anti-estrogen therapies.

Emphasizing this fact, a recent phase III study showed that lapatinib in combination with the anti-estrogen aromatase inhibitor letrozole, significantly reduced the risk of disease progression, while increasing the progression free survival of metastatic breast cancer patients that had ER+/HER+ disease [294, 295]. Furthermore, the ability of lapatinib to resensitise breast cancer cell lines to anti-estrogens is dependent on FOXO3a-mediated transcription of the estrogen receptor [291], highlighting the important role FOXO3a plays in modulating cell fate in response to cytotoxic agents. Further understanding of the regulation of FOXO3a is needed so that its role in the efficacy of combinatorial therapies can be exploited more effectively.

1.9.2 HDACs in cancer

Disruption of the acetylation profile of cellular components through the disruption of HATs and HDACs is thought to lead to the development of cancer. The loss of aceylation of H4K16 is frequently observed in cancer development (In total 25 cancer cell lines were tested, including breast, colon, lung and leukaemic cell lines relative to the nontransformed controls) [296], suggesting that increased HDAC expression/activity or reduce HAT expression/activity is a feature of tumourigenesis. Complementing this research, another study performed by Suzuki *et al* showed that H4 hypoacetylation is associated with breast cancer progression from normal epithelium to invasive cancer, and that interestingly this was most marked between normal epithelium samples and the development of a ductual carcinoma *in situ* (DCIS) suggesting that loss of H4 acetylation is an early even in cancer development [297]. Interestingly sirtuins, predominantly SIRT1, are the only family of HDACs that have been found to deacetylate H4K16 suggesting that their increased activity or expression could be a frequent event in cancer development [298].

The increased expression of class I HDACs is associated with cancer development. Conversely, the expression of class II HDACs is reduced in

tumours. Moreover, those that had higher expression of class II HDACs had a more favourable outcome [299].

Sirtuins SIRT1, -2, -3 and -7 have all be found to be upregulated in tumours [300]. SIRT1 is the most rigouously studied sirtuin in cancer and has found to be upregulated in breast, skin, colon and prostate cancers compared to untransformed tissues [258]. Regulators of SIRT1 expression have also been found to be lost or silenced in cancer development. The expression of hypermethylated in cancer 1 (HIC1) is lost in 60% of breast cancers, as well as other types of cancer such as brain, renal and colon. The loss of expression is the result of the methylation of a region of chromosome 17p13.3 [301]. HIC1 has been found to bind to the SIRT1 promoter and inhibit transcription, suggesting that its loss in tumourigenesis leads to the upregulation of SIRT1, promoting cancer development [302]. Deleted in breast cancer 1 (DBC1) is known to bind to the SIRT1 protein, inhibiting its catalytic activity and also disrupt its interaction with its substrates. DBC1 is located in chromosomal region that is deleted in human breast cancer (chromosome 8p21) [303] and whose expression is also lost in lung and colon cancer cell lines [304] suggesting that its loss leads to an increase in SIRT1 activity. This overexpression of SIRT1 leads to the deacetylation and suppression of down-stream targets, such as p53, and the decreased ability of inducing apoptosis in response to genotoxic stress [304]. A positive regulator of SIRT1 activity, active regulator of SIRT1 (AROS) has also been identified, whose expression positively correlates with SIRT1 in a range of cancer cell lines (kidney, colon, lung, bone and blood) and suppresses p53-induced apoptosis in a SIRT1-dependent manner, suggesting that positive regulators of SIRT1 are upregulated in cancer [305]. Taking this further, the activity of SIRT1 is also regulated by its posttranslational status. Studies have shown that the sentrin-specific protease 1 (SENP1) desumoylates SIRT1 in response to genotoxic stress, which dramatically reduces its activity, resulting in the acetylation and activation of p53, resulting in the induction of apoptosis [306].

This research suggests that proteins that regulate the sumoylation status of SIRT1 could also be important in cancer development.

Recently, SIRT2, -5 and -7 were found to be elevated in pancreatic cancer [258]. SIRT7 was also shown to be upregulated in breast cancer, compared to normal tissue. Moreover, increased SIRT3 and SIRT7 expression was linked to lymph node-positive breast cancer, suggesting that they play a role in tumourigenesis and metastasis [307].

Further studies are required to assess the role of sirtuin expression and activity in cancer development. To have a full understanding of the dynamics of regulating the acetylation status of key modulators of cell fate, the expression and activity of HATs and HDACs have to be explored. Although sirtuin expression may not by statistically elevated in tumour samples, its activity could be increased or its cellular competitor could be lost or inhibited, making the understanding of sirtuin proteins roles in tumour biology infinitely more complex but ultimately more important.

1.9.3 Targeting HDACs in cancer

The study of HDACs has increased dramatically in recent years, mainly due to the discovery of their role in cancer but also due to the development of small molecules that can specifically target HDACs *in vivo*, influencing their activity. Although advances in HDAC inhibitors have been made, clinical development has been largely with class I and II proteins, the most notable compound being the small molecule inhibitor SAHA (Vorinostat), which was the first HDAC inhibitor to be approved by the FDA in 2006 for the treatment of cutaneous T-ell lymphoma (CTCL) [308]. No class III HDAC inhibitor, to date, has progressed from preclinical research into clinical trials for cancer treatment. What defines the interest in sirtuins is their intracellular targets, histone and non-histone proteins that are involved in a plethora of cellular processes, but also the fact that

established class I and II inhibitors like TSA do not effect sirtuin activity and newly developed sirtuin inhibitors appear to be selective for class III HDACs meaning sirtuin proteins provide a novel and selectable target in cancer [309].

The investigatation into sirtuin inhibitors and their efficacy in different disease states is still in its infancy. To date, the compounds cambinol and tenovin-6 have been the most rigorously tested sirtuin inhibitors. Both compounds have been shown to target multiple sirtuin proteins, predominantly SIRT1 and SIRT2 (and possibly others) [310]. Cambinol is a dual sirtuin inhibitor, suppressing tumour growth in nude mice xenographed with burkitt lymphoma and human melanoma cells, respectively [311, 312]. Moreover, the antiproliferative function of cambinol was facilated by the acetylation of p53, promoting its activation and the acetylation of the B-cell lymphoma 6 protein (BCL6) oncoprotein, resulting in its inhibition [311]. Tenovin-6 was also shown to be a potent activator of p53 through the inhibition of SIRT1 and SIRT2, promoting the induction of apoptosis in tumour cell lines and reduced tumour growth in xenograft mice [312].

Other inhibitors such as sirtinol and EX527 have also been shown to successfully inhibit cancer cell growth. Sirtinol is known to induce senescent-like growth arrest in breast and lung cancer cell lines through inhibition of SIRT1 and SIRT2 [313]. Furthermore, sirtinol treatment increased the efficacy of cisplatin in prostate cancer cell lines [314]. EX527 is the most potent SIRT1 inhibitor identified, having an IC50 of 38nM [315] (although in our own study we found it to be 10-fold less, it is still considered to be the most potent SIRT1 inhibitor identified [316]), although the effect EX527 monotreatment has on cell fate has not been fully elucidated as most publications only use it in combination with cytotoxic agents such as etoposide [315].

THESIS AIMS AND OBJECTIVES

The aim of this thesis was to investigate the importance of FOXO3a and sirtuins in breast cancer. As well as investigate whether they played important roles in the development of resistance, and more importantly, whether this interaction could be exploited therapeutically.

Also, I aimed to elucidate the mechanism of action of known sirtuin inhibitors and whether it could be exploited to increase the efficacy of anticancer therapies, predominantely those that are known to indirectly effect the activation of FOXO3a.

CHAPTER 2

MATERIALS AND METHODS

2.1 CELL CULTURE

The human breast carcinoma cell lines MCF-7, MDA-MB-231, ZR-75-1, BT474 and SkBr3 originated from the American Type Culture Collection and were acquired from Cancer Research UK, in which they were tested and authenticated. These procedures include cross-species checks, DNA authentication, and quarantine. Cell lines used in this thesis were in culture for <6 months and were maintained in Dulbecco's modified eagle medium (DMEM)(Sigma Aldrich, UK) containing 10% foetal calf serum (FCS) and 2 mmol/L glutamine and 100 U/mL penicillin/streptomycin in a humidified incubator at 37°C with an atmosphere of 10% CO_2.

The colon carcinoma cell line DL23, previously described [170], were grown in RPMI-1640 medium which was supplemented similarly as described above.

The mouse embryonic fibroblasts (MEF) were derived from *wildtype*, *p53-/-* and *FOXO-/-* (FOXO1, FOXO3a and FOXO4) double knockout mice. Cells were cultured in DMEM and supplemented similarly as described above.

MEF *FOXO-/-* were a kind gift from Professor Boudewijn Burgering, University Medical Centre, Utrecht.

Cisplatin Resistant MCF-7 cells (CisR) and Lapatinib resistant SkBr3 cells (R5) were generated by myself and were cultured in the same conditions as the parental cell lines, apart from the exception that, when resistant, were supplemented with 1 μM/ml of cisplatin and 0.5 μM/ml of lapatinib, respectively.

2.1.1 Cell line maintenance

Cells were grown and split at approximately 80% confluency, twice a week (Cells were split at defined ratios MCF-7 1:5, SkBr3 1:5, DL23 1:7, ZR-75-1 1:5, MDA

231 1:6, BT474 1:5, NIH 3T3 WT 1:8, NIH 3T3 p53-/- 1:8 and MEF FOXO-/- 1:8). Media was aspirated and the cell monolayer was washed once in warm PBS and then detached by the addition of 1× trypsin-EDTA. After the cells were detached by the addition of trypsin, they were resuspended in the appropriate media and seeded into a new flask or into the appropriate cell culture plasticware. Cells were cultured for approximately 35 passages before discarding.

2.1.2 Long-term maintenance of cells

Cells of good health and a low passage number were detached from the monolayer with trypsin, as described above, and washed in media. They were then spun at 500× g for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in FCS with 10% dimethyl sulphoxide (DMSO). 1 ml aliquots were transferred to cryotubes and slowly frozen in a Mr. Frosty (VWR International) at -80°C overnight before being transferred to storage in liquid nitrogen.

For defrosting cells, cryotubes were defrosted rapidly in a waterbath (~37°C) and the defrosted solution was added to complete media and spun at 1000× g for 5 minutes. The DMSO containing supernatant was removed and cell pellets were redissolved in fresh supplemented medium and placed in a 25cm^2 culture flask to grow.

2.2 CHEMICALS

Cisplatin (Onco-tain DBL, Leamington Spa) was maintained as a stock solution (3.3M) at room temperature and diluted in fresh media prior to treatment. **Paclitaxel** (Imperial College Healthcare, UK) was maintained as a stock solution. **Lapatinib** (LC laboratories, USA) was dissolved in DMSO and stored at -20°C at a concentration of 1mg/µl. **Cycloheximide** (Sigma-Aldrich, Dorset, UK) was dissolved in DMSO and stored at -20°C at a concentration of 10mg/µl. **Nicotinamide** (SigmaAldrich, UK) was dissolved in DMSO at concentration of 10mM and stored at -20°C. SIRT inhibitors **EX527** (6-Chloro-2,3,4,9-tetrahydro-1Hcarbazole-1-carboxamide), **Sirtinol** (2-[(2-Hydroxynaphthalen-1-ylmethylene)amino]-N-(1-phenethyl)-benzamide), and **Salermide** (N-{3-[(2-hydroxynaphthalen-1-ylmethylene)-amino]-pheny-2-phenylpropionamide) were prepared according to reported procedures (synthesized by Sebastian Kroll and Paolo Di Fruscia), stock solutions of 10mM were dissolved in DMSO and stored at -20°C.

2.3 *IN VITRO* SIRTUIN ACTIVITY ASSAY

The Biomol hSIRT1/hSIRT2 activity assay (AK-555/ AK-556) was used to deduce the activity potential of SIRT inhibitors. These *in vitro* assays use only a few components and allow the study of inhibition or activation of protein activity.

The assay was done essentially as described [317] and according to the manufacturer's instructions (Biomol International). All compounds were preincubated with the hSIRT1/hSIRT2 before commencing the reaction through the addition of the “Fluor de Lys” deacetylase substrate. Deacetylation of K382-p53/K320-p53 was used as a marker of HDAC activity. All compounds were prepared fresh in DMSO to 10 mmol/L, 24 h before the assay was preformed. Fluorescence was read (excitatory, 360; emission, 460) using the Fluoroskan Ascent FL fluorometer (Thermo Fisher) using the Ascent software. The dose

concentrations were done in triplicate. The amounts of acetylation were derived from the levels of fluorescence (displayed in arbitrary fluorescence units). In addition, the percentages of inhibition were calculated from the arbitrary fluorescence unit of the treated assays and nontreated controls.

2.4 THREE-DIMENSIONAL MODELLING

To prepare the docking-related files including protein and inhibitors, the three dimensional structure of Human SIRT1 was modeled by the Protein Homology/ analogy Recognition Engine (Phyre; [318]). The modeled structure of SIRT1 is based on the structure of yeast Sir2 (PDB entry is 2HJH) already containing nicotinamide and Zn, with the original human SIRT1 amino acid sequence from residues 212 to 529. The threedimensional structure of SIRT2 used for docking was taken from the human Sirtuin C-pocket (PDB entry is 1J8F). The three inhibitor structures, Sirtinol, Salermide, and EX527, were generated by the Dundee PRODR G2 Server [319]. The docking studies were done using the site feature docking algorithm from the LibDock [320] software of the Discovery Studio package (version 2.1). The force field was applied to the SIRT1 and SIRT2 using the in-built CHARMm force field calculation. The binding region for the docking of SIRT2 is the same a previously described [321], which was a 10-Å sphere around the Gln167. The docking region used for SIRT1 was designed in a 10-Åf sphere around the residue of Gln345, which is genetically conserved to the Gln167 Q7 of SIRT2. All the docking studies were carried out by default setting of the LibDock software and then the generated results of these docking studies were subjected to the “Score Ligand Poses” built-in function of the Discovery Studio to evaluate and rank the docking results.

The best pose of each docking study was chosen based on the LigScore2 [322]. Furthermore, the best pose of each docking study was energy minimized with the protein structure by the “Ligand Minimization” function of the Discovery Studio.

All the structural presenting figures were created with the PyMOL program (DeLano Scientific).

2.5 PROTEIN ANALYSIS

2.5.1 Preparation of total protein lysates

Whole cell extracts prepared by harvesting cells by using 1×trypin-EDTA, once detached medium was added to inactivate the trypsin and samples were spun 500× g for 5 minutes. The supernatant was discarded and cell pellets were washed in PBS and spun for an additional 5 minutes (500× g). The supernatant was discarded and the cell pellet was frozen at -80ºC until lysis was performed. Frozen pellets were lysed in lysis buffer (0.1% Triton, 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.4), 10 mmol/L NaF, 1 mmol/L sodium orthovanadate, 30 mmol/L Na β-glycerophosphate, and protease inhibitors ("Complete" protease inhibitor mixture, as instructed by the manufacturer, Roche Applied Science)) on ice for 10 min. Insoluble material was removed by centrifugation (13000rpm 4^{O}C 5 minutes). The supernatent was then transferred to a clean eppendorf tube and protein concentration was determined by Bio-Rad Dc protein assay.

2.5.2 Determination of protein concentration

Protein concentration was determined using the Bio-Rad D_c protein assay (Bio-Rad Laboratories), as instructed by the manufacturer. 20µl of reagent S was added to 1 ml of reagent A. 100µl of this mixture were added to 2µl of protein solution. After 2 minutes, 800µl of reagent B was added to the mixture, the samples were shaken gently and then covered to protect them from direct sunlight. After 30 min incubation, absorbance was read at 750nm for each of the experimental samples as well as the non-protein containing control. Protein concentrations were determined by the equation absorbance x 25 = µg/µl.

2.5.3 Western blotting or Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Gels for elucidating protein expression were made using the acrylmide/bis stock solution. Twenty five percent ammonium persulphate (APS) and Tetramethylethylenediamine (TEMED) were used as polymerisation catalysts. SDS Gels consisted of a lower resolving gel and an upper stacking gel. The percentage of the resolving gel used depended on the size of the protein of interest. Lower percentage gels allowed better examination of proteins of higher molecular weight (mW) and vice versa (see table for the differing amount of constituents for each gel percentage).

	Resolving gel				Stacking gel
	7%	**10%**	**12%**	**14%**	**5%**
dH_20-ml	5.02	4.02	3.35	2.68	3.67
1.5M Tris pH 8.8	2.5	2.5	2.5	2.5	-
1.5M Tris pH 6.8	-	-	-	-	0.42
30%Acrylamide/ 0.8% Bis mix (37.5:1)	2.33	3.33	4.00	4.67	0.83
10% SDS -µl	100	100	100	100	50
25% APS -µl	40	40	40	40	20
TEMED-µl	10	10	10	10	10
Total-ml	10	10	10	10	5

Table 2.1: SDS-PAGE percentages and their constituents

To separate the proteins, 20µg of protein lysate was added to an equal volume of 2× SDS loading buffer (4% (w/v) SDS, 62.5mM Tris-HXL (pH 6.8), % (v/v) glycerol, 0.01% (w/v) bromophenol blue) and boiled for 5 minutes at 100ºC. Samples were then spun at 1000rpm for 1 minute and the appropriate volume of

sample was loaded into the sacking wells. SDS-PAGE gels were run in running buffer (25mM Tris-Base, 250mM glycine, 0.1%(w/v) SDS) at 60V through the stacking gel and then 100V through the resolving gel.

Once the proteins had been separated by SDS-PAGE, proteins were electro-transferred on to Protlan nitrocellulose membranes (Schliecher and Schuell) using a wet tank blotting system (Bio-Rad Laboratories Trans-Blot Cell) in transfer buffer (25mM Tris, 190 mM glycine and 20% (v/v) ethanol) for 90 minutes at 90V. PLEASE NOTE the tanks were packed in ice to ensure they did not overheat.

Membranes were then blocked in either 5% milk or 2.5% Bovine Serum albumin (BSA) in TBS plus 0.5% Tween (TBST) for 1 hour at room temperature and then incubated with a specific primary antibody (see table 2.2) for 16 hours at 4 degrees (Table 2). Membranes were then washed every 15 minutes with 50mls TBST for one hour at room temperature. They were then treated with their respective horseradish peroxide conjugated secondary antibody (anti-rabbit or anti-mouse – see table 2.2) for one hour at room temperature. The membranes were then washed every 15 minutes with 50mls TBST for one hour at room temperature. The membrane was then developed using the enhanced chemiluminescence detection system (GE Healthcare, UK).

Antibody	*Product number*	*Species*	*Company*
EGFR	2232	rabbit	Cell signalling technology
p-EGFR (tyr1068)	2234	rabbit	Cell signalling technology
HER2/neu (C-18)	sc-284	rabbit	Santa cruz
P-HER2/ERB2 (y1248)	44-900	mouse	Biosource
HER3/ERB3 (c-17)	sc-285	rabbit	Santa cruz
Total AKT	9272	rabbit	Cell signalling technology
Total AKT (5G3)	2966	mouse	
p-AKT(473)	9271	rabbit	Cell signalling technology
p-AKT (308)	9275	rabbit	Cell signalling technology
MAPK	4695	rabbit	Cell signalling technology
p-MAPK (tyr202/tyr204)	9101	rabbit	Cell signalling technology
FOXO1 (C29H4)	2880	rabbit	Cell signalling technology
FOXO3a	H-144 sc-11351	rabbit	Santa Cruz
FOXO4	834	rabbit	Gift - Prof. BM Burgering
FOXM1 (k-19)	(k-19)	rabbit	Santa cruz
p53	Sc-98	mouse	Santa cruz
Gadd45alpha (c-20)	Sc-792	rabbit	Santa cruz
MDR1 (C219)	517310	mouse	Calbiochem
p27 (C-19)	sc-528	rabbit	Santa cruz
c/caspase-3 (Asp175)	9661	rabbit	Cell signalling technology
SIRT1	Ab32441	rabbit	Abcam
SIRT2	sc-20966	rabbit	Santa cruz
SIRT3	2627	rabbit	Cell signaling
SIRT4	s0948	rabbit	Sigma Aldrich
SIRT5	SA464	rabbit	Biomol
SIRT6	2950	rabbit	Cell signaling
SIRT7	Ab62748	rabbit	Abcam
FLAG	F1804	mouse	Sigma Aldrich
Beta-tubulin	Sc-9104	rabbit	Santa cruz

Table 2.2: Antibodies used for western blotting

2.6 TOTAL RNA EXTRACTION

Total RNA was isolated from mammalian cells using the RNeasy Kit (Qiagen, Crawley, UK). The protocol was performed in line with the manufacturers instructions.

Frozen cell pellets were resuspended in 350µl of RLT buffer (containing 10% ß-mercaptoethanol) and homogenised by pippetting with a 1000 µl pipette. 350 µl of 70% ethanol was added and the total mixture was added to the provided pin column and place in a 2ml collection tube and spun in a benchtop centrifuge for 15s at 10,000rpm. The flow through was discarded and 700 µl of buffer RW1 was added to the column and was spun at 10,000rpm for 15s, the flow through was again discarded. Next, 500 µl of RPE buffer was added to the column and microcentrifuged for 15s, 10,000 rpm, the low through was discarded and the column was spun for an additional 2 minutes at 10,00 rpm to extract any waste from the column. The column was then transferred to a clean, steralize epindorf tube. 30 µl of RNase-free water was carefully added to the centre of the column and was spun for 1minute at 10,000 rpm to elute the extracted RNA. The purity and concentration of the RNA was then determined using Nanorop, which measures the spectrometric absorption at 260nm and 280nm. RNA samples were then stored at -80ºC or immediately used to make cDNA, using first strand cDNA synthesis.

2.6.1 First strand cDNA synthesis

2 µg of total RNA was reverse transcribed into first strand cDNA using Superscript III first strand cDNA synthesis system (Invitrogen, Paisley, UK).
One microlitre of random primers and 1 µl of 10mM dNTPs mix (containing the four bases adenine, cytosine, guanine and thymine) were added to 2 µg of total RNA to make a total volume of 14 µl (using sterile RNase free water). The sample was heated for 5 minutes at 65ºC and then placed immediately on ice for

1 minute. Four microlitres of 5× first strand buffer, 1µl of 0.1M DTT, 1 µl RNaseOUT and 1 µl of the reverse transcriptase Superscript III were then added to make a total volume of 20 µl. The mixture was spun for 15s at 500 rpm the placed on a thermocycler (GeneAmp PCR system 9700, Applied Biosystems) where they were incubated for 5 minutes at 25ºC for 5 minutes, then heated to 50ºC for 50 minutes. The reaction was terminated by heating the mixture to 70ºC for 15 minutes.

2.6.2 Real-time quantitative PCR (RT-qPCR)

cDNA (synthesised as stated above) were used as template and gene expression was measured using RT-qPCR, which was performed using an ABI PRISM 7700 sequence detection system (Applied Biosystems), using the SYBR Green Mastermix (Applied Biosystems) according the the manufacturers instructions. Gene specific primers were designed using Primer Express Software (Applied Biosystems).

Primers for FOXO3a (Human FOXO3a-sense TCTACGAGTGGATGGTGCGTT and FOXO3a-antisense CGACTATGCAGTGACAGGTTGTG) and L19 (Human L19-sense GCGGAAGGGTACAGCCAAT and L19-antisense GCAGCCGGCGCAAA) were optimised by previous members of the lab.

Analysis of gene expression was performed using the relative standard curve method, where 2µg of cDNA from each sample was mixed and diluted into serial dilutions (1/4, 1/16, 1/64 and 1/256). As a control the established housekeeping gene L19 was used to normalise gene expression between samples.

The reaction mix contained 2µl of sample and 23µl of SYBR Green master mix, primers and water were added to a final volume of 25µl. The TAQMAN 7700 real-time PCR machine was used to measure gene expression. All measurements were performed in triplicate.

2.7 TRANSFECTION OF PLASMID DNA

Cells were seeded into 10cm^3 dishes to confluency of approximately 60%. Plasmid DNA was transfected using Fugene. The ratio 3:1 (ul of fugene: µg of DNA) was used, as recommended by the manufacturer.

To begin, 975 µl of optimem (sterile DMEM containing no supplements can also be used) was pipetted into a plastic bijou. 6 µl of fugene were then carefully added and the mixture was shaken slightly and left at RT for 5 minutes. After which 2 µg of plasmid DNA was added, gently mixed and left at room temperature for 20 minutes. The mixture was then pippetted dropwise into the 10cm3 dish while gently being shaken, ensure the mixture dispersed eveny. Overexpression of desired proteins were confirmed 24 h after transfection, after cell harvesting and western blot analysis.

2.8 RNA INTERFERANCE (RNAi)

RNA interference was used to specifically repress the expression of a chosen gene or genes.

In this study, all SMARTpool siRNAs used were purchased from Dharmacon, RNA technologies. The SMARTpool siRNAs used were: FOXO3a (L-00307-00), siSIRT1 (L-003540-00), siSIRT2 (L-004826-00).

Oligofectamine (Invitrogen LT) was used as the transfection reagent. 200,000 cells per well of a 6-well plate, were plated 24h prior to transfection. For each well, 70µl of Optimem, was mixed with 5µl oligofectamine and incubated at room temperature for 10min. This solution was then mixed with 250µl Optimem and 7.5µl (50nM final concentration) siRNA oligos specific for each gene. After 25min incubation, 160µl OPTIMEM was added to each mixture, reaching a final volume

of 500µl, which was then added to the wells that have been previously washed with warmed PBS. The plates were then incubated for 4 h at 37ºC, after which 2 ml of DMEM, containing 10%FCS, was added to each well and the plates were incubated for a further 24 h.

Cells were then treated with cytotoxic agents and cell cycle analysis was performed. Also protein expression was confirmed using western blot.

As control, cells were also treated with just 1× universal buffer (used as the siRNA vector) containing no siRNA. This ensured that any effect the transfection procedure had on the cells would be detected. Also cells were transfected with a non-specific siRNA (D-001210-01-05, Dharmacon), which does not target any known proteins, ensuring that any non-targeting effects of siRNA delivery and interaction with cellular machinery would be measured.

2.9 CELL CYCLE ANALYSIS

The cell cycle was analysed using flow cytometry. The media was removed and the cells were washed with 1ml PBS (the media and wash was retained). Cells were then scraped in cold PBS on ice and then spun at 4000rpm (4^{o}C) for 10 minutes (using a Sorvall Legend RT). The supernatant was discarded and the pellet was resuspended and washed in 500µl PBS and spun at 4000rpm (4^{o}C) for 10 minutes and then resuspended and fixed in 5ml of 90% ethanol at 4^{o}C for 16 hours. Cells were then spun at 4000rpm (4^{o}C) for 10 minutes. The supernatant was discarded and the cells were washed in 1ml PBS and then spun at 4000rpm (4^{o}C) for 10 minutes. The supernatant was discarded and then the cells were stained using 500µl propidium iodide (50µmol/L) for 1 hour and then analysed using a FACS canto and FACSDiva software (BD Biosciences).

2.10 SULFORHODAMINE B (SRB) ASSAY

The SRB assay is used as a measurement of cell proliferation/viability, the dye binds to and stains the protein content of the cell. Because the binding of the SRB is stoichiometric, the amount of dye extracted from the stained cells is directly proportional to cell mass and is representative of cell number [323]. Approximately 3000 cells were seeded in each well of the 96-well plates. After culture, 100μL of trichloroacetic acid (TCA) was added to each well and incubated for 1 h at 4°C. The plates were then washed with deionized water three times before incubation at room temperature for one hour with 0.4% sulforhodamine B in 1% acetic acid. The plates were then washed with 500 μL 1% aceitc acid or until unbound SRB was washed away. The plates were then left to air-dry overight and then stored. To measure the plate, 100 μL Tris (10 mmol/L) was then added to each well to solubilize the bound sulforhodamine B dye and placed on a rotator for 30 minutes to evenly distribute the dye. The plates were then read at 492 nm using the Sunrise plate reader (Tecan Group Ltd).

2.11 CASPASE-GLO 3/7 ASSAY

Caspase assays were done using the Caspase-Glo 3/7 assay kit (Promega) according to the manufacturer's protocol.

Cells were culture as described previously in 96-well CulturPlates (6005680, PerkinElmer, UK). After cells were treated with the appropriate regimes of cytotoxic compounds, 50μl of the 100μl medium/compound mix was removed from each of the wells. 50μl of caspase-glo assay was added to each well; plates were covered with foil and were gently mixed for 30 minutes at RT. Luminescence was read by the PHERAstar Plus (BMG Labtech). Experiments were performed in triplicate.

CHAPTER 3

SIRT INHIBITORS INDUCE CELL DEATH AND p53 ACETYLATION THROUGH TARGETING BOTH SIRT1 AND SIRT2

3.1 INTRODUCTION

The development and progression of cancer involves both epigenetic and genetic changes leading to the alteration of gene expression and thus cell phenotype. It is well established that alteration in the epigenome of the cell through promoter hypermethylation and histone deacetylation is a common facet of tumorigenesis. Indeed, previous studies have reported that the promoters of tumour suppressor genes, such as $p16^{INK4A}$ and $p15^{INK4B}$, frequently demonstrate increases in DNA methylation and/or histone deacetylation, thereby leading to gene silencing [324]. However, emerging evidence also suggests that the role of acetylation as a protein post-translational modification, independent of histone modification, may also play a critical role in cell fate and thus tumorigenesis. For example, the activities of the tumour suppressors' p53 and FOXO3a are both in part regulated by their acetylation status. The degree of acetylation is largely mediated by a balance between histone acetyl transferase (HATs) and histone deacteylase (HDAC) activity, in a substrate specific manner.

Sirtuins (also called SIRTs) are NAD+-dependent class III HDACs that share extensive homologies with the yeast histone deacetylase Silent Information Regulator 2 (Sir2) [325]. In yeast [233], nematode worms [234] and fruit flies [326] SIRT/Sir2 activity has been shown to be crucial for lifespan extension in response to metabolic and other environmental stresses [327-330]. In mammals, seven Sir2 homologues (Sirtuin 1-7 or SIRT1-7) have been identified, which possess primarily histone deacetylase (SIRT1, -2, -3, -5) or mono-ribosyltransferase activity (SIRT4 and SIRT6) [225, 226], which target histone and various non-histone proteins in distinct subcellular locations.

The mammalian SIRT1 is the direct homologue of the yeast Sir2 and has a wide range of substrates and cellular functions. SIRT1 can induce chromatin silencing through deacetylation of histones H1, H3 and H4 [331], and can modulate cell survival by regulating the transcriptional activities of p53 [332], NFκB [333],

FOXO proteins [223, 265] and p300 [334]. In contrast to SIRT1, SIRT2 is predominantly a cytoplasmic protein and has been shown to be able to deacetylate a number of cytoplasmic substrates, including α-tubulin [243, 335].

Both SIRT1 and SIRT2 may have a role in the development of cancer. For example, SIRT1 can activate stress defence and DNA-repair mechanisms, thus allowing the preservation of the genomic integrity [237]. Conversely, it has also been demonstrated that SIRT1 overexpression can enhance tumour growth, and promote cell survival in response to stress and drug resistance. Nevertheless, SIRT1 has been shown to be up-regulated in a spectrum of cancers, including lymphomas, leukaemia and soft-tissue sarcomas, prostate cancer, lung and colon carcinomas [336-338]. Overexpression of SIRT2 can significantly prolong the mitotic (M) phase and delay mitotic exit. In consequence, it has been proposed that SIRT2 might function as a mitotic checkpoint protein in G2/M to prevent the induction of chromosomal instability, particularly in response to microtubule inhibitor (MTI) mediated mitotic stress [244]. Consistently, tumours with high levels of SIRT2 are refractory to chemotherapy, especially microtubule poisons [339].

The dual role of SIRTs in modulating the acetylation of tumour suppressor proteins and chromatin renders them attractive therapeutic targets for anti-cancer drug development [237]. However, despite the development of a number of effective SIRT inhibitors, little is known regarding the specific mechanisms of action and cellular targets of these proteins. In the present study, we have used breast cancer cells as a model system, and profiled the *in vitro* and *in vivo* activity of several recently developed potent SIRT inhibitors. Moreover, we have identified the tumour suppressor p53 as the critical target of the SIRT-inhibitor-induced cell death in breast cancer cells.

3.2 RESULTS

3.2.1 Sirtinol and Salermide induce cell death while EX527 triggers G1 arrest

To gain insights into the anti-cancer potential of the three novel synthetic SIRT inhibitors EX527 [340], Sirtinol [309] and Salermide [321] (Figure 3.1), we first investigated the effects of these SIRT inhibitors on the proliferation of the breast carcinoma cell line MCF-7. The natural SIRT inhibitor nicotinamide was also included as a reference compound. SRB results showed that concentrations of 25μM and over of either Sirtinol or Salermide could significantly inhibit MCF-7 cell proliferation (≥80% of inhibition), whereas only ≥100μM of EX527 could effectively repress MCF-7 cell proliferation (Figure 3.2). Even at 100μM, nicotinamide failed to elicit any substantial inhibitory effect on MCF-7 cells (Figure 3.2). To confirm these proliferative results, we next examined the effects of these SIRT inhibitors on cell cycle progression and treated cycling MCF-7 cells with 50μM of each of the four SIRT inhibitors for 0, 24 and 48 h. Flow analysis results showed that both Sirtinol and Salermide effectively induced cell death, while EX527 treatment caused an increase in G1 cell population, with no detectable cell death (Figure 3.3A). These cell proliferation data were supported by morphological examination using light microscope demonstrating that only Sirtinol and Salermide could induce extensive cell death at 50μM (Figure 3.3B).

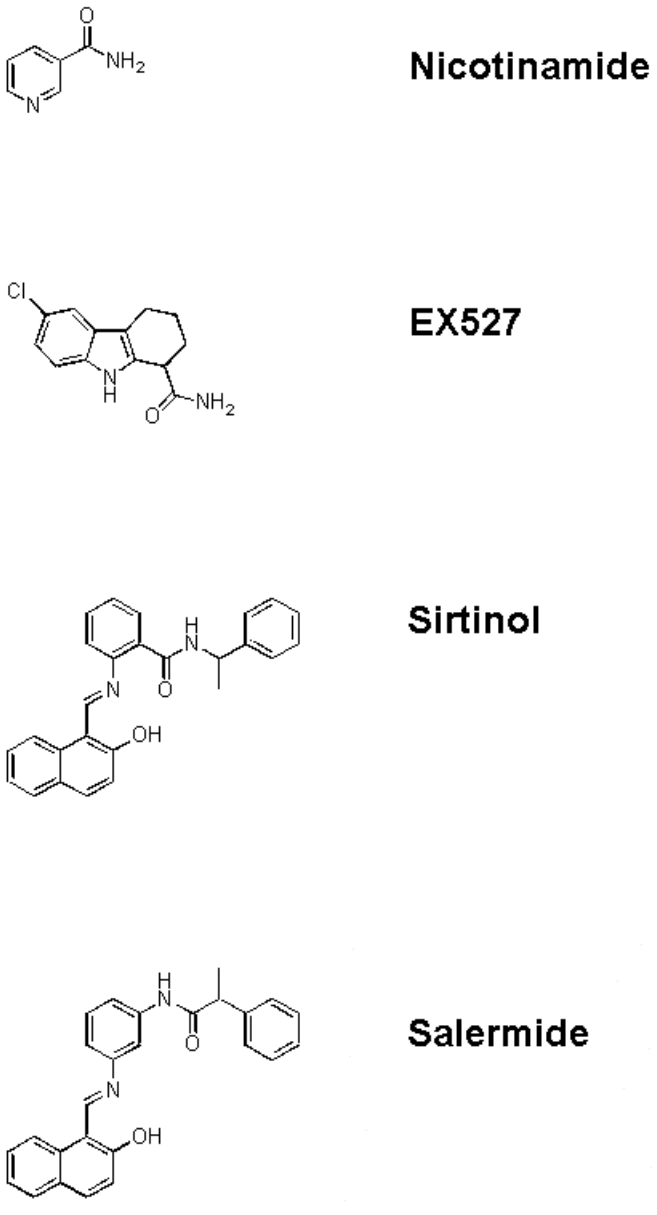


Figure 3.1 Sirtuin Inhibitors. The molecular structure of the sirtuin inhibitors, Nicotamide, EX527, Sirtinol and Salermide.

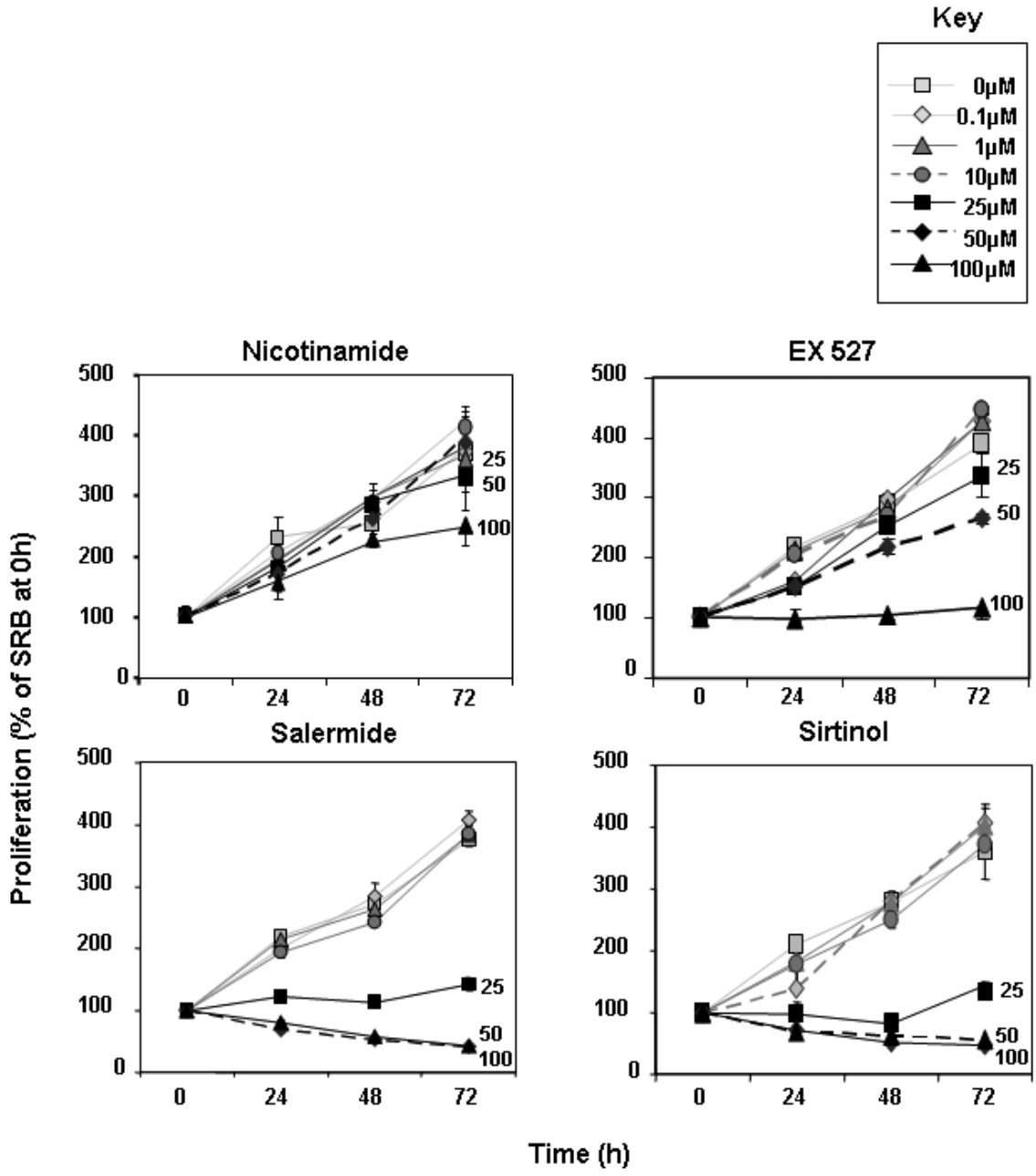


Figure 3.2 Effects of SIRT inhibitors on MCF-7 cell proliferation. MCF-7 cells were treated with 0-100μM of nictotinamide, EX527, Sirtinol or Salermide for 0, 24, 48 and 72 h. Cell proliferation was determined by sulforhodamine B assay. Results shown are mean ± SD of three independent experiments.

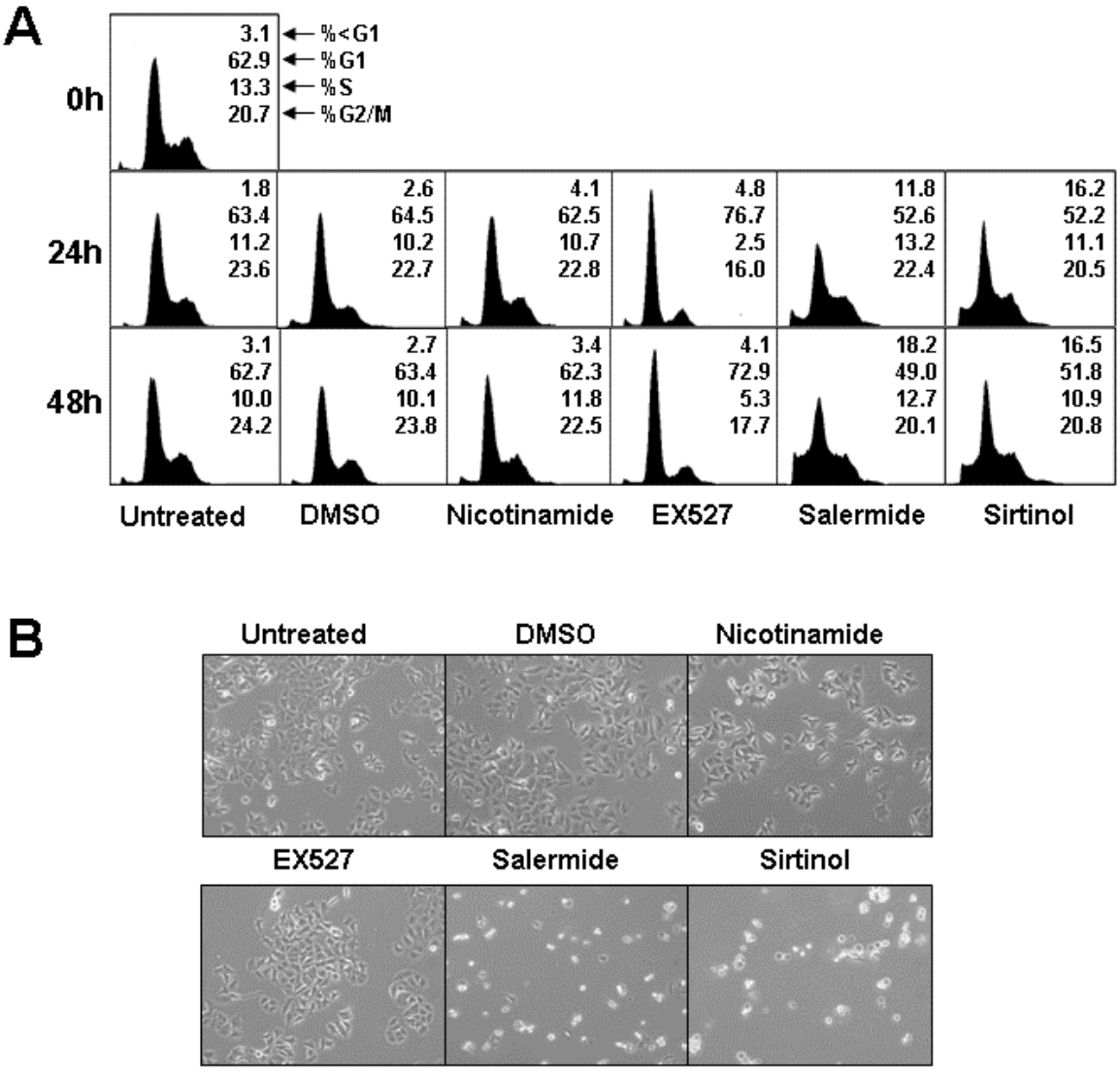


Figure 3.3 Effects of SIRT inhibitors on MCF-7 the cell cycle and mophology A) MCF-7 cells were either untreated or treatmented with 50 μM of nicotinamide, EX527, Salermide or Sirtinol for 0, 24 and 48 h and flow cytometric analysis of DNA content was performed after propidium iodide staining. Percentage of cells in each phase of the cell cycle (sub-G1, G1, S, and G2/M) is indicated. Representative data from three independent experiments are shown. **B)** Representative phase-contrast microscopy images of MCF-7 cells untreated or after treatment with 50 μM of nicotinamide, EX527, Salermide or Sirtinol (magnification 100X).

3.2.2 G1 arrest exhibited by EX527 is reversible

To examine whether the cytotoxic effects of sirtinol and salermide were reversible, MCF7 cells were treated with 50 μM of nicotinamide, EX527, Salermide or Sirtinol for 0, 24, 48 and 72 hours. At 24 hours cells were also washed and culture in inhibitor-free medium for 24 and 48 hours. The effect on proliferation was assessed using SRB (Figure 3.4).

The removal of sirtinol and salermide had no effect on the viability of the MCF7 cells, compared to the cells that had been continually exposed to the sirtuin inhibitors, suggesting that short treatment of sirtinol and salermide resulted in non-reversible apoptosis and cell death (Figure 3.4). Interestingly, the cytostatic effects of EX527 were reversible; cells washed at 24 hours were able to continue proliferating in inhibitor-free medium similar to the rate of proliferation of the untreated controls (Figure 3.4). Moreover, the effect on proliferation of cells continually treated with EX527 compared to washed cells was statistically significant ($p<0.001$). These results strengthen the findings that sirtinol and salermide are more cytotoxic that EX527, and that the cytostatic function of EX527 is reversible.

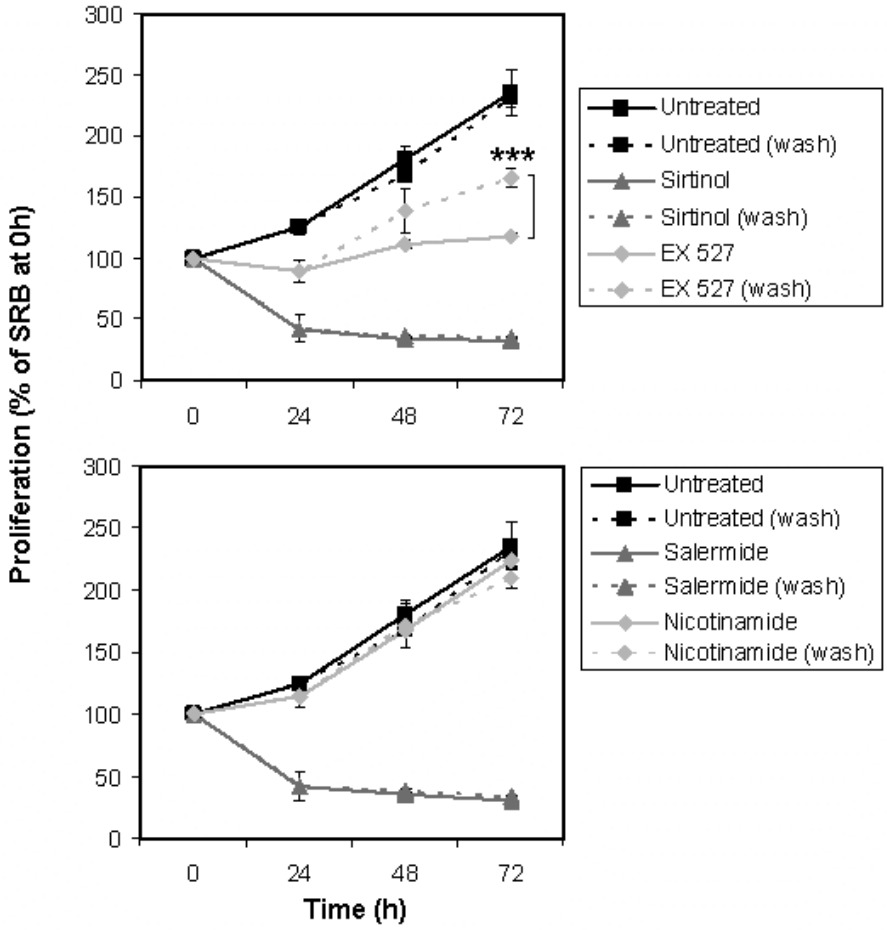


Figure 3.4 The cytostatic effect of EX527 is reversible. MCF-7 cells were treated with 50μM of nictotinamide, EX527, Sirtinol or Salermide for 0, 24, 48 and 72 h.. Cells were also treated for 24 h with each compound and then washed with PBS, and cultures in inhibitor-free media. Cell proliferation was determined by sulforhodamine B assay. Results shown are mean ± SD of three independent experiments.

3.2.3 SIRT inhibitors display potent in vitro SIRT1/2 inhibitory activities

To unravel the molecular basis for the difference in cytotoxicity between these SIRT inhibitors, we examined the *in vitro* inhibitory potential of these SIRT inhibitors on SIRT1 and SIRT2 using an *in vitro* HDAC activity fluorescent assay. The percent values of SIRT activity at different doses (0-100 μM) of inhibitors were first determined against human recombinant SIRT1 and SIRT2 using a synthetic p53 derived peptide as a substrate, and from these data the IC50 values for the SIRT inhibitors were established (Figure 3.5). The *in vitro* assay revealed that Sirtinol displayed a higher degree of inhibitory activity towards SIRT1 than SIRT2, with IC50 of 37.6 and 103.4 μM, respectively. Salermide also exhibited comparable SIRT1 and SIRT2 inhibitory activity (IC_{50}=76.2 and 45.0 μM, respectively). EX527 showed the higher SIRT1 inhibitory activity (IC_{50}=0.38 μM) and SIRT2 inhibitory activity (IC_{50}=32.6 μM) compared to Sirtinol and Salermide, while nicotinamide had relatively low potency against SIRT1 with an IC50 of 85.1 but potently inhibited SIRT2 (IC_{50}=1.16 μM). These *in vitro* SIRT1/2 inhibition results are generally in consensus with the previously published data on the efficacy of some of these SIRT inhibitors on SIRT1 and -2 [309, 315, 340]. However, these *in vitro* data could not completely explain for the failure of EX527 to induce cell death, as EX527 has higher SIRT2 inhibitory activity than Sirtinol, and higher SIRT1 inhibitory activity compared to Sirtinol and Salermide, yet does not induce cell death.

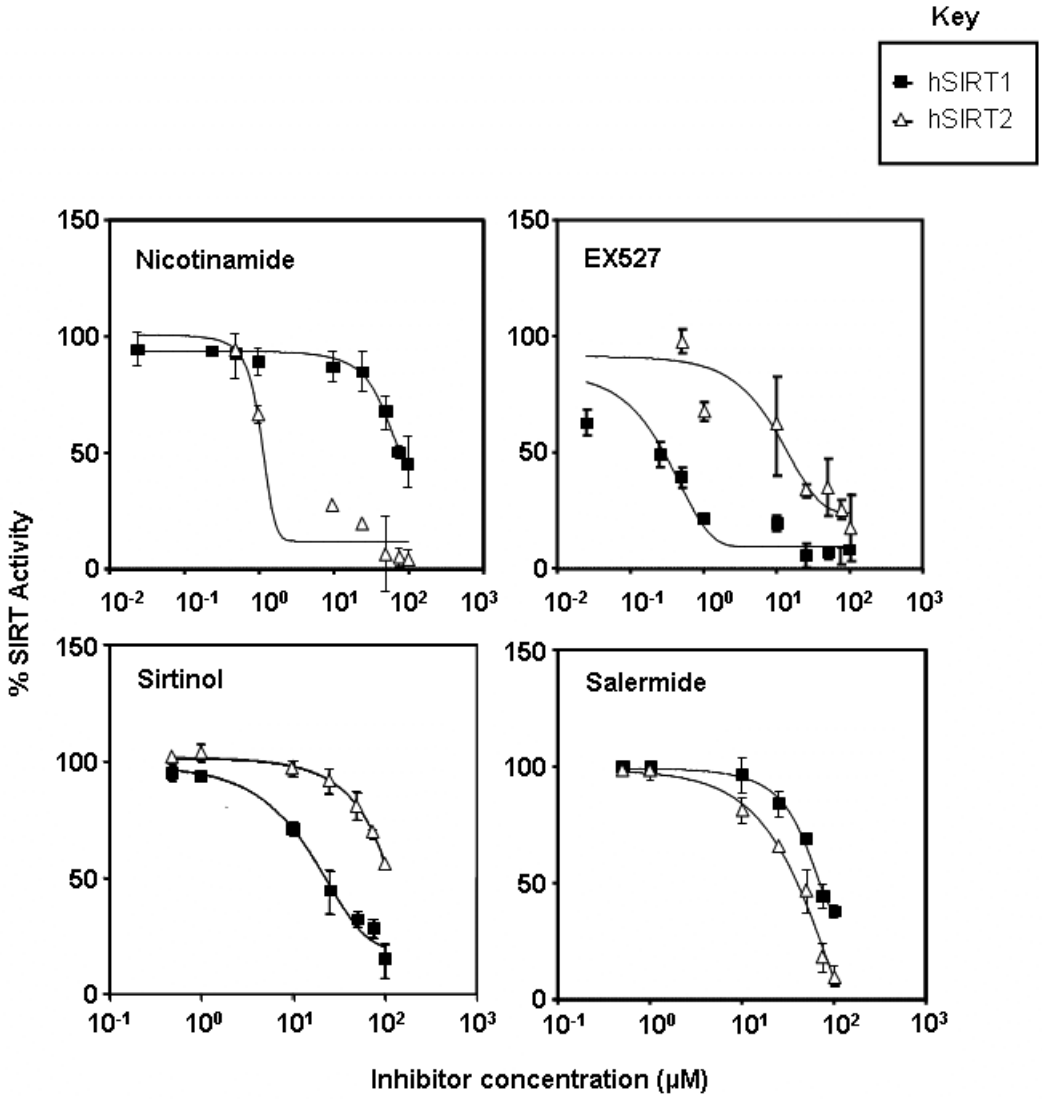


Figure 3.5 *In vitro* SIRT1 and SIRT2 inhibition assays. Recombinant human SIRT1 and SIRT2 were treated with various concentrations of Sirtinol, Salermide, EX 527 and nicotinamide as indicated and their relative inhibitory potential (against DMSO as controls) was analysed and displayed as % of SIRT activity. Results shown are mean ± SD of three independent experiments.

3.2.4 Modelling of SIRT1/2 docking predicts Sirtinol and Salermide binding efficiently to SIRT1/2, and of EX527 to SIRT1 alone

Next, we analysed the inhibitory functions of these SIRT inhibitors using computational docking studies (Modelling was performed by Chun-Yuan Chen, Hong Kong University). Preliminary, computational docking studies were carried out on Sirtinol, Salermide and EX527 using both Gold and LibDock in an effort to rationalize the observed SIRT1/SIRT2 selectivity differences. For these studies, the crystal structure of hSIRT2 (PBD code: 1J8F) was used along with a hSIRT1 model, and the inhibitors docked into the C-pocket (Figure 3.6A). The hSIRT1 model was obtained from the Phyre website (structure 2HJH) and based on the homologous yeast protein Sir2 (Figure 3.6A). In terms of SIRT1 binding, both Sirtinol and Salermide show significant hydrogen bonding interactions with Gln 345 and His 363 (Figure 3.6B). EX527 also displays hydrogen bonding to Gln 345, with additional π- π interactions with His 363 and Phe 273. In terms of SIRT2 binding, Sirtinol and Salermide show comparable hydrogen bonding interactions with Gln 167 (Figure 3.6B). Sirtinol also displays π- π interactions with His 187 and Phe 119, whereas Salermide has a hydrogen bond with His 187 and no interaction with Phe 119. For EX527, we did not find any significant interactions with SIRT2, the closest interaction identified being a van der waals force between EX527 and Ala 85. On the basis of these results, it is plausible that the residues Phe 273, Phe 297, Gln 345, and His 363 of SIRT1 and residues Phe 96, Phe 119, Gln 167, and His 187 of SIRT2 may play critical roles in inhibitor binding. Our docking studies suggest that both Sirtinol and Salermide can form key interactions with these residues in the requisite binding pocket, whereas EX-527 only forms strong interactions with SIRT1. In conclusion, the computational modelling and docking studies suggest that that both Sirtinol and Salermide have high degrees of selectivity for SIRT1/2, while EX527 has a high specificity for SIRT1 but not SIRT2.

The present computational study is in general consistent with the *in vitro* HDAC assay in that both Sirtinol and Salermide target SIRT1 and SIRT2, while EX527 has a high specificity for SIRT1. However, whilst EX527 demonstrated strong *in vitro* inhibitory activity towards SIRT2, the docking assay suggested little affinity between EX527 and SIRT2. Taken together, these findings suggest that Sirtinol and Salermide have a high affinity for SIRT1 and SIRT2, while EX527 is selective for SIRT1 but not SIRT2. These findings also suggested that both SIRT1 and SIRT2 have to be inhibited in order to induce cell death *in vivo* as they target SIRT1 and SIRT2, while EX527 induced cell cycle arrest at G1 because it targets only SIRT1.

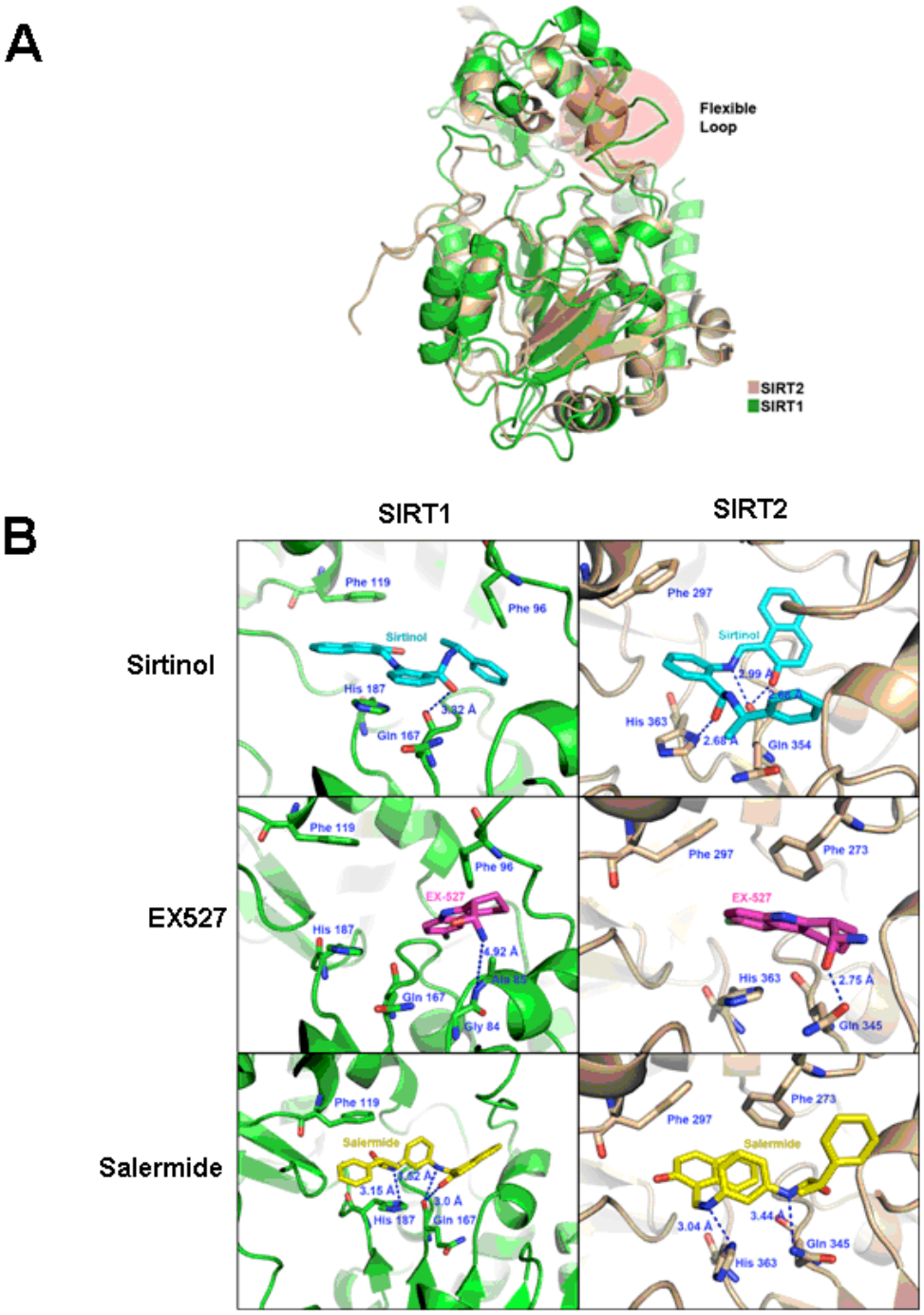


Figure 3.6 The three-dimensional structures of SIRT1 and SIRT2. A) The crystal structure of hSIRT2 (PBD code: 1J8F) was used along with a hSIRT1 model, and the inhibitors docked into the C-pocket. The hSIRT1 model was obtained from the Phyre website (structure 2HJH) and based on the homologous yeast protein Sir2. **B)** Structures of the complexes of inhibitors with SIRT1 and SIRT2 modelled by docking the inhibitors onto the C-pocket of SIRT1 and SIRT2 structure after minimization (Modelling was performed by Chun-Yuan Chen, Hong Kong University).

3.2.5 Sirtinol and Salermide but not EX527 can induce p53 acetylation *in vivo*

To investigate further the distinct effects of the SIRT inhibitors on cell fate, we performed Western blot analysis on MCF-7 cells treated with 50 μM for 0, 24 and 48 h, to study their effects on the acetylation status of the SIRT1/2 target p53 (Figure 3.7A). Interestingly, Sirtinol and Salermide but not EX527 could induce p53 acetylation at lysine 382, which has been shown to correlate with p53 activation. Consistently, the p53 acetylation was also accompanied by an induction of p53 stability. The control nicotinamide was ineffective in inducing p53 acetylation at the concentrations studied. To examine the effects of the compounds on SIRT2 activity, we investigated the expression levels of acetylated tubulin, which has been shown to be a specific SIRT2 target [243]. The results demonstrated that only Sirtinol and Salermide but not EX527 and nicotinamide could induce tubulin acetylation. We next treated MCF-7 cells for 24 h with a range of concentrations of SIRT inhibitors and harvested the cells for Western blot analysis (Figure 3.8B). Similar to earlier data, the results showed that at concentrations of $\geq$ 50 μM, Sirtinol and Salermide, but not EX527 and nicotinamide, are able to induce p53 acetylation and that only Sirtinol and Salermide but not EX527 and nicotinamide can induce tubulin acetylation. Notably, there was also a general decrease in protein levels in MCF-7 cells after treatment with 100 μM Salermide and was likely to be a result of protein degradation due to cell death after drug treatment. The failure of EX527 to induce p53 and tubulin acetylation was not because of its lack of SIRT-inhibitory activity as it could effectively cause global protein acetylation as revealed by the acetyl-Lysine antibody. Together these results suggest that while both Sirtinol and Salermide target SIRT1 and -2, EX527 only inhibits SIRT1 and not SIRT2 effectively and that p53 acetylation requires inhibition of both SIRT1 and -2.

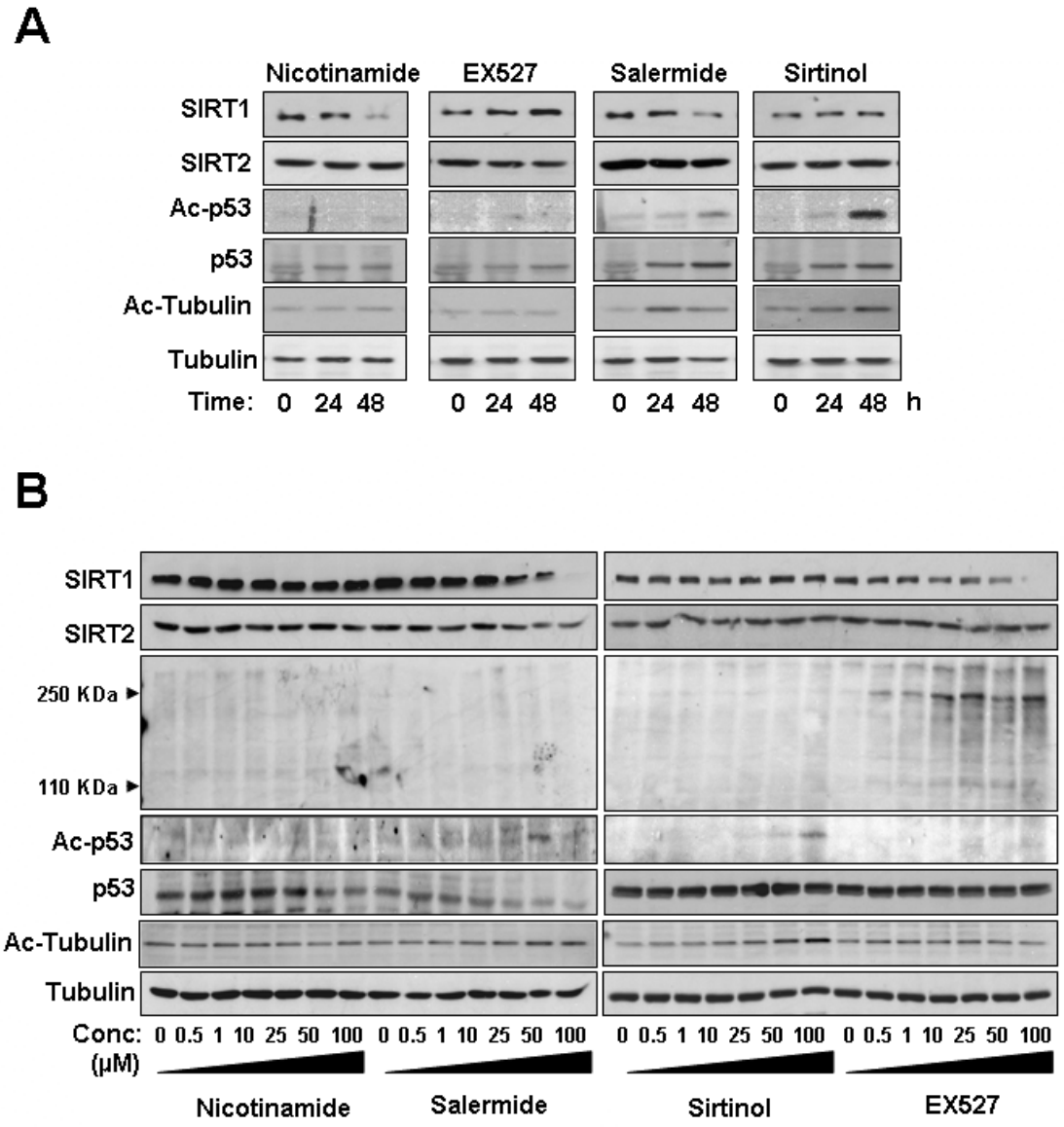


Figure 3.7 Effects of SIRT inhibitors on p53, tubulin, and global lysine acetylation. A) MCF-7 cells were treated with 50 μM of SIRT inhibitors for 0, 24 and 48 h of nicotinamide, Salermidem Sirtinol or EX527. The expression of SIRT1, SIRT2, acetylated-lysine, acetylated-p53, p53, acetylated-tubulin and tubulin was analysed by western blotting. **B)** MCF-7 cells were treated with increasing concentrations (0-100μM) and western blot analysis was performed to determine the protein expression levels of SIRT1, SIRT2, acetylated-p53, p53, and tubulin.

3.2.6 The cytotoxic function of sirtinol and salermide is dependent on the presence of functional p53

To examine the role of p53 in mediating the cytotoxic and cytostatic effects of the SIRT inhibitors, we treated breast carcinoma cell lines [341], MCF-7 (wild-type p53), MDA-MB-231(mutant p53), BT474 (mutant p53), and ZR-75-1 (very low p53 expression) with 50 μM of SIRT inhibitors for 0, 24 and 48 h. Cell cycle analysis results showed that both Sirtinol and Salermide effectively induced cell death in MCF-7 cells, but the ability of Sirtinol and Salermide to induce cell death is compromised in other breast cancer cell lines without functional p53 (Figure 3.8). Notably, EX527 treatment caused a substantial increase in G1 cell population with little cell death in all breast carcinoma cell lines, with the exception of BT-474. To further confirm these results, we also studied the effects of the SIRT inhibitors on the cell cycle status of mouse embryo fibroblasts (MEFs) derived from wild-type and p53-deficient mice (Figure 3.9). Consistent with the breast carcinoma cell line data, the flow cytometric results indicated that while EX527 treatment caused a substantial increase in G1 population with little cell death in both the wild-type and *p53*$^{-/-}$ MEFs, Sirtinol and Salermide only induced cell death efficiently in wild-type but not *p53*$^{-/-}$ MEFs (Figure 3.9A). We next examined the expression of acetylated-lysine, acetylated-p53, acetylated-tubulin in the wild-type and *p53*$^{-/-}$ MEFs following SIRT inhibitor treatment (Figure 3.9B). The western blot results showed that Sirtinol and Salermide, but not EX527 , are able to induce p53 acetylation in the wild-type MEFs, and that Sirtinol and Salermide but not EX527 can induce tubulin acetylation in both the wild-type and *p53*$^{-/-}$ MEFs. Western blotting using the acetyl-Lysine antibody again revealed that EX527 is more effective than Sirtinol and Salermide in inducing the acetylation of some proteins and some of these EX527 targets might account for its ability to induce G1 arrest. Cleaved caspase-3 was used as a marker of apoptosis, and the western blot results showed loss of p53 decreases the cleavage and activation of caspase-3, reiterating our findings from the cell cycle analysis (Figure 3.9B). Together these results suggest that functional p53

is required for the cytotoxic function of Sirtinol and Salermide but is dispensable for the cytostatic effects of EX527.

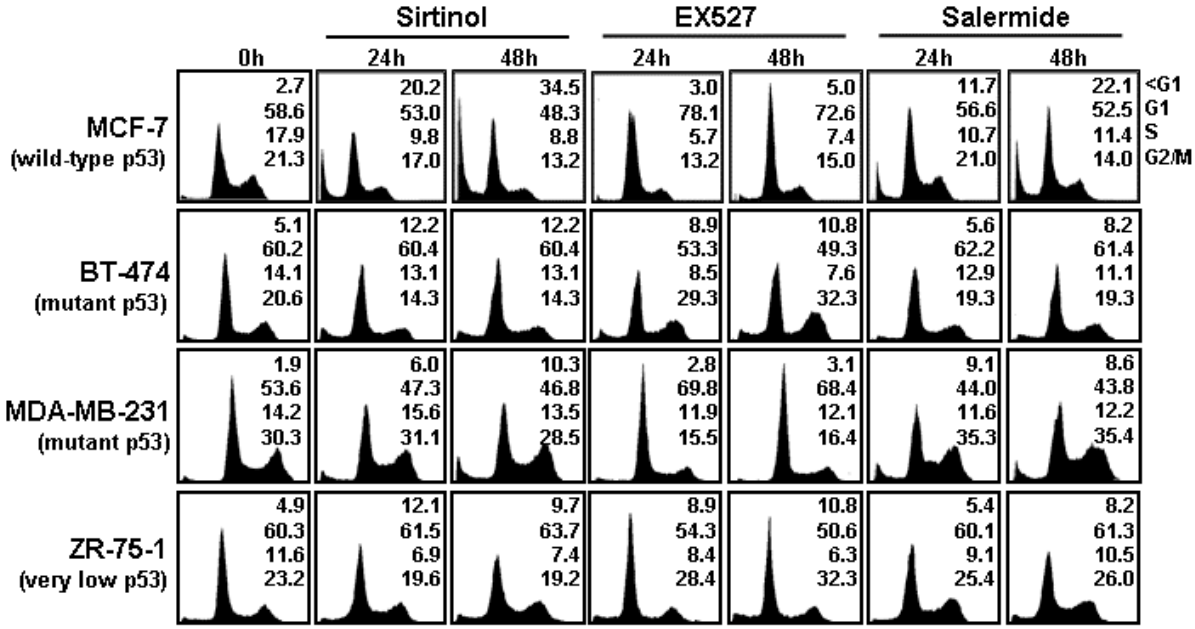


Figure 3.8 The effect of SIRT inhibitors on the cell cycle and dependence on functional p53 A) MCF-7, MDA-MB-231, BT474, and ZR-75-1 breast carcinoma cells were treated with 50 μM of SIRT inhibitors for 0, 24 and 48 h of Sirtinol, EX527 and Salermide. Flow cytometric analysis of DNA content was performed after propidium iodide staining. Percentage of cells in each phase of the cell cycle (sub-G1, G1, S, and G2/M) is indicated. Representative data from three independent experiments are shown.

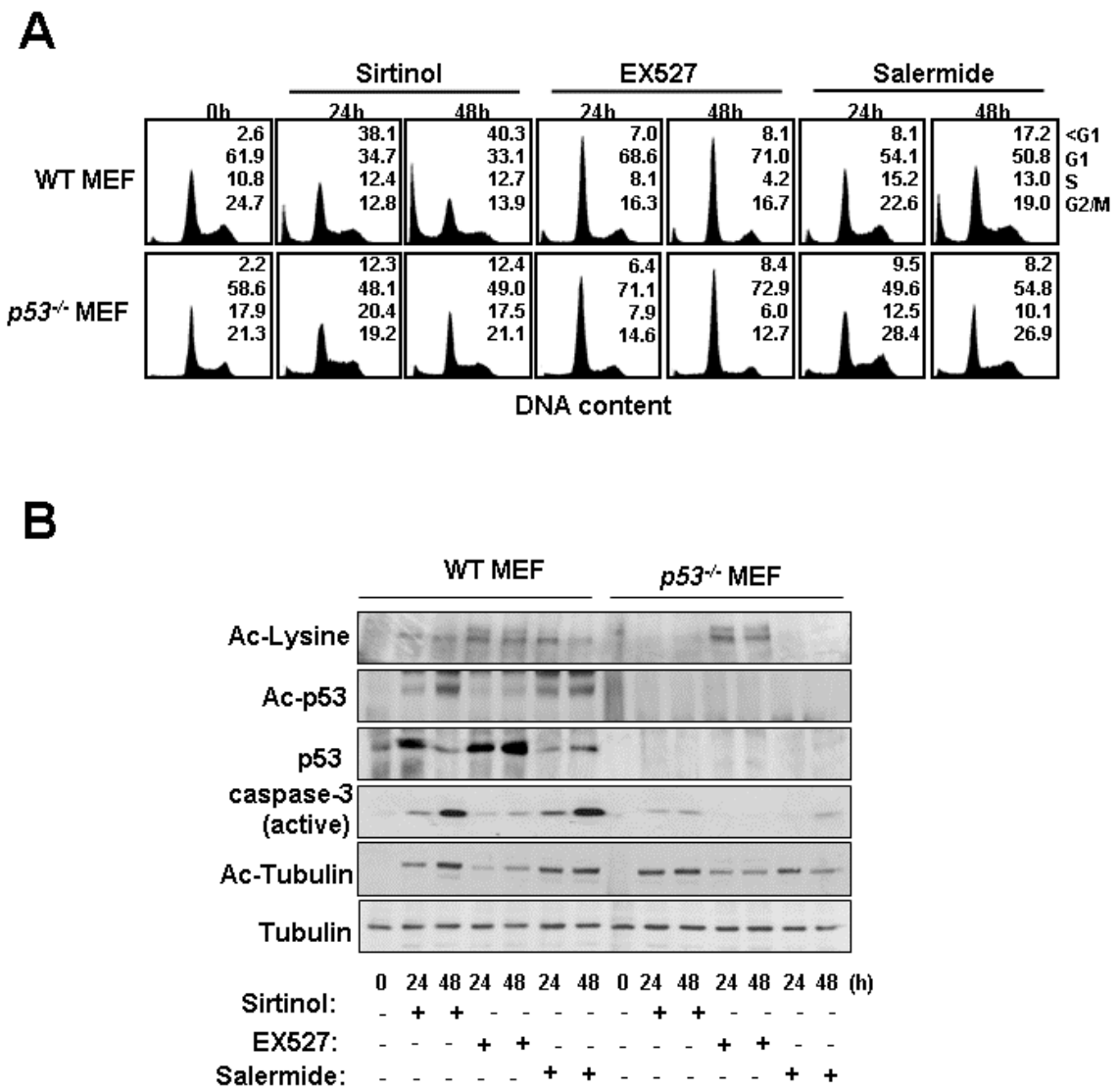


Figure 3.9 Effects of SIRT inhibitors on cell cycle status and p53, tubulin, and global lysine acetylation. A) wild-type (WT) and *p53*$^{-/-}$ MEFs were treated with 50 μM of SIRT inhibitors for 0, 24 and 48 h of Sirtinol, EX527 and Salermide. Flow cytometric analysis of DNA content was performed after propidium iodide staining. Percentage of cells in each phase of the cell cycle (sub-G1, G1, S, and G2/M) is indicated. Representative data from three independent experiments are shown. B) The expression of cleaved (active) caspase-3, acetylated-lysine, acetylated-p53, p53, acetylated-tubulin and tubulin was analysed by western blotting in the SIRT inhibitor-treated wild-type (WT) and *p53*$^{-/-}$ MEFs.

3.2.7 EX 527 protects MCF-7 cells from paclitaxel treatment

To access whether combination of sirtuin inhibitors could increase the proapoptotic potency of an established cytotoxic agent, 50μM of sirtinol, salermide and EX527 were treated alone or in combination with 10nM of paclitaxel for 0-72 hours, cell proliferation was assessed using SRB assay (Figure 3.10A). Treatment of paclitaxel resulted in a decrease in cell viability, suggesting that the cells were undergoing apoptosis. As previously observed Sirtinol treatment was the most cytoxic of the sirtuin inhibitors but no combination of paclitaxel with sirtinol or salermide resulted in a marked decrease in cell viability compared with paclitaxel treatment alone. Interestingly EX527 treatment was able to protect cells from paclitaxel treatment. To assess this further, MCF-7 cells were treated with sub-lethal concentrations of paclitaxel (0-2.5nM) alone or in combination with Sirtinol, EX-527 and Salermide for 72 hours, cell viability being determined using SRB (Figure 3.10B).

A decrease in cell viability was observed as you increased the concentration of paclitaxel (287.8% ± 10.2, 219.1% ± 25, 64.3% ± 2.9 and 68.8% ± 9.3 for 0, 0.1, 1 and 2.5nM paclitaxel treatment respectively) (Figure 3.10B). The treatment of Sirtinol and Salermide (50μM) alone resulted in reducing cell viability below 100% meaning cell cycle arrest and apoptosis had occurred. Sirtinol and Salermide were able to reduce cell viability without paclitaxel. Interestingly, cotreatment of EX527 with paclitaxel at all concentrations protected MCF-7s from paclitaxel treatment. This effect was especially marked at 1nM treatment of paclitaxel with EX527, where single treatment with paclitaxel reduced the cellular population to 64.3% (SD ± 2.9), single treatment of EX527 had no effect (277% ±16.1 compared to 287.8% ± 10.2 of the vector treated control) whereas combination with EX 527 resulted in 226.4% (SD ±21.7), a marked protection in cell viability.

This highlights that therapeutically, some sirtuin inhibitors could actually decrease the efficacy of established chemotherapeutic drugs when used in combination, the converse to what would be expected.

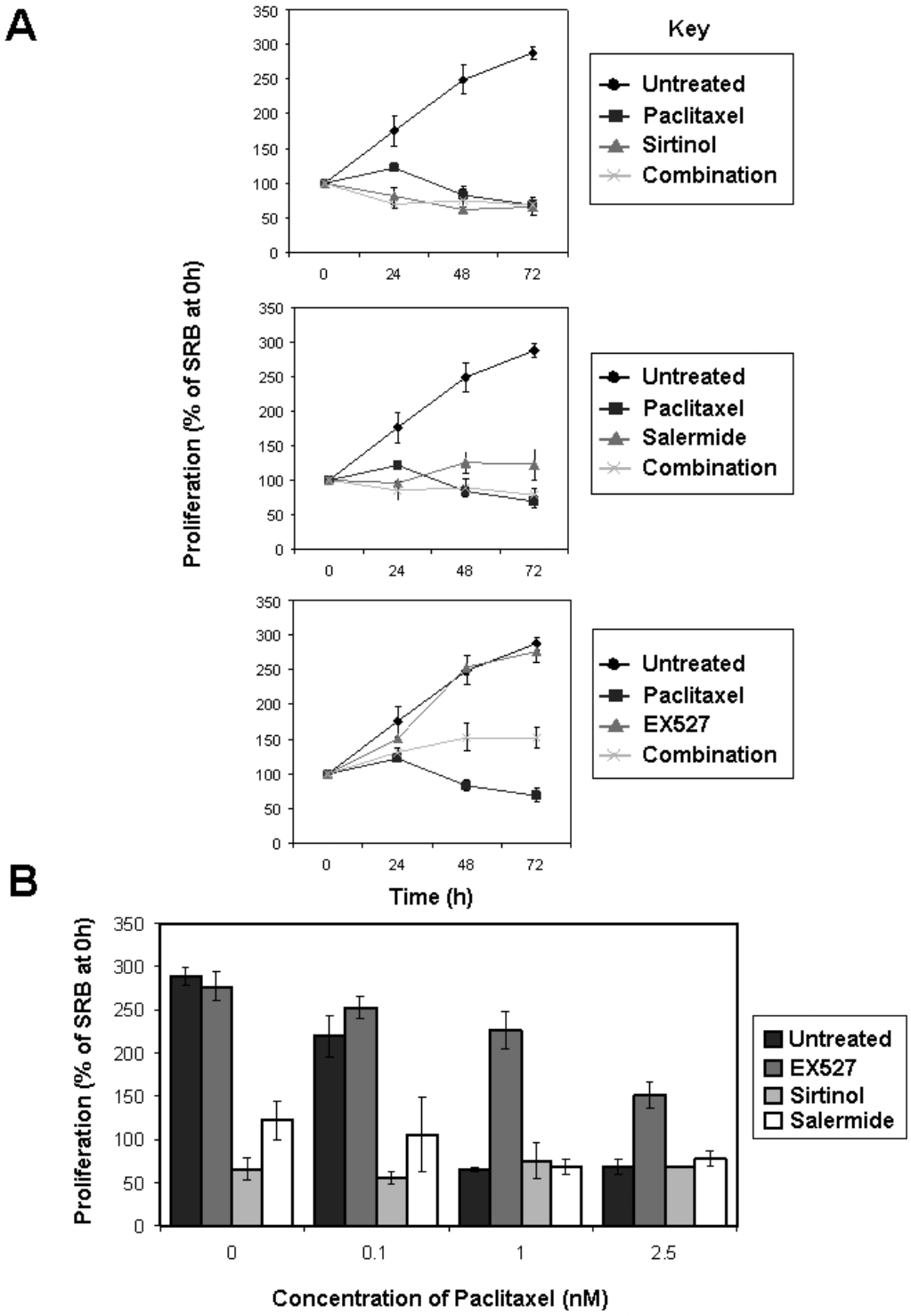


Figure 3.10 EX527 protects against paclitaxel treatment. A) MCF-7 cells were treated with 10nM of Paclitaxel, 50μM of SIRT inhibitors alone or in combination for 0, 24, 48 and 72 h. **B)** MCF-7 cells were treated with 0, 0.1, 1 and 2.5nM of Paclitaxel alone or in combination with 50μM of SIRT inhibitors. Cell proliferation was determined by sulforhodamine B assay. Results shown are mean ± SD of three independent experiments.

3.2.8 Silencing of both SIRT1 and SIRT2 is required for inducing MCF-7 cell death

Our earlier results suggested that induction of cell death by SIRT inhibitors *in vivo* requires the inactivation of both SIRT1 and SIRT2. To examine this conjecture, MCF-7 cells were transfected with either control siRNA or siRNA against SIRT1, SIRT2 or SIRT1 and SIRT2. The transfected cells were then incubated with or without 10nM of the chemotherapeutic drug paclitaxel for 24 h before subjected to cell cycle and Western blot analyses. The cell cycle analysis showed that siRNA-mediated knockdown of endogenous SIRT1 or SIRT2 individually had no discernable effect on the cell cycle status of MCF-7 cells, but silencing of endogenous SIRT1 and SIRT2 simultaneously induced a noticeable G2/M phase cell cycle arrest as well as cell death (Figure 3.11A). Interestingly, treatment of MCF-7 with 10nM paclitaxel also elicited a cell death and cell cycle arrest profile similar to that induced by SIRT1/2 knockdown. In addition, we discovered that silencing of SIRT2 alone actually abolished the paclitaxel-induced G2/M cell cycle arrest and cell death. The Western blot analysis confirmed successful SIRT1 and/or SIRT2 knockdown and showed that the SIRT1/2 knockdown-induced G2/M arrest and cell death was associated with an increase in p53 acetylation (Figure 3.11B).

Intriguingly, paclitaxel treatment also resulted in a similar increase in p53 acetylation, and this p53 hyperacetylation was again accompanied by cells undergoing G2/M arrest and cell death. In addition, we discovered that silencing of SIRT2 alone by specific siRNA suppressed paclitaxel-induced cell cycle arrest and cell death as well as p53 acetylation. Together these results suggest that paclitaxel mediates the G2/M arrest-associated cell death through inducing the acetylation and activation of p53.

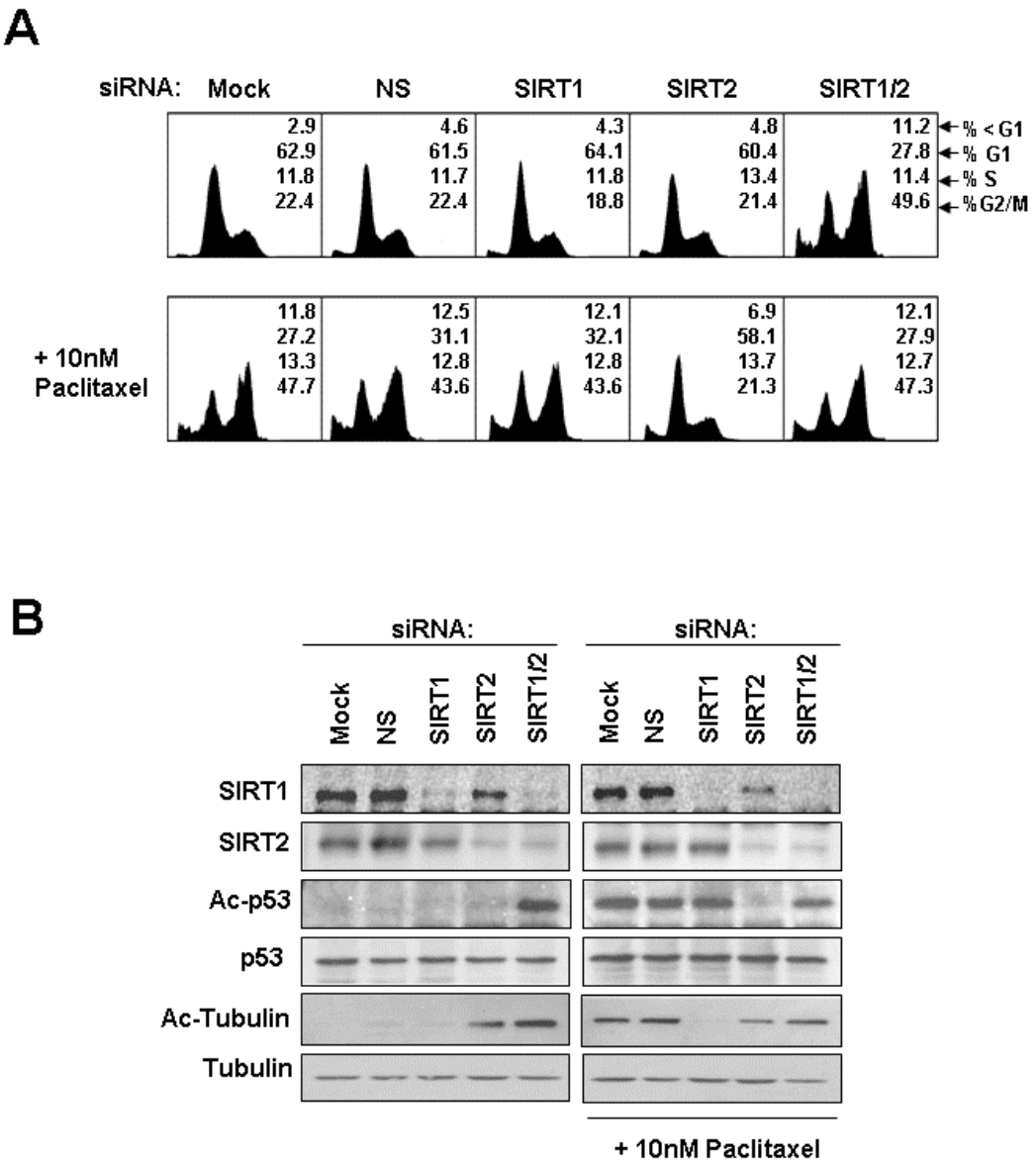


Figure 3.11 Effects of SIRT1/2 silencing and paclitaxel on cell cycle progression and p53 acetylation. MCF-7 cells were either untransfected (Mock), transfected with non-specific (NS) siRNA (100 nM) or siRNA smart pool against SIRT1 (100 nM) , SIRT2 (100 nM), or SIRT1 plus SIRT2 (100 nM) for 24 h and then incubated for another 24 h in the absence or presence of 10nM paclitaxel. A) Flow cytometric analysis of DNA content was performed after propidium iodide staining. Percentage of cells in each phase of the cell cycle (sub-G1, G1, S, and G2/M) is indicated. Representative data from three independent experiments are shown. B) The expression levels of SIRT1, SIRT2, acetylated-p53, p53, and tubulin were determined by Western blotting.

3.3 DISCUSSION

In this study, we have for the first time compared the potencies, mechanisms of action, and cellular targets of the three recently developed SIRT inhibitors, Sirtinol, Salermide and EX527. From cell proliferation and cell cycle assays, it was found that while Sirtinol and Salermide can effectively induce cell death, EX527 does not trigger cell death, but a cell cycle arrest. The *in vitro* SIRT inhibitory assay shows that all three compounds potently inhibited SIRT1, with EX527 at least two orders of magnitude more potent than Sirtinol and Salermide. This finding is supported by computational docking studies demonstrating that all three inhibitors bind SIRT1 with high affinity. However, despite the *in vitro* SIRT inhibitory assay showing high degrees of inhibitory activity against SIRT2 for all three compounds, only Sirtinol and Salermide exhibit high affinity for SIRT2 in the structural studies. It is important to note that the structural studies do have their own limitations - providing a qualitative examination of SIRT2 inhibition using sirtuin small molecule inhibitiors - but the results are supported by the *in vivo* finding that EX527 failed to cause the re-acetylation of tubulin, a critical cellular target of SIRT2 [342]. This finding is also consistent with our *in vivo* results indicating that inactivation of both SIRT1 and SIRT2 is required for induction of cell death and p53 acetylation and that EX527 neither triggers cell death nor p53 acetylation in MCF-7 cells. The structural-*in vitro* activity discrepancy could be due to the fact that in the *in vitro* SIRT2 inhibitory assay the recombinant human SIRT2 is the single predominant Sirtuin species existing at high concentrations and the specificity might be lost as a result. Alternatively, EX527 could have an altered selectivity towards the recombinant SIRT2 protein under *in vitro* experimental conditions. Moreover, the *in vitro* assays used a short synthetic p53 peptide instead of the full-length wild-type protein. These observations showed that the *in vitro* SIRT assays are not always consistent with findings *in vivo*, which may be based upon cell cycle analysis, analysis of global acetylation profiles, SIRT isoform-specific target acetylation, and docking studies. Nevertheless, this also highlights the fact that the *in vitro* SIRT assay data has to

be interpreted with caution and should not be use alone in studying SIRT or inhibitor activity. It is important to note however, that while the structural and computational docking studies provide a qualitative appreciation of possible sources of inhibitor selectivity, further detailed biological study is required (for example, site-selective mutations) to verify these results *in vivo*. For example, while the results provide qualitative comparisons with a recent report by Lara *et al* [321], the precise binding modes of the inhibitors differ. Moreover, computational studies by Huhtiniemi *et al* [343] suggest that the conformational freedom of the 'flexible loop' region of the C-pocket may play a crucial role in substrate binding, something that is not taken into account using static docking techniques. Pacholec *et al* have recently employed novel NMR techniques to elucidate sirtuin-targeted compounds *in vivo* to great success, suggesting that the discrepancies that we have observed between structural modeling and *in vitro* assays experiments could be investigated further using such techniques [344].

It is notable that in this study we examined the cellular potency and targets of the SIRT inhibitors using the MCF-7 breast carcinoma cell line because they have substantial levels of SIRT1 and SIRT2 as well as a functional p53. In mammalian cells, the transcription factor *p53* functions as a tumor suppressor and has been shown to be a target of acetylation as well as SIRT1 and SIRT2. Acetylation of functional p53 leads to its activation and is usually triggered in response to oncogenic as well as environmental stress signals, including oxidative stress and chemotherapeutic drug treatments [345]. Once activated, p53 triggers the apoptotic programme to induce cell death. In cell based *in vivo* studies, both Sirtinol and Salermide but not EX527 can efficiently induce p53 acetylation and stabilization, and this was associated with the induction of cell death in MCF-7 cells. Moreover, further studies using breast carcinoma cell lines with different p53 status and wild-type and p53-deficient MEFs showed that Sirtinol and Salermide are ineffective in inducing apoptosis of cell lines that lacked p53 expression. These findings suggest that the tumour suppressor p53 is integral to

the cytotoxic activity of the SIRT inhibitors Sirtinol and Salermide, and the failure of EX527 to trigger cell death is because of its inability to cause the acetylation and activation of endogenous p53. Lain *et al.* [312] also reported that they found no acetylation of p53 in response to EX527 monotreatment, although the data was not shown in the publication. A cooperative role for SIRT1 and SIRT2 to regulate stress-induced cell death pathways in a p53-independent manner has also been described [339]. It is also likely that SIRT inhibitors, like Sirtinol and Salermide, also target SIRTs other than SIRT1 and -2. Nevertheless, SIRT1 and -2 will probably have a more prominent role in controlling cell growth and survival as they exist in the same intracellular compartments (ie. nucleus and cytoplasm) as most of the cell cycle and death regulators [229]. The reason why Sirtinol and Salerimide are ineffective in causing global lysine acetylation is unclear. However, since these global lysine acetylation patterns are associated with treatment with the very potent SIRT1 inhibitor EX527, it could be that these acetylated patterns might represent proteins preferentially targeted by SIRT1 and/or other HDACs.

Data from SIRT inhibitor experiments also suggest that both SIRT1 and SIRT2 have to be inactivated in order to induce cell death *in vivo,* at least in SIRT1 and SIRT2 positive cells. This is confirmed by the siRNA-silencing experiments showing that not one but both SIRT1 and SIRT2 have to be deleted to cause cell death. This observation is consistent with a recent study demonstrating that despite the interaction of SIRT1 with p53, deletion of SIRT1 in MEFs fails to induce p53 acetylation and activate the apoptotic function of p53 [346]. Notably, cell cycle profiles of MCF-7 cells are different in response to treatment with EX-527 compared to SIRT-1 siRNA, and this might suggest that additional mechanisms of induction of cell cycle arrest by EX-527, which could be mediated by inhibiting other histone deacetylases (HDACs). Intriguingly, treatment with the chemotherapeutic drug paclitaxel also induces a G2/M-arrest associated cell death indistinguishable from that triggered by silencing of SIRT1 and SIRT2. The fact that this was also accompanied by p53 acetylation suggest that SIRT

inhibitors, such as Sirtinol and Salermide, and paclitaxel function through common pathways and mediate their cytotoxic effects through targeting p53 and acetylation. Unexpectedly, silencing of SIRT2 is sufficient to repress the cytotoxic effects of paclitaxel and abolish p53 hyperacetylation. Similarly, SIRT1 knock-down could also prevent paclitaxel from causing the reacetylation of tubulin. The molecular mechanisms responsible are unknown, and might involve compensatory mechanisms between different SIRT proteins. It is also likely that SIRT2 silencing will cause cell cycle arrest and chromatin condensation as revealed by *sirt2*-deficient mouse studies [244]. As a consequence, SIRT2 silencing alone may lessen the potency of chemotherapeutic drugs, such as paclitaxel, which preferentially target proliferating cells. Examining whether the differing sirtuin inhibitors could complement paclitaxel treatment, MCF7 cells were treated the sirtuin inhibitors alone and in combination with paclitaxel. There was no perceived additive or synergistic effect of paclitaxel with sirtinol or salermide, reiterating that their have already reached the maximum potency with which they can induce apoptosis. EX527 treatment did reduce the potency of paclitaxel treatment both when treated with increasing concentrations and over a time course, protecting cells from apoptosis, most likely due to the potent arrest at G1. Moreover, the potent G1 arrest observed with EX527 treatment is overcome when the compound is withdrawn, allowing cells to proliferate normally. Unlike sirtinol and salermide treatment, whose effect on the cellular phenotype cannot be reversed. These findings add caution to using any sirtiun inhibitor in combination with established chemotherapeutic regimes, without investigating their exact effect on the cellular phenotype. Compunds that are not fully illucidated could have undesirable effects.

In summary, we have used a combination of *in vitro* SIRT inhibition assay, structural studies and *in vivo* cell based systems to investigate the substrate specificity and efficacy of three novel SIRT inhibitors, Sirtinol, Salermide and EX527. Together the results also provide evidence to show that the cytotoxic effect of SIRT inhibitors is mediated predominantly through the inactivation of

both SIRT1 and -2 and the subsequent acetylation of the tumour suppressor p53. These data also suggest that a redundancy of functions may exist between SIRT1 and SIRT2, and that SIRT1 and SIRT2 cooperate to deacetylate the tumour suppressor protein p53 to attenuate cell death.

3.4 FUTURE WORK

To elucidate further the cytotoxic functions of sirtinol and salermide, it would be interesting to make resistant cell lines to these compounds to observe the exact mechanism that regulate sirtuin mediated apoptosis. They are still able to initiate apoptosis in a p53 independent manner, albeit less efficiently. Understanding these mechanisms will increase the potency of sirtuin inhibitors and possibly their clinical relevance.

Due to the fact that EX527 treatment results in the acetylation of a different subset of genes to other sirtuin inhibitors, investigating further the exact genes that are acetylated with EX527 treatment could represent a new pool of genes that are only deacetylaed by SIRT1. Because SIRT1 is found to be upregulated in many cancers, these posttranslational targets could be important in tumour progression and could be impart responsible for SIRT1s role in tumourigenesis.

The cell cycle profile observed with SIRT1 and SIRT2 knockdown displays a marked increase in the G2-M population, as well as an increase in sub G1 cells. This is different to the cell cycle profile of sirtinol and salermide treated cells containing WT p53 where a marked increase in sub G1 cells is observed, meaning that although SIRT1 and SIRT2 inhibition results in the induction of apoptosis, sirtinol and salermide are executing their cytotoxic actions by additional mechanism, possibly by inhibiting other sirtuin family members. The development of SIRT1-/- and SIRT2-/- knockout MEFs would allow for the study of the addition functions of sirtinol and salermide.

CHAPTER 4

RESISTANCE TO LAPATINIB THROUGH LOSS OF FOXO3a IN SKBR3 CELLS

4.1 INTRODUCTION

The human epidermal growth factor (HER) family consists of four members, EGFR, HER2, HER3 and HER4. They are type I transmembrane growth factor receptor tyrosine kinases, EGFR and HER2 being the most well defined members [347]. HER2 (neu), the oncogenic tyrosine kinase is overexpressed in 25% of breast cancer cases; unfortunately these tumours are more prevalent in premenopausal women and ultimately lead to a poorer clinical outcome. This makes HER2 a promising therapeutic target [14]. The study of 5,232 breast cancer cases showed 48% to be EGFR positive [99]. Although EGFR expression does not inversely correlate with patient survival, like HER2 overexpression, recent publications are starting to unravel its importance, suggesting that its subcellular localisation is important in breast cancer, showing nuclear accumulation of EGFR is inversely correlated with patient survival [102].

Lapatinib (also known as GW572016 and Tykerb) is an orally administered potent small molecule inhibitor that functions as a dual inhibitor, binding reversibly to EGFR and HER2 tyrosine kinases, inducing growth arrest or tumour cell apoptosis in EGFR dependent or HER2-depedent tumour cell lines or xenographs [290, 348]. It is currently the most clinically developed dual tyrosine kinase inhibitor (TKI) and was found to increase progression-free survival in patients with advanced HER2-postive tumours when given in combination with the antimetabolite chemotherapy drug capecitabine (Xeloda) compared with capecitabine alone, in patients who had already failed previous treatment regimes with anthracyclines, taxanes and trastuzumab [349].

Although Lapatinib shows promise, some patients develop resistance and eventually relapse [350, 351]. Unfortunately, these mechanisms of resistance are poorly understood. Elucidating the mechanisms of acquired resistance to TKIs will provide essential information that will lead to advances in novel therapeutic strategies and in turn, lead to increased clinical efficacy.

We and others have shown previously the importance of FOXO3a in eliciting the proapoptotic potency of TKI therapy [291-293]. FOXO3a a member of the forkhead family of transcription factors and is negatively regulated by the PI3K/AKT signaling cascade [126]. HER2 overexpression in cancer leads to the up-regulation of PI3K activity and phosphorylation of AKT. Upon activation, AKT enters the nucleus where it phosphorylates FOXO3a, causing its nuclear export and subsequent inhibition of transcriptional activity [352, 353]. FOXO3a is known to regulate a plethora of target genes important for cell cycle regulation (e.g., *p27Kip1*, *p130(RB2)*, *cyclin D*), apoptosis (e.g., *Bim, Fas-ligand* and *TRAIL*) and oxidative stress (e.g., *mnSOD and Catalase*) [84, 151, 156, 165, 170, 171, 173, 176], so inhibiting its transcriptional activity results in growth, survival and development of cancer cells.

It has been shown recently that low expression of FOXO3a in ovarian cancer [354] and the nuclear exclusion of FOXO3a in Acute Myeloid Leukemia is linked with a poorer clinical outcome [355]. In this study we report the loss of FOXO3a expression in the *in vitro* derived lapatinib resistant cell lines from SkBr3 breast cancer cells. Moreover, loss of FOXO in NIH 3T3 mouse embryonice fibroblasts confers resistance to lapatinib and FOXO knock out cells can be resensitised to lapatinib by reinstating FOXO3a expression, suggesting modulation of FOXO3a expression with combined therapy could circumvent lapatinib resistance and improve clinical outcome.

4.2 RESULTS

4.2.1 Generation and characterisation of Lapatinib resistant cells

SkBr3 breast cancer cell lines were continually exposed to increasing concentrations of lapatinib *in vitro* for two months, after which resistance developed and cells were actively proliferating in the presence of lapatinib. Resistant cells (termed SkBr3 R5) were then maintained in 0.5μM of lapatinib. SRB assay was used to confirm resistance, WT and R5 cells were treated with increasing concentrations of lapatinib for 72 hours, the IC50 for WT and R5 cells were 0.42 and 1.23μM respectively (Figure 4.1A). R5 cells tolerated concentrations ~3 times higher than that of the parental cell lines. Cell cycle analysis was also performed; WT and R5 cells were treated with 1μM of lapatinib for 24 hours (Figure 4.1B). WT cells treated will lapatinib resulted in a reduction of cells in the G1 phase of the cell cycle (66.9 to 53.9%) and an increase in sub G1 (7.2 to 30.2%) indicating that they were undergoing apoptosis. Conversely a reduction in cells in the G1 phase of the cell cycle was not observed in R5 cells treated with lapatinib (65.8 to 67.2%). Although there was an increase in the sub G1 population, this was not as marked as observed in the parental cell lines. Together these results confirm that SkBr3 R5 cells are more resistant to lapatinib compared to their parental cell line.

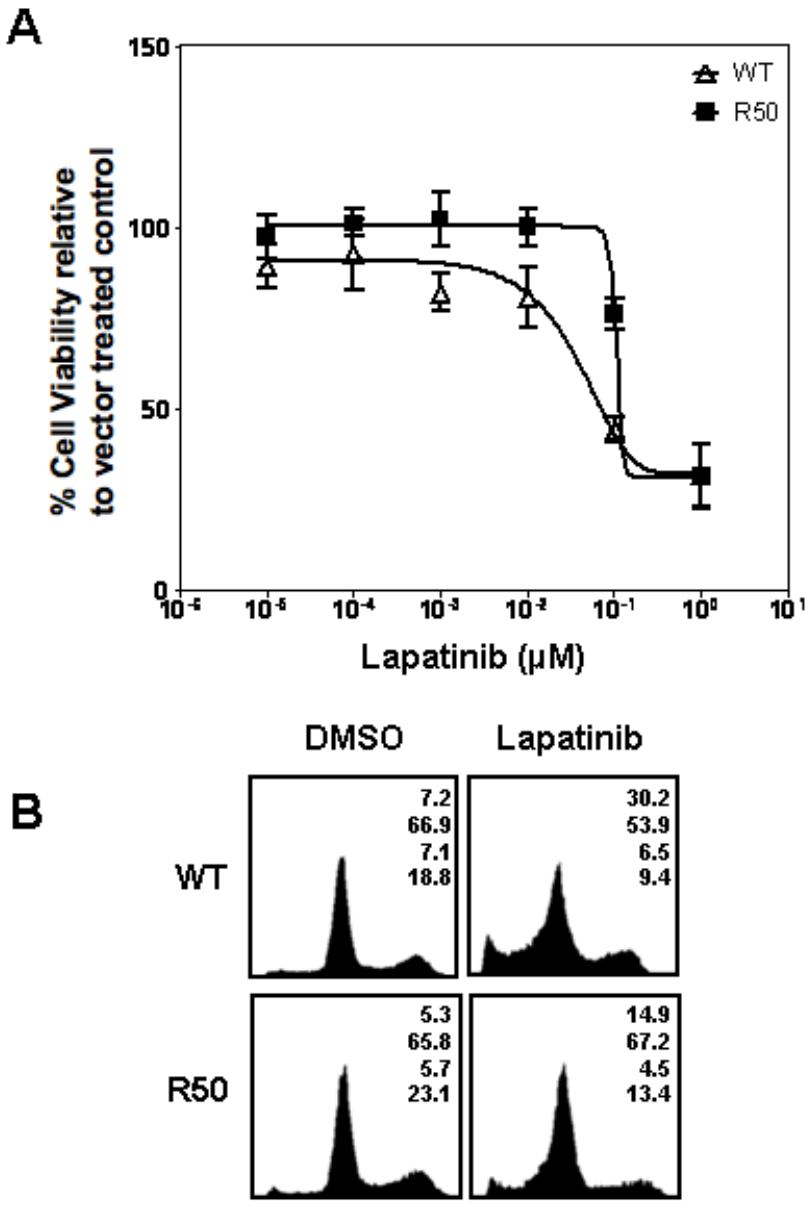


Figure 4.1 SkBr3 R5 cells are resistant to lapatinib compared with parental cell lines. **A** Resistant R5 and parental SkBr3 cells (WT) were treated with 0-10μM of lapatinib for 72 h. Cell viability was determined by SRB assay. Points, mean of three independent experiments; bars, SD. **B** Resistant R5 and WT cells were treated with DMSO or 1μM of Lapatinib for 24 h, flow cytometric analysis of DNA content was done after propidium iodide staining. Percentage of cells in each phase of the cell cycle (sub-G 1, G1, S, and G2-M) is indicated. Representative data from three independent experiments are shown

We and others have previously shown FOXO3a to be important in eliciting the proapoptotic potency of EGRF/HER2 targeted therapies [291-293]. To examine the effect of the development of resistance on FOXO3a and other proteins important in PI3K signaling, protein expression was elucidated using western blot (Figure 4.2A). The protein expression of the EGFR family members, EGFR, HER2 and HER3 did not change between WT and R5 cells, although an increase in EGFR phosphorylation is observed. In keeping with this, a slight increase in the phosphorylation of AKT (308 & 473) was also observed, as well as a marked decrease in P-ERK (202/204). Strikingly, there was a significant reduction in the protein expression of FOXO3a (as well as P-FOXO3a Thr32). We have shown previously that there is an inverse correlation between the expression of FOXO3a and the pro-proliferative transcription factor FOXM1 [98]. This observation is reiterated in the R5 cells, where there is an increase in expression of FOXM1. We also checked the expression of MDR1, which we have previously shown to be important in the FOXO3a-dependent development of chemoresistance in leukemic cells [356]. In this setting, there was no change in the expression of MDR1.

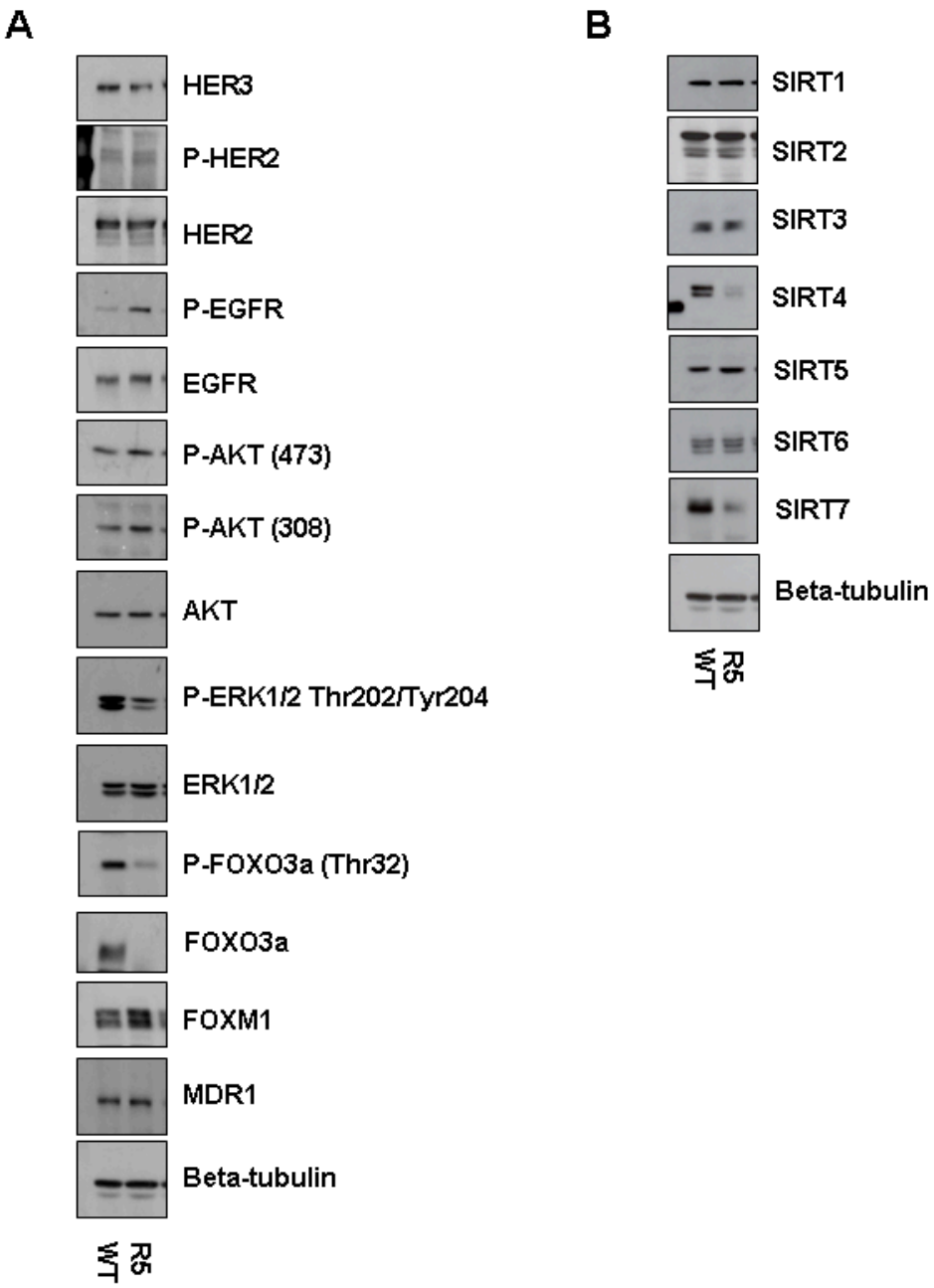


Figure 4.2 Characterisation of the R5 cells and their parental cell line SkBr3. Lapatinib resistant SkBr3 cells were made by exposing WT cells to increasing concentrations of lapatinib over a period of 2 months. Resistant cells were defined as those able to actively proliferate in lapatinib-suplemented medium. Expression of proteins involved in PI3K signalling and their relative phosphorylation levels (**A**) and the HDAC class III Sirtuins (**B**) were determined in WT and R5 cells by western blot of total cell lysates (Normally cycling cells were harvested ~70%)

4.2.2 Loss of FOXO3a confers resistance to lapatinib

To confirm the importance of FOXO3a in lapatinib mediated apoptosis in SkBr3 cells, the endogenous pool was reduced using targeted siRNA specific for FOXO3a and cell viability and cell cycle analysis were performed. Successful knockdown was confirmed using western blot (Figure 4.3A). After 24 hours treatment with 0-1μM lapatinib, an increase in cell viability was observed in the siFOXO3a cells compared with the mock and non-specific treated control (Figure 4.3C). This is most evident at 1μM treatment where the cell viability increases from 39.1 and 32.9 to 52.4% for the mock ($p<0.01$), non-specific control ($p<0.05$) and siFOXO3a treated cells, respectively. Cell cycle analysis reaffirmed this observation, showing a reduction in sub G1 population of lapatinib treated cells, compared to the controls (32.4, 38.3 to 26% for the mock, non-specific and siFOXO3a, respectively)(Figure 4.3B).

Due to the fact that obtaining 100% knockdown is difficult to achieve via transient transfection, FOXO negative (FOXO-/-) and wild type MEFs were used to examine the conjecture further (Figure 4.4A). Both FOXO-/- and WT MEFs were treated with increasing concentrations of lapatinib for 72 hours (Figure 4.4B), cell viability was observed using SRB. FOXO-/- MEFs were ~5 times more resistant to lapatinib than their parental counterparts (37.24 to 6.47μM, respectively). When treated with 0-10μM of lapatinib for 24 h (Figure 4.4C), viability in WT MEFs reduced from 100% to 74.4%, indicating cytotoxicity, whereas treatment on FOXO-/- cells resulted in an increase in cell viability from 100% to 114.1%, suggesting the cells were actively proliferating. Statistical analysis (two-way ANOVA Bonferonni post-test) revealed a significant protection against lapatinib treatment in the FOXO-/- at concentrations of 5 and 10μM ($p<0.01$).

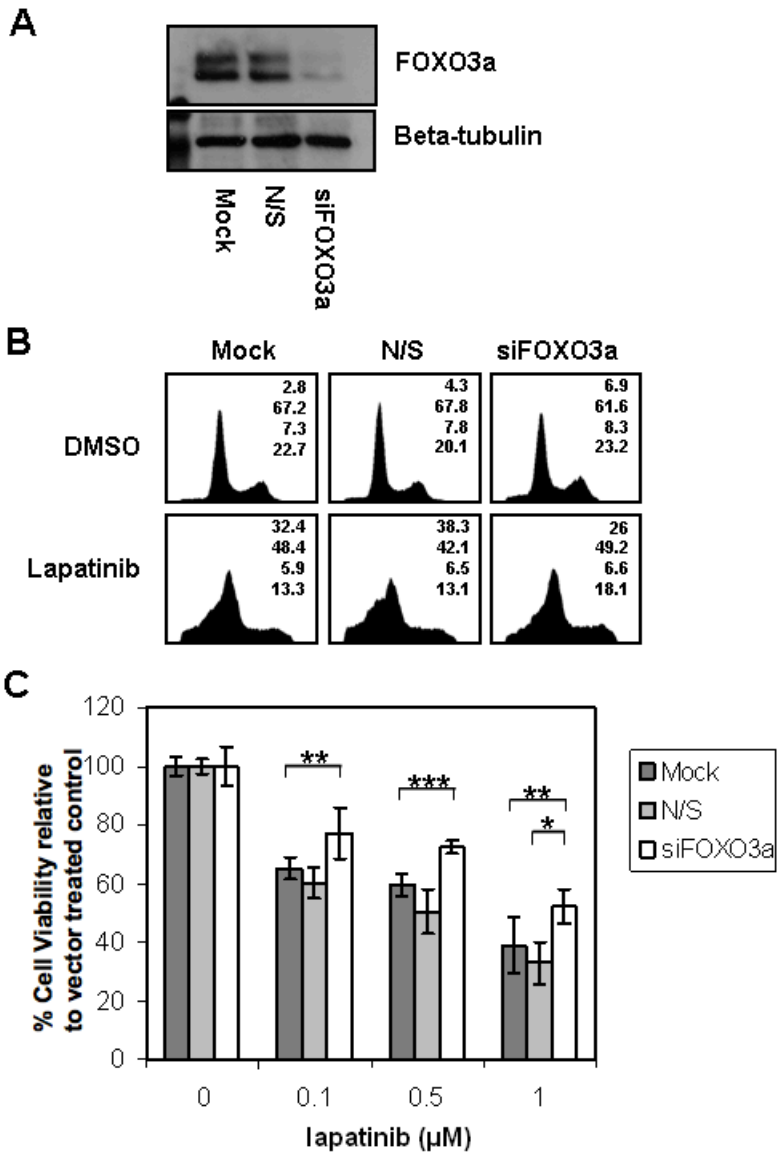


Figure 4.3 FOXO3a targeted knockdown protects SkBr3 cells from lapatinib treatment SkBr3 cells were treated with mock, non/specific and FOXO3a targeted siRNA for 24 h and then treated for another 24h with 1μM of lapatinib. Successful knockdown was confirmed by western blot of total cell lysates **(A)** and flow cytometric analysis of DNA content was performed after propidium iodide staining **(B)**. Percentage of cells in each phase of the cell cycle (sub-G 1, G1, S, and G2-M) is indicated. Representative data from three independent experiments are shown. SRB assay was also performed **(C)**. Mock, non-specific and siFOXO3a treated cells were seeded into a 96 well plate and 24 h later treated with 0-1μM for a further 24h. Statistical analysis was performed using two way ANOVA Bonferonni post-test (* = $p<0.05$, ** = $p<0.01$ *** = $p<0.001$).

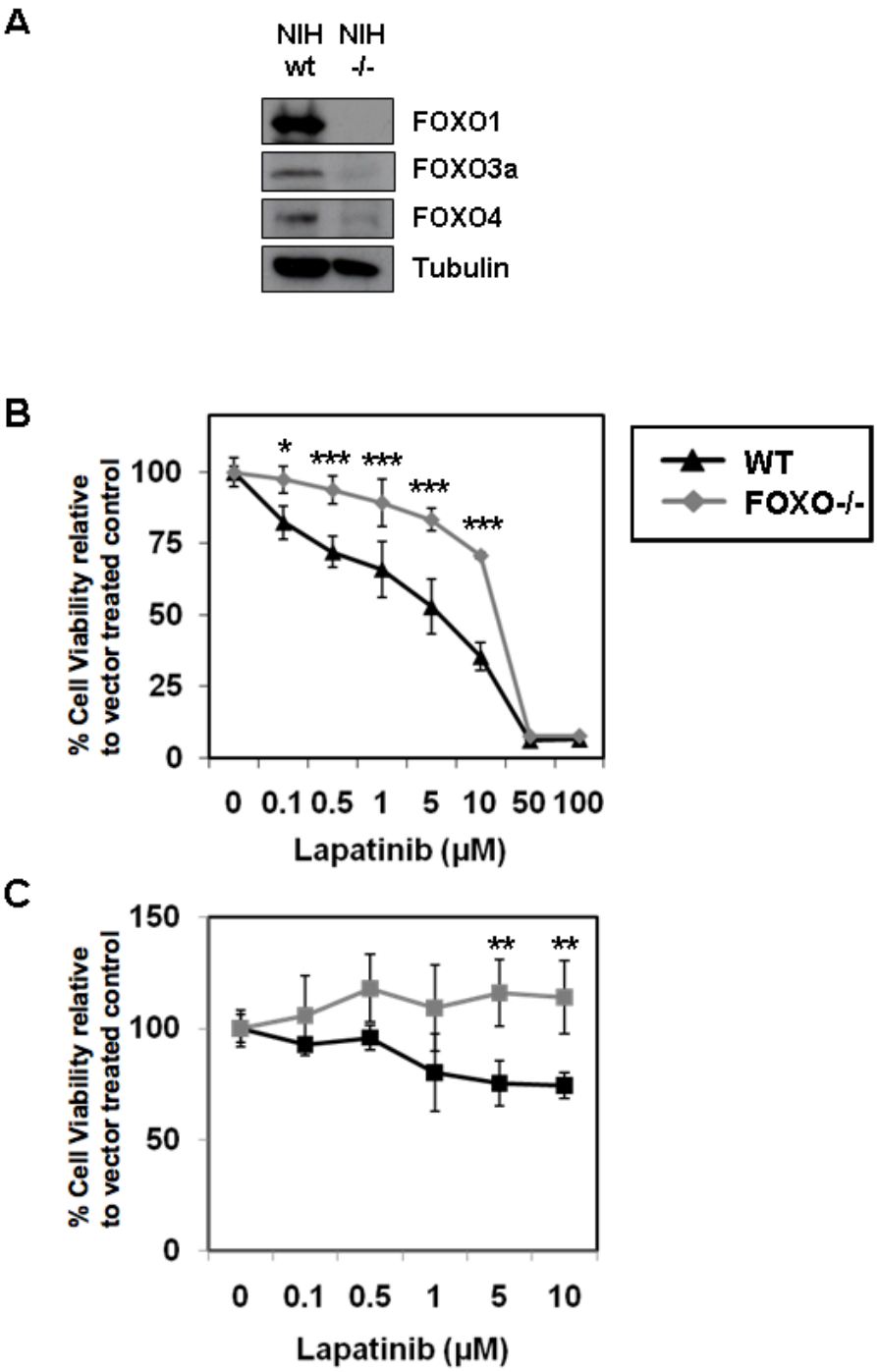


Figure 4.4 Loss of FOXO reduces the potency of Lapatinib. NIH 3T3 cells WT and FOXO null MEFs **(A)** were treated for 72 h with 0-100μM of Lapatinib **(B)** and for 24 h with 0-10μM **(C)** Cell proliferation was determined by SRB assay. Results shown are mean ± SD of three independent experiments. IC50s were also calculated for WT and FOXO-/- cells (6.47 & 37.24μM respectively). Statistical analysis was performed using two way ANOVA Bonferonni post-test (* = $p<0.05$, ** = $p<0.01$ *** = $p<0.001$). Part A performed by Miss Stephanie Guest

4.2.3 Overexpressing FOXO3a in FOXO-/- MEFs resensitises cells to lapatinib treatment.

To assess the ability of FOXO3a to resensitize FOXO-/- MEFs to lapatinib, FOXO3a was overexpressed and treated with 5μM of lapatinib and DMSO vector control for 24 hours (previously shown to have no negative effect on viability at 24h treatment). Overexpression was confirmed by western blot (Figure 4.5A) and cell cycle analysis was also performed (Figure 4.5B). Treatment of the FOXO-/- MEFs with lapatinib resulted in the dephosphorylation of EGFR showing that lapatinib does effected EGFR activity but there was little effect on the cell cycle compared with the vector control and no marked increase in the percentage of sub G1 cells (4 to 5.9%, DMSO, lapatinib treatment, respectively) (Figure 4.5B). Reinstating FOXO3a expression causes a reduction of cells in the G1 phase and an increase of the sub G1 population, indicating that the cells are undergoing apoptosis. Treatment with lapatinib resulted in the dephosphorylation of EGFR and a further increase in the sub G1 population from 18.1 to 26% respectively (Figure 4.5B). It is unsurprising that the sole addition of FOXO3a can induce apoptotis, as one of the marked effects of lapatinib treatment is the increased stabilisation and cellular expression of FOXO3a. This overexpression is merely mimicking one of the mechanisms of action of lapatinib.

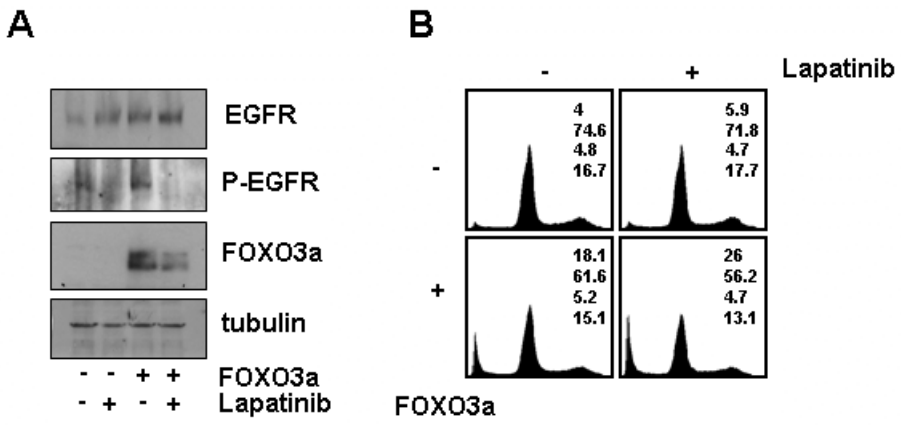


Figure 4.5 Reinstating FOXO3a results in sensitivity to lapatinib NIH 3T3 FOXO-/- were transfected with FOXO3a or backbone vector and were treated with 5μM of lapatinib or DMSO control for 24 h. Protein expression was determined using western blot of total cell lysates **(A)** and flow cytometric analysis of DNA content was done after propidium iodide staining **(B)** Percentage of cells in each phase of the cell cycle (sub-G 1, G1, S, and G2-M) is indicated. Representative data from three independent experiments are shown.

4.3 DISCUSSION

We and others have shown previously the importance of FOXO3a in eliciting the proapoptotic potential of EGRF/HER2 targeted therapies [291-293]. To take this line of study further and examine the role of FOXO3a in acquired resistance to TKIs we developed the lapatinib resistant cell line SkBr3 R5. Confirming resistance, R5 cells were shown to tolerate ~3 times higher concentrations of lapatinib compared to the parental cell lines using the SRB growth assay. The effect of lapatinib treatment on WT and R5 cells was also shown to have a differing effect on the cell cycle. WT cells displayed a reduction of cells in the G1 phase of the cell cycle and an increase in the sub G1 population. R5 cells did not show a reduction of cells in G1 but a slight increase, and the induction of apoptosis was markedly decreased compared to WT cells treated with lapatinib (14.9%compared with 30.2% respectively).

The resistant cells displayed increased phosphorylation of EGFR and preferential activation of the PI3K signaling cascade and a decrease in ERK activation. This highlights the important of PI3K signaling in tumour development and resistance. On further inspection R5 cells were shown to have a marked decrease in expression of FOXO3a.

To confirm this conjecture, WT cells were transfected with FOXO3a targeted siRNA. The knockdown of FOXO3a conferred resistance to lapatinib and provided statistically significant protection from lapatinib ($p<0.05$). To examine the effect of complete loss of FOXO3a and its other family members FOXO1 and FOXO4, FOXO-/- derived NIH 3T3 MEFs (and WT) were treated with an increasing concentration of lapatinib for 24 and 72 hours. Treatment of lapatinib for 24 hours resulted in a decrease in cell viability in the WT but not the FOXO-/- cells. The FOXO-/- negative cells were also shown to be ~5 times more resistant to lapatinib treatment (72h). This suggests that there is some redundancy among FOXO proteins in the R5 cells, i.e. FOXO1 and 4 can partially compensate, albeit

less efficiently, for the loss of FOXO3a in the R5 cells. This has been previously reported in FOXO deficient (CRE-mediated disruption) mice [357].

With this study we show for the first time, from *in vitro* derived lapatinib resistant cell lines (SkBr3 R5), that loss of FOXO3a occurs during the development of resistance to lapatinib. Mechanisms of resistance to lapatinib have previously been reported [291, 358], but this has always been in the ER-positive breast cancer cell line BT-474 and did not show any effect on or loss of FOXO3a. Although interestingly, their study does support our findings, due to the fact that in the resistant BT474 cell line (rBT474), lapatinib treatment was still found to cause the dephosphorylation of HER2, HER3, AKT and MAPK, suggesting a loss of function of proteins further down the signaling cascade, for example FOXO3a, resulting in an inability to elicit the proapoptotic response [291]. Moreover, because this lack of efficacy could be a result of low levels or loss of FOXO3a - the cytoplasmic localisation of FOXO3a is associated with poor survival in patients [359] - compounds that stabilise FOXO3a, increase its expression and/or causes its derepression could be used to resensitise lapatinib-resistant tumours, maximising its proapoptotic potency and efficacy.

Recently it was shown that FOXO3a expression is lost in human lung adenocarcinoma (LAC) (~60%) and that this was predominantly though gene deletion, highlighting the role of FOXO3a as a tumour suppressor, promoting carcinogen-induced apoptosis, inhibiting carcinogenesis [285, 360].

To summarise, FOXO3a is known to be responsible for the cytotoxic function of a range of chemotoxic agent such as Paclitaxel, Imatinib and Trastazumab [102, 182, 183, 361], This strengthens its importance as a marker for responsiveness to targeted therapies, since overexpression of HER2 does not necessarily correlate to a positive clinical outcome. Also, FOXO3a status could prove to be an important diagnostic factor in other groups of patients such as ER+/HER- negative, who could benefit from combination therapy with lapatinib [362].

4.4 FUTURE WORK

To show further that FOXO3a is important in the efficacy of lapatinib and that loss of FOXO3a is a clinically relevant mechanism of resistance, patient samples that have been treated with lapatinib could be investigated to examine FOXO3a expression and whether it correlated with reduction in tumour size. Current trials, such as the neo-ALTTO (Neoadjuvant Lapatinib and/or Trastuzumab Treatment Optimisation) study, which assesses the ability of paclitaxel in combination with lapatinib, trastuzumab or both, to reduce tumour size before surgery would provide a unique opportunity to investigate the role of FOXO3a in lapatinib cytotoxicity prior to the treatment of established chemotherapeutic agents such as FEC (5FU, epirubicin and cyclophosphamide) [363]. Although the patients have been treated with lapatinib and paclitaxel, we have shown previously that FOXO3a is also important in eliciting the proapoptotic potency of paclitaxel [183, 364]. Investigating clinical samples would strengthen the importance of FOXO3a expression in eliciting the cytotoxic action of many chemotherapeutic agents.

CHAPTER 5

THE ROLE OF SIRT1 IN CISPLATIN RESISTANCE

5.1 INTRODUCTION

Cisplatin (cis-diamminedichloro-platinum), a platinum-based chemotherapeutic agent has been an established and important treatment for many cancers, including ovarian, bladder, testicular and non-small lung cancer, as a single agent and in combination with other anticancer agents, reducing tumour burden and increasing life expectancy of patients [365, 366].

Although cisplatin is not used as a first-line therapy in breast cancer, recent studies have shown that at least in a subset of patients termed, 'triple negative' – lacking the expression of estrogen, progesterone and HER2 receptors – who account for 15% of all breast cancer cases [367], showed response to neoadjuvant cisplatin treatment. 22% of patients achieved pathological complete response and 64% of patients had a clinical complete or partial response [368]. In another study, patients displayed a reduction in the risk of progression by 47%, when treated in combination with cisplatin and gemcitabine, compared with non-triple negative metastatic breast cancer patients [369]. These studies highlight the possible use of cisplatin as a viable therapy for some breast cancer patients.

Platinum-based compounds mediate their cytotoxicity by forming platinum intrastrand and interstrand DNA adducts activating nuclear excision repair and homologous recombination pathways [370]. The inability to activate appropriate DNA repair mechanisms lead to the accumulation of DNA strand breaks, and ultimately the induction of apoptosis and cell death [371].

Resistance to cisplatin is a major clinical obstacle, after an initial favorable response the majority of patients relapse with cisplatin-resistant disease [372]. Several pathways and mechanisms have been associated with the acquisition of cisplatin resistance, including increased cell proliferation, an increase in drug-efflux pumps, increased response to DNA damage repair, and evasion of pro-

apoptotic pathways [365, 372]. Several targets have been implicated in the development of resistance in breast cancer cell lines, including amphiregulin [373], Dicer [374], cyclin D1 and BCL2 [375]. However, a better understanding of the molecular mechanisms that cause chemotherapeutic resistance are needed so that the benefit of platinum-based treatments to patients can be prolonged, as well as increasing their efficacy with possible combinatorial therapies.

Recently it has been shown that the PI3K/PKB/FOXO pathway is important in the cellular response to cisplatin treatment in ovarian cancer cells [376] and mediates its cytotoxicity in colon carcinoma cells [377]. The histone deacetylase SIRT1 has also been shown to be important in the development of cisplatin resistance in prostate cancer cell lines [314], epidermiod adenocarcinoma and hepatoma cell lines [378, 379]. Moreover, SIRT1 knock down was shown to sensitize the p53-deficient chicken cell lines, DT40 to cisplatin treatment [339].

In this study we evaluate the effect cisplatin resistance has on proteins involved in the PI3K/PKB/FOXO pathway and also the HDAC class III proteins, termed sirtuins. Using MCF-7 derived cisplatin resistant (termed CisR) and parental cell lines we found that SIRT1 was upregulated, and that this upregulation protected CisR cells from cisplatin treatment. Also, SIRT1 suppressed the activation of FOXO3a. These findings highlight the role of SIRT1 in the development of chemoresistance but more importantly the effect the upregulation of SIRT1 has on suppressing the proapoptotic potency of chemotoxic agents that indirectly active FOXO3a.

5.2 RESULTS

5.2.1 Development and characterisation of cisplatin resistant cells

The breast cancer cell line MCF-7 was continually exposed to increasing concentrations of cisplatin *in vitro* for two months, after which, resistance developed and cells were actively proliferating in the presence of cisplatin. Resistant cells (CisR) were then maintained in 1μM of cisplatin. The growth assay SRB was used to confirm resistance. MCF-7 wildtype (WT) and CisR cells were treated with increasing concentration of cisplatin (0-1μM) for 72 hours (Figure 5.1). MCF7 WT cells showed a dose-dependent decrease in cell viability, whereas little effect on proliferation was observed in the cisR cells. At 1 μM treatment cell viability was 38.5% (SD±5.2) and 81.6% (SD±5.1) for WT and cisR cells respectively, and this difference in viability was shown to be statistically significant ($p<0.001$) (Figure 5.1). To confirm cisplatin resistance using another method, MCF-7 WT and CisR cells were treated with 1μM of cisplatin for 0-48 hours and cell cycle analysis was performed (Figure 5.1). WT cells displayed an increase in G2-M population at 24 h treatment compared to the zero hour control (46.4% compared with 26.9%). At 48h post treatment there was a decrease in G2-M cells and G1 population but an increase in the sub G1 cells, indicating cells they were undergoing apoptotis. Whereas CisR cells displayed no significant change in cell cycle distribution when treated with cisplatin, showing that they are indeed resistant to cisplatin treatment (Figure 5.1).

It has been shown recently that FOXO3a is important in modulating the cytotoxicity of cisplatin in colon carcinoma cells [377], to examine whether there were any changes in protein expression between WT and CisR cells we checked the expression of FOXO3a and proteins important in its regulation (Figure 5.2). We found that FOXO3a expression had decreased in the CisR cells compared with WT parental cell lines. There was also an increase in phosphorylation of

AKT (473) which has been shown to be important in regulating the activity of FOXO3a and a possible mechanism of resistance [380].

Due to the decreased expression of FOXO3a we characterized the expression of the class III HDAC proteins, termed sirtuins (Figure 5.2). SIRT1, SIRT2 and SIRT3 have previously been shown to interact with and regulate the subcellular localisation and activity of FOXO3a [223, 269, 270]. Profiling all seven sirtuins, there were changes in expression. SIRTs 6 and 7 appeared to be down-regulated although not markedly. The most striking change was a ~3 fold increase in the protein expression of SIRT1 (Figure 5.2), which is known to effect the activation of downstream targets by FOXO3a, inhibiting its ability to activate genes involved in the pro-apoptotic response, instead activating a subset of genes involved in stress resistance [223].

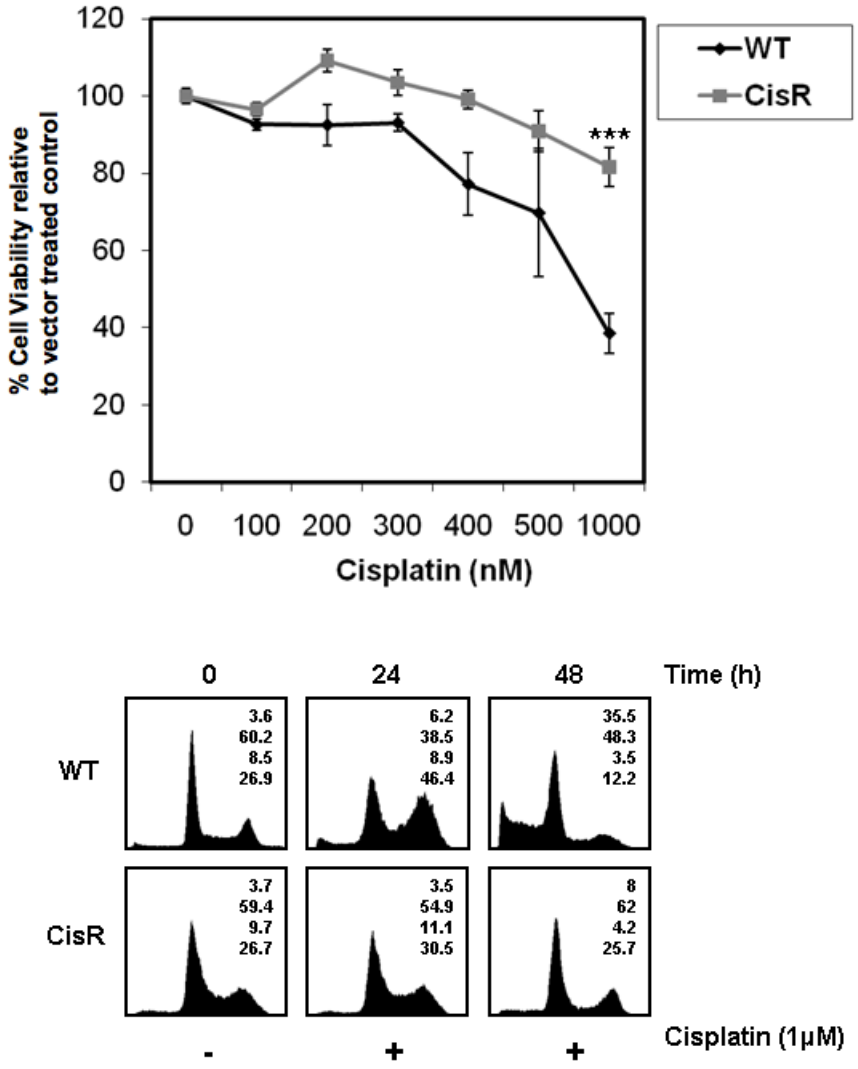


Figure 5.1 CisR cells are resistant to cisplatin compared with the parental cell line, MCF7 MCF7 WT and cisR cells were treated with increasing concentrations of cisplatin for 72 hours. Proliferation was determinded by sulforhodamine B assay. Results are in triplicate. Mean ± S.D. MCF7 cells were treated with cisplatin (1μM) for 0, 24 and 48 hours. Cells were fixed in ethanol and the DNA content was analyzed after propidium iodide staining by flow cytometry

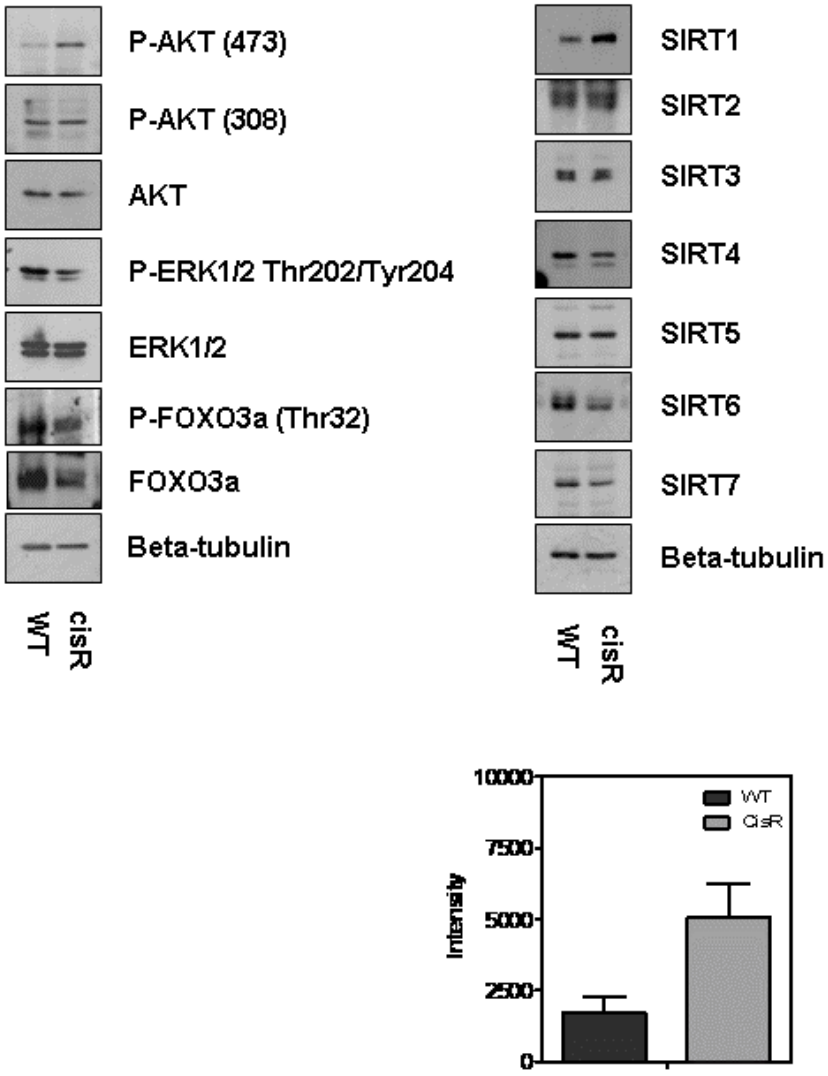


Figure 5.2 Characterisation of CisR cells compared with their parental cells MCF-7 Expression of proteins involved in PI3K signaling and their relative phosphorylation levels (**A**) and the HDAC class III Sirtuins (**B**) were determined in WT and CisR cells by western blot of total cell lysates (Normally cycling cells were harvested ~70%)

5.2.2 FOXO3a is not activated in CisR cells

To assess the effect of cisplatin treatment on FOXO3a expression we treated WT and CisR cells for 0-48h with 1μM of cisplatin (Figure 5.3). Cisplatin treatment in WT cells showed an increase in the cellular expression of FOXO3a and an increase in expression of the canonical downstream target, p27. In CisR cells, cisplatin treatment resulted in an increase of SIRT1 at 24h but no effect on FOXO3a or p27, suggesting that SIRT1 upregulation is suppressing the activation of FOXO3a (Figure 5.3).

To expand this conjecture further, WT and CisR cells were transfected with mock control, control siRNA or siRNA against SIRT1 (Figure 5.4). The transfected cells were then incubated with or without 1 μM of cisplatin for 24 hour before the cells were subjected to cell cycle and Western blot analyses. The cell cycle analysis showed that the siRNA-mediated knockdown of endogenous SIRT1 in both untreated WT and CisR cells had no discernable effect on the cell cycle. Strikingly, treatment of cisplatin when SIRT1 expression was reduced in CisR cells displayed a marked increase in the sub G1 population compared with mock and non-specific siRNA treated controls (37.2% compared to 9.9% 10.1% respectively)(Figure 5.4). WT cells treated with cisplatin showed an increase in the sub G1 population compared to the untreated control (14% compared to 2.1%), this population was markedly increased when SIRT1 expression had been reduced, from 14.3% and 13.3% to 26.9% (mock, non-specific and siRNA SIRT1 respectively)(Figure 5.4). This data highlights the role of SIRT1 in mediating the cytotoxicity of cisplatin in WT MCF-7 cells, showing that its expression negatively affects the efficacy of cisplatin. Also it suggests the SIRT1 overexpression plays a major role in development of resistance to DNA damaging-inducing agents, such as cisplatin.

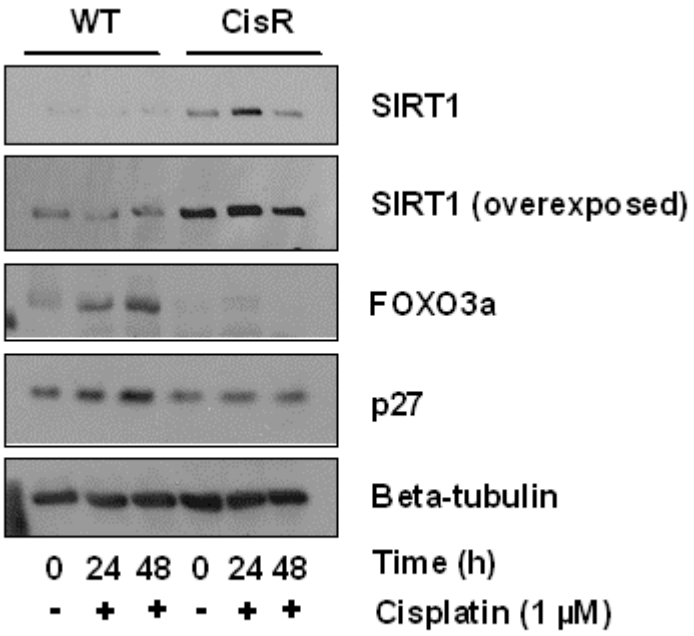


Figure 5.3 Cisplatin treatment activates FOXO3a in WT but not CisR cells WT and CisR cells were treated with 1μM of cisplatin for 0-48 h. Protein expression was determined using western blot of total cell lysates.

5.2.3 SIRT1 can protect cells from cisplatin treatment

To examine whether the sole overexpression of SIRT1 can protect cells from cisplatin treatment MCF-7 cells were transfected with empty expression vector or expression vector encoding FLAG tagged SIRT1 for 24 h, then reseeded into a 96 well plate for 24 h and treated with increasing concentrations of cisplatin (0-21μM) for a further 24 h, proliferation was determined using the growth assay SRB and overexpression was confirmed using western blot (Figure 5.5).

SIRT1 overexpression protected MCF-7 cells from cisplatin treatment at all concentrations tested showing no statistical effect on cell viability ($p<0.001$)(Figure 5.5). This protection being most prominent at 21μM cisplatin treatment where the empty expression vector control had 48.1% viability compared with the SIRT1 transfected cells that had 90.5% cell viability ($p<0.001$). This demonstrates that the sole overexpression of SIRT1 can protect cells from cistplatin treatment and that SIRT1 plays a vital role in the development of cisplatin resistance, protecting cells from the cisplatin induced activation of apoptosis, favouring a prosurvival outcome.

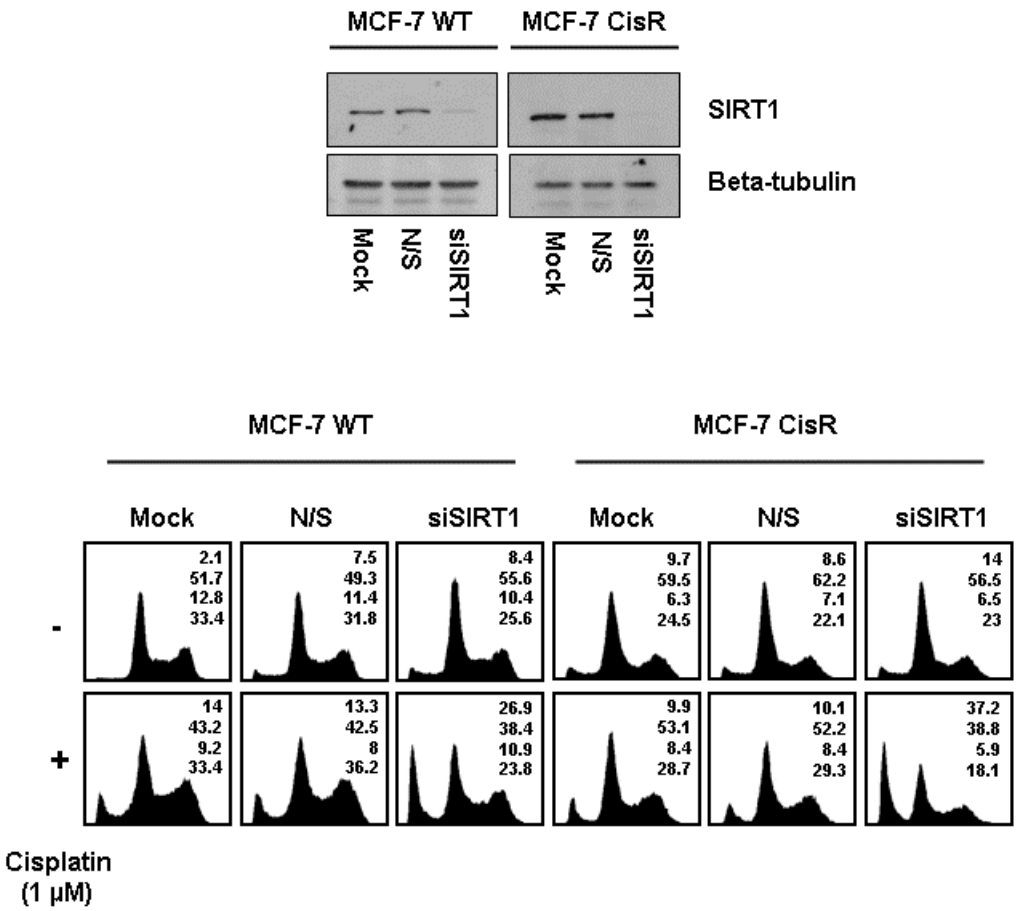


Figure 5.4 Knocking down SIRT1 sensitizes WT and cisR cells to cisplatin treatment MCF7 WT and cisR cells were transfected with mock, control siRNA pool or SIRT1 targeted siRNA for 24 h and then treated with DMSO or cisplatin (1μM) for 48 hours. Sufficient knock-down was confirmed using western blot of total cell lysates. For cell cycle analysis cells were fixed in ethanol and the DNA content was analyzed after propidium iodide staining.

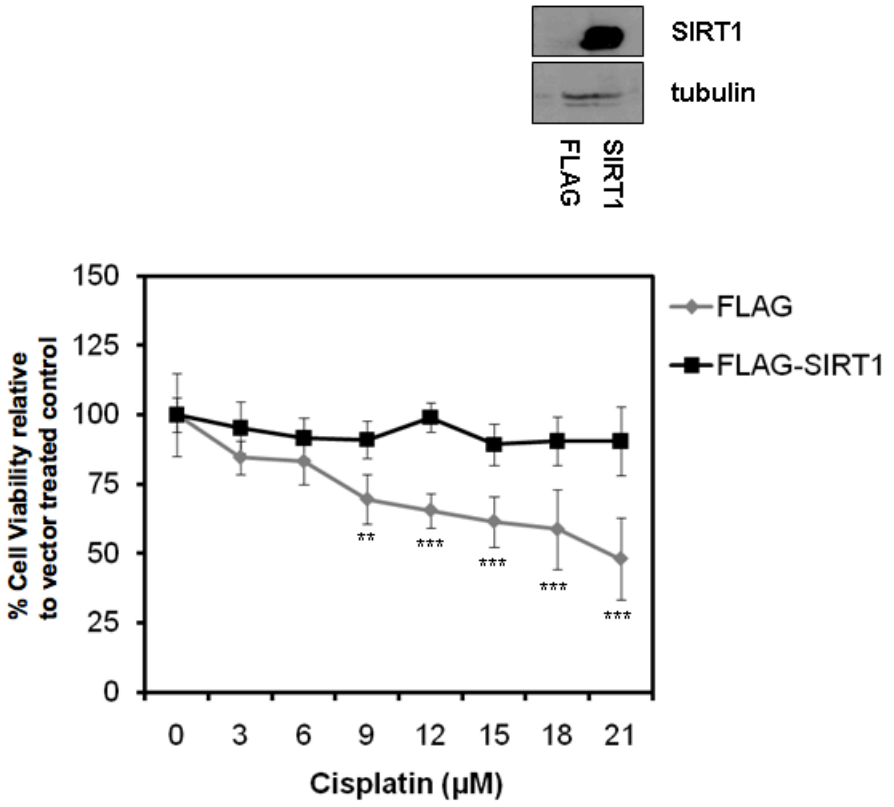


Figure 5.5 SIRT1 overexpression protects MCF-7s from cisplatin MCF-7 cells were transfected with FLAG and FLAG-SIRT1 for 24h then treated with increasing concentrations of cisplatin (0-21μM) for a further 24 hours. Overexpression was confirmed using western blot of total cell lysates. Proliferation was determined by sulforhodamine B assay. Results are in triplicate. Mean ± S.D

5.3 DISCUSSION

The role of the PI3K signalling pathway and its components are known to be important in a range of cellular processes and has been shown to be important in many human malignancies. Changes in the regulation of these components are known to be important in tumour development, for example, the overexpression of HER2 leads to the activation of the PI3K and its downstream effectors, resulting in increased cell cycle progression, proliferation and survival [381]. In ovarian cancer, dysregulation of the PI3K cascade through increased activity of AKT leads to the development of resistance to cisplatin treatment [382].

In this study, we show for the first time that SIRT1 is upregulated in derived cisplatin resistant breast cancer cell lines and that the specific upregulation of SIRT1 protects resistant cells from cisplatin induced apoptosis possibly by suppressing the activation of FOXO3a.

We and others show that the activation of FOXO3a in cisplatin-resistant cells is incomplete [377]. The lack of activation of FOXO3a in the cisplatin resistant cells suggests that this suppression of activity is due to the upregulation of SIRT1. SIRT1 overexpression in cisplatin sensitive cells was sufficient to protect MCF7 cells from cisplatin treatment.

An earlier passage of the CisR cells were used by Kwok *et al* [383] and were found to have increased expression of the DNA damage repair genes XRCC1 and BRCA2. We have previously shown that FOXO3a represses the expression of pro-survival transcription factor FOXM1 in response to the tyrosine kinase inhibitor gefitinib [98]. A likely mechanism of resistance could be the suppression of FOXO3a activity by SIRT1 allows FOXM1 to upregulate DNA damage repair genes, allowing continued proliferation and evasion of apoptosis.

Moreover, SIRT1 -/- DT40 chicken cells, which are deficient of p53, displayed increased sensitivity to cisplatin, highlighting that SIRT1s role in chemoresistance is possibly independent of its interaction and regulation of p53, and must be due to other cell cycle regulatory genes such as FOXO3a to regulate its cytotoxicity [339].

The silencing of SIRT1 expression was sufficient to resensitise resistant cells to cisplatin treatment, as well as increasing the cytotoxicity of cisplatin in WT cells. Interestingly there is a ~4 fold increase in phosphorylated AKT on residue 473 whereas the phosphorylation of 308 was unchanged. It is interesting to note that the fold change observed in P-AKT (473) expression is similar to the fold change in SIRT1 expression. It has previously been shown that deregulation of P-AKT (473) leads to a deficient phosphorylation of specific downstream targets. Genetic ablation of sin1 abolished phosphorylation of AKT on serine 473 and disrupted its interaction with rictor-mTOR, but phosphorylation of AKT on threonine 308 was maintained. AKT was unable to phosphorylate FOXO3a (Thr32) but was still able to interact and phosphorylate other targets, such as TSC2 and GSK3 [384]. Guertin *et al* reiterated these findings, showing that loss of mTORC2 function, through loss of *mLST8* and *rictor* in mouse embryonic fibroblasts differentially affected the phosphorylation status of AKT downstream targets, showing that AKT could no longer phosphorylate FOXO3a but was still able to phosphorylate other proteins targets [83]. Expanding these findings, it is logical to presume that the contrary exists where increased phosphorylation of AKT (473) preferentially represses the activity of FOXO3a. Further research is needed to understand whether SIRT1 overexpression and the development of resistance suppresses FOXO3a activity by directly regulating the post-translational status of FOXO3a, or indirectly via regulating the activity of other proteins in the PI3K signalling cascade, or both.

Irrespective of the mechanism of SIRT1s regulation of FOXO3a these findings have implications on treatment regimes for patients who are being treated with

cisplatin or who have developed resistance to cisplatin treatment. Combining cisplatin with sirtuin targeted therapy should circumvent resistance and increase the efficacy of cisplatin treatment, as well as allowing the lowering of the concentration of cisplatin used, reducing toxicity, side-effects, increasing patient quality of life.

5.4 FUTURE WORK

Because cisplatin is used in triple-negative tumours, a specific subset of breast cancer patients, to confirm the important of SIRT1 in the development of cisplatin resistance additional cisplatin resistant cell lines such as MDA-MB-231, BT20 and Cal51, which are model cell lines that are negative for ER, PR and HER2, should also be made to strengthen the findings of MCF CisR cells and show that it is not a cell line specific finding. Investigating SIRT1 expression in these cell lines would produce data that would be more clinically significant, especially if specific SIRT1 inhibitors could be used to resensitise them to cisplatin treatment.
Also it would be interesting to examine whether SIRT1 is upregulated in therapies that are known to elicit their cytotoxic effect through indirectly activating FOXO3a, such as paclitaxel [183, 364].

CHAPTER 6

SIRTINOL TREATMENT ENHANCES THE PRO-APOPTOTIC POTENCY OF LAPATINIB IN A FOXO3a-DEPENDENT MECHANISM

6.1 INTRODUCTION

Lapatinib, also known as GW572016 and Tykerb, is an orally administered potent small molecule inhibitor that functions as a dual inhibitor, binding reversibly to EGFR and HER2 tyrosine kinases, inducing growth arrest or tumour cell apoptosis in EGFR dependent or HER2-dependent tumour cell lines or xenographs [290, 348]. We and others have shown previously that the tyrosine kinase inhibitors (TKIs) Lapatinib and Gefitinib (a specific EGFR inhibitor), modulates their anti-proliferative potency through the transcription factor, FOXO3a, highlighting its crucial role in maintaining the pro-apoptotic efficacy of these targeted therapies [291-293] . FOXO3a is a member of the FOXO subfamily of forkhead transcription factors, which contains three other members, FOXO1, FOXO4 and FOXO6 in humans, and regulate a plethora of gene involved in many cellular including cell cycle regulation (e.g., *p27Kip1*, *p130(RB2)*, *cyclin D*), apoptosis (e.g., *Bim, Fas-ligand* and *TRAIL*) and oxidative stress (e.g., *mnSOD and Catalase*) [84, 126, 151, 156, 165, 170, 171, 173, 176].

Histone deacetylases (HDACs) and histone acetytransferases (HATs), namely the class III HDACs termed sirtuins, control the aceytylation status of histones and non-histone proteins. The regulation of the posttranslational status of non-histone targets such as p53 has been shown to affect its activity, DNA-binding affinity and stability [212, 385-387]. There are seven mammalian sirtuins (SIRT1-7) and unlike the other classes of HDACs are dependent on the coenzyme NAD+ [388]. All sirtuins contain a catalytic domain of ~275 amino acids [300]. SIRT1 being the most studied due to its homology to the yeast deacetylase Sir2 [223]. Sir2 is evolutionary conserved and is known to regulate longevity and aging in lower organisms, such as, *Caenorhabditis elegans* [389].

The development of HDAC inhibitors (HDACi) as viable therapeutic regimes has mainly focused on inhibitors of class I and II HDACs [390], SAHA (Vorinostat) being the first HDACi approved by the Food and drugs administration (FDA) for

the treatment of cutaneous T-cell lymphoma (CTCL). Since many other compounds have undergone or are undergoing clinical development [308, 391], but none to date have targeted the sirtuins, although mouse models have been used to show compounds that target sirtuins, such as the tenovins, namely tenovin-6, reduce tumour growth [312]. Sirtinol, a sirtuin inhibitor, was found to have an antiproliferative effect, through inhibition of SIRT1 and SIRT2 in breast cancer cells [313]. We have shown previously that the antiproliferative effect of sirtinol was less efficient in p53 null mouse embroyonic fibroblast (MEFs) and cell lines containing mutant p53 [316].

Recent clinical trials have shown that lapatinib is effective at treating patients overexpressing HER2, and more interestingly lapatinib in combination with capecitabine has been shown to prolong progression-free survival in patients that were previously receiving trastazumab [349, 392], An emerging problem with TKIs is the development of resistance. Understanding the molecular mechanisms of how this resistance arises would provide knowledge on whether patients would initially be receptive to targeted therapy and the likely success of combinatorial treatment.

Here, we demonstrate the importance of FOXO3a in the proapoptotic potency of lapatinib, and sirtinol treatment causes the stability and cellular accumulation of FOXO3a. In combination, lapatinib and sirtinol display synergism at inducing apoptosis, this synergy being dependent on the presence of FOXO, due to its stabilization by sirtinol treatment and its activation by lapatinib.

6.2 RESULTS

6.2.1 The antiproliferative function of sirtuin inhibitors is dependent on the presence of FOXO proteins

We have previously shown that the proapoptotic potency of the sirtuin inhibitors sirtinol and salermide are dependent on the presence of functional p53 [316]. Because there are similarities between the regulation of p53 and FOXO, we took NIH 3T3 mouse embryonic fibroblasts (MEFs) wild-type (WT), p53 null (p53-/-) and FOXO null (FOXO-/- lacking FOXO1, FOXO3a and FOXO4), and treated them with 0-100μM of EX527, sirtinol and salermide for 72 hours and assessed their effect using the proliferation assay SRB. The antiproliferative effect of EX527 was impaired by the lack of FOXO, suggesting that it elicits its cytostatic action through the FOXO family members ($p<0.001$) (Figure 6.1). Moreover, it is logical to suggest that the unknown acetylated protein seen with EX527 treatment (Figure 3.7) could be involved in directly or indirectly (via the PI3K cascase) regulating FOXO activity. Although this would need to be investigated further.

Interestingly, we also found that between 10-40μM of sirtinol treatment the loss of FOXO proteins significantly decreased its efficacy ($p<0.001$), suggesting that at lower concentrations, the cytotoxic mechanism of sirtinol is modulated through FOXO proteins (Figure 6.1). Moreover, the presence of FOXO proteins was more important than the presence of p53 at influencing cell viability when treated with the three sirtuin targeted compounds (Figure 6.1).

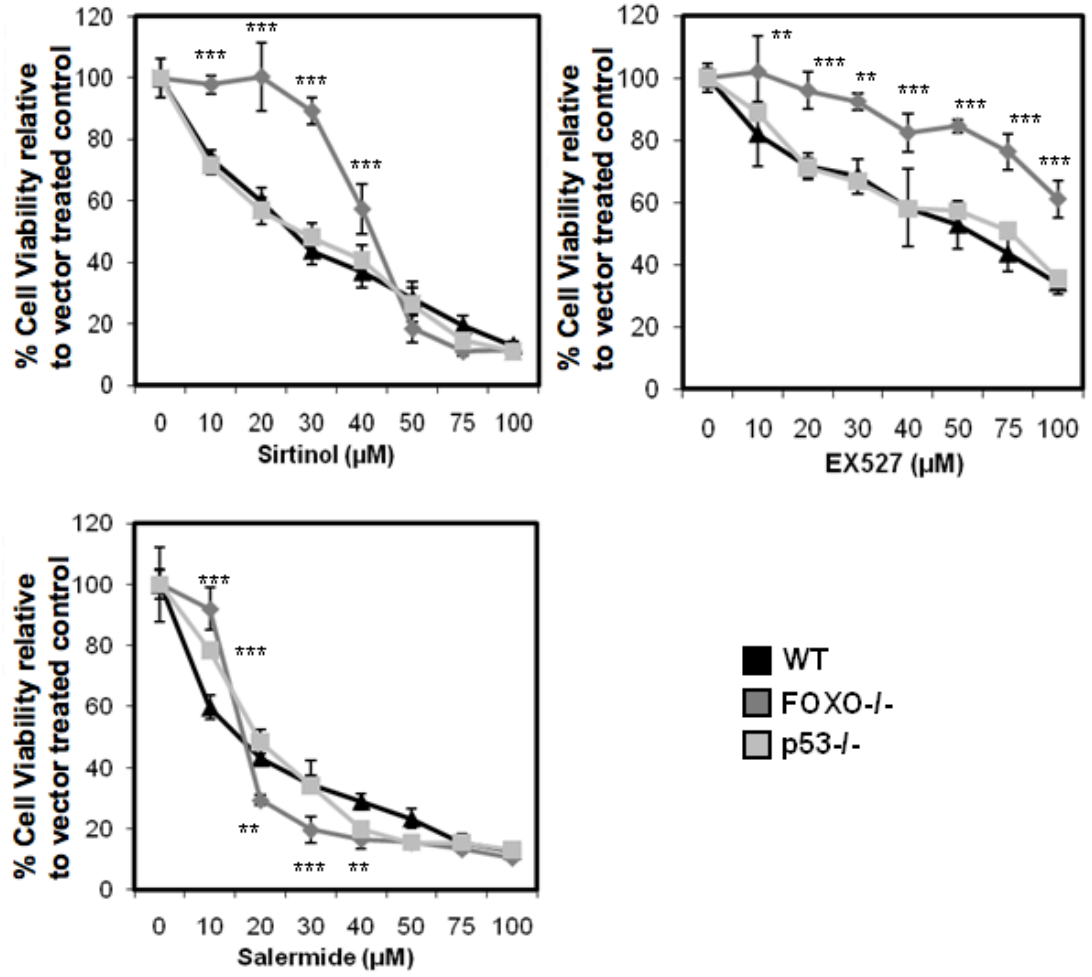


Figure 6.1 The antiproliferative effects of Sirtinol and EX527 are dependent on the presence of FOXO A NIH 3T3 cells WT, p53 null and FOXO null mouse embryonic fibroblasts were treated for 72 hours with 0-100μM of Sirtinol, EX527 and Salermide. Cell proliferation was determined by sulforhodamine B assay. Protein expression was confirmed using western blot analysis of total cell lysates. Results shown are mean ± SD of three independent experiments. Statistical analysis was performed using one way ANOVA (* = $p<0.05$, ** = $p<0.01$ *** = $p<0.001$).

6.2.2 Sirtinol treatment stabilizes FOXO3a, increasing its cellular expression

Because sirtinol is the most cytotoxic sirtuin inhibitor that we tested, we decided to access the effect of sirtinol treatment on FOXO3a protein expression. To distinguish that the effects on FOXO3a and the cell phenotype by sirtinol treatment was independent of the presence of p53, SkBr3 cells – which contain mutant p53 - were taken and treated with 0-100μM of sirtinol for 24h; protein expression was analysed using western blot (Figure 6.2A). Sirtinol treatment resulted in the increased cellular expression of FOXO3a compared with the DMSO treated control. Interestingly sirtinol treatment had no effect on the phosphorylation of AKT (308) suggesting that sirtinol treatment was not affecting the canonical PI3K signalling cascade upstream of FOXO3a (Figure 6.2A) but by another mechanism of regulation, possibly via changes in mTORC2 activity or mdm2-dependent ubiquitination and degradation [193, 393].

To assess the impact of sirtinol treatment in a setting containing WT p53, MCF-7 cells were treated with 25μM of sirtinol for 0-8h (Figure 6.2B). Treatment resulted in the increase in cellular expression of FOXO3a. MCF-7s were treated with 25μM of sirtinol for 24 h and there was no statistically significant increase in FOXO3a mRNA expression (Figure 6.2C), suggesting that sirtinol treatment causes the stabilization of FOXO3a, similar to its effect on p53, but that this effect was p53-independent as it was observed in both p53 WT and mutant cell lines.

To confirm the conjecture whether the effect on FOXO3a was posttranslational, MCF7 cells were taken and WT FOXO3a-FLAG and 294D FOXO3a-FLAG (294D being a phosphorylation mimic that is ubiquinated and degraded) were overexpressed for twenty four hours and then treated with CHX for 0-8h with or without sirtinol (Figure 6.3). The degradation of FOXO3a when treated with DMSO was similar to what was previously reported [193], its cellular expression

reduced 50% after 8 hours of cyclohexamide treatment (Figure 6.2A). The treatment of 25μM of sirtinol caused the stabilization of WT FOXO3a when treated with cyclohexamide, the cellular abundance of FOXO3a being the same as 0 hour untreated cells (Figure 6.2B). But sirtinol was unable to stabilize the phosphorylation mimic 294D FOXO3a (Figure 6.2C), its degradation rate being similar to FOXO3a WT. This suggests that sirtuin inhibition caused by sirtinol treatment results in the acetylation and stabilization of FOXO3a and that this acetylation dysregulates the phosphorylation of FOXO3a on serine 294 and its subsequent ubiquitination and degradation. There is an evolutionary conserved lysine residue at 290, suggesting that acetylation here, sterically hinders phosphorylation of FOXO3a, but this needs to be investigated further through site-specific mutations of FOXO3a – unacetylatable and acetylation mimics – to confirm the exact lysine residues responsible for modulating FOXO3a protein expression.

To confirm the stabilization only occurred with sirtinol treatment, EX527 was also tested and was unable to protect WT FOXO3a from degradation (Figure 6.3D), suggesting that more than one sirtuin needs to be inhibited to cause the stabilization of FOXO3a.

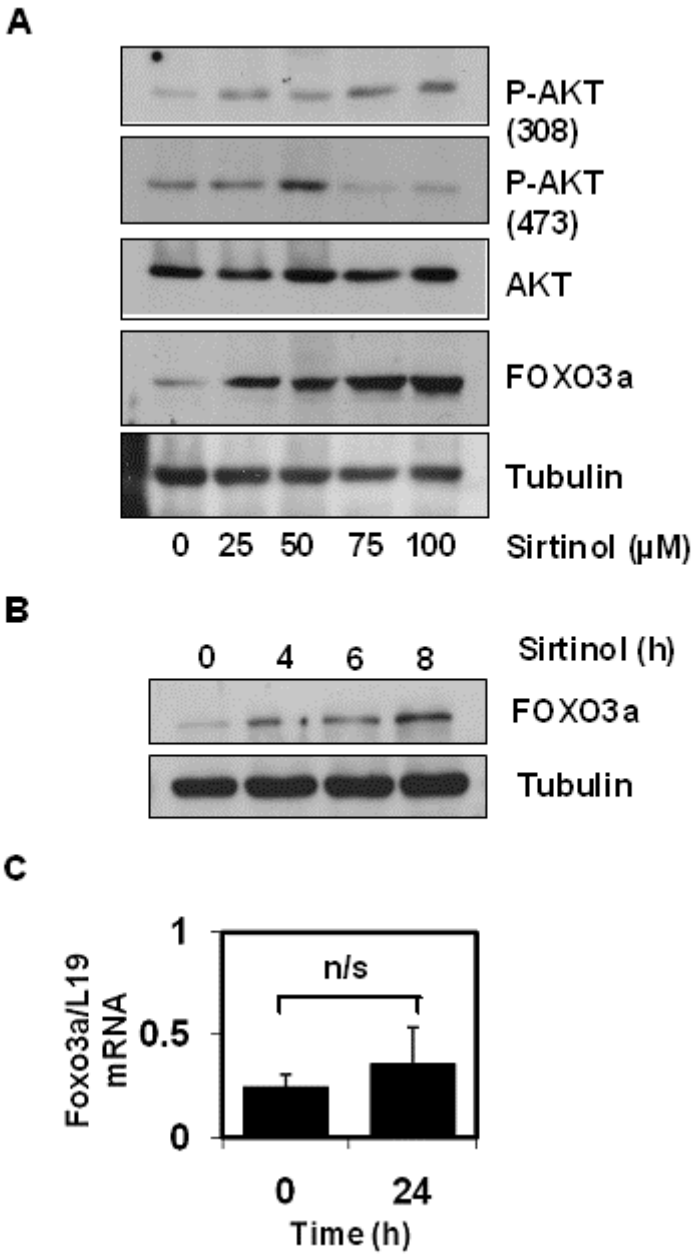


Figure 6.2 Sirtinol treatment stabilizes FOXO3a A SkBr3 cells were treated with 0-100μM sirtinol for 24 h. Protein expression was analysed using western blot. **B** MCF-7 cells were treated with 25μM of Sirtinol for 0-8h; protein expression was determined using western blot. **C** MCF-7 cells were treated with 25μM sirtinol for 24 h. Cells were collected and total RNA was isolated. FOXO3a mRNA levels were analyzed by real-time quantitative PCR normalized with L19 mRNA levels. Experiments were done in triplicate. Mean ± SD

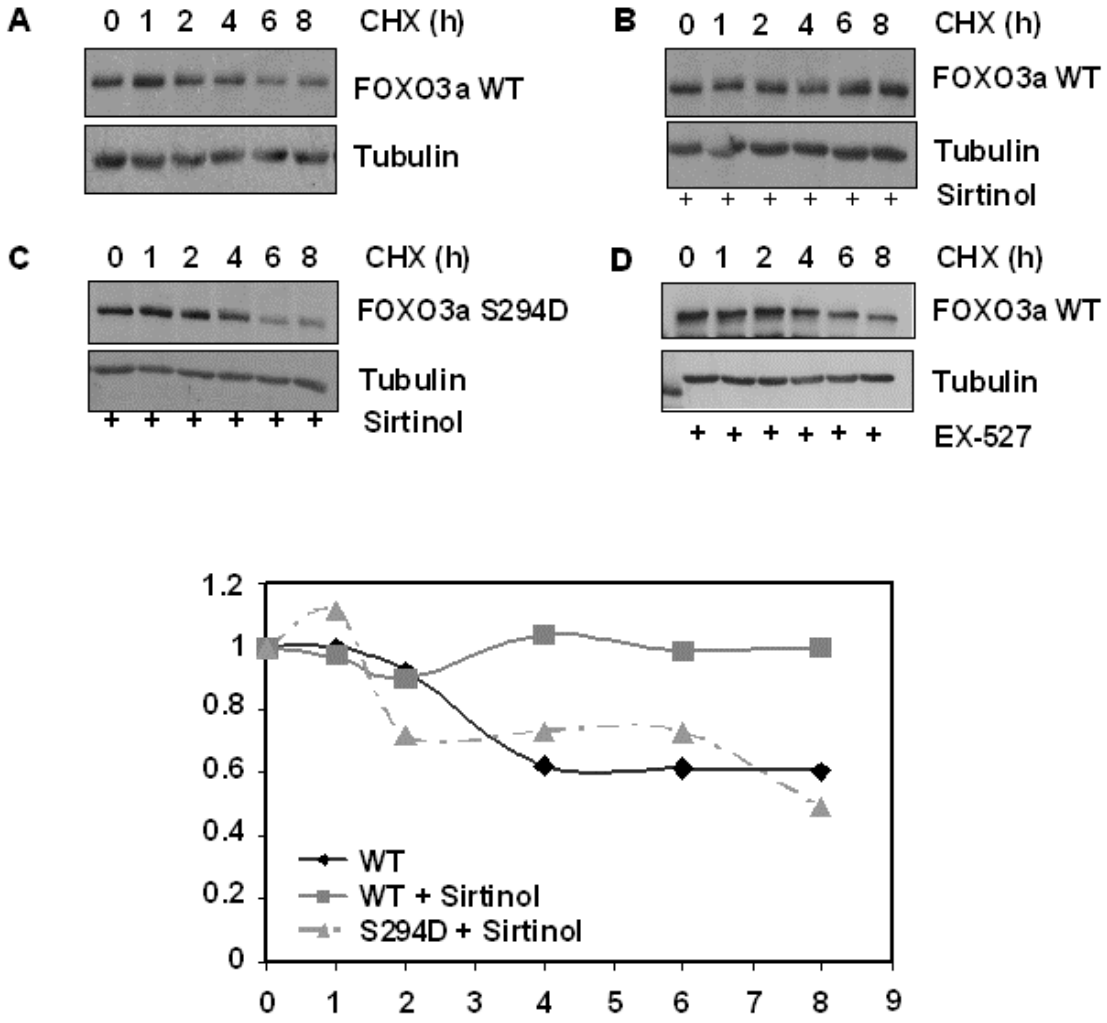


Figure 6.3 Sirtinol treatment stabilizes FOXO3a A MCF-7 cells were transfected with WT FOXO3a-FLAG and treated with cyclohexamide for 0-8h alone or in combination (**B**) with 25μM of sirtinol, protein expression was determined using western blot **C** MCF-7 cells were transfected with FOXO3a-FLAG 294D mutant and treated with cyclohexamide and sirtinol for 0-8h, protein expression was determined using western blot **D** MCF-7 cells were transfected with WT FOXO3a-FLAG for 24h and then treated with cyclohexamide and 50 μM EX527 for 0-8h. Protein expression was analyses using western blot**.** Protein expression was analysed using imageJ.

Increasing the protein level of FOXO3a is important in the cytotoxic function of sirtinol, to see if activating FOXO3a can increase this cytotoxcity. WT, A3 (a constitutively active/nuclear localised FOXO3a, previously described [182]) and 294D were over-expressed and treated with and without sirtinol (Figure 6.4). Successful uniform over-expression was confirmed using western blot and assessment of the effect on the cell cycle was observed using flow cytometry. Overexpression of FOXO3a did result in an increase in the sub G1 population from 2.1 to 9.4, 13.8 and 7.2% (Untransfected, WT, A3 and 294D respectively), showing that the A3 unphosphorylated mutant is the most pro-apoptotic form of FOXO3a, mostly likely due to its nuclear localisation (Figure 6.4). Sirtinol treatment resulted in an increase in the sub G1 population of the untransfected cells from 2.1 to 15.4%, similar to what was previously observed (Figure 3.3A). Overexpression of WT FOXO3a and sirtinol treatment increased this apoptotic population to 22.8% but strikingly, overexpression of the A3 mutant and sirtinol treatment caused a marked increase to 39.6%. The 294D mutant with sirtinol treatment induced a sub G1 population of 18.6%, suggesting that the increase potency of FOXO3a is due to its stabilization and activation (Figure 6.4).

To confirm these observations, the colon carcinoma cell line DL23, expressing a constitutively active FOXO3a where the three AKT phosphorylation sites have been mutated to alanines, is fused to the ligand-binding domain of the estrogen receptor (FOXO3a:ER) protein that is activated and translocated to the nucleus upon tamoxifen treatment [170], was taken and treated for 0-24 hours with tamoxifen, sirtinol and in combination (Figure 6.5A). Western blot analysis confirms that tamoxifen activates FOXO3a (a change in the migration of FOXO3a in the gel), Sirtinol treatment stabilizes the FOXO3a:ER construct, and combination treatment results in the stabilization and activation of FOXO3a:ER (Figure 6.5A). The effects on FOXO3a are posttranslational, as no differential effect on FOXO3a mRNA is observed with any of the treatments performed (Figure 6.5B). To assess the effect of combination treatment on cell proliferation DL23 cells were treated with tamoxifen, sirtinol and in combination for 24 hours,

cell viability was observed using the growth assay SRB. Combination treatment was shown to be more cytotoxic than treatment of tamoxifen and sirtinol alone, cell viability being 14.9, 47.9 and 37.6% respectively (24 h, $p<0.001$) (Figure 6.5D). This enhance cytotoxicity was also observed when examining the effect of combination treatment on proliferation (Figure 6.5C). These results suggests that sirtinol treatment could increase the proapoptotic potency of targeted therapies that indirectly activate FOXO3a [337]. The regulation of the FOXO3a downstream targets mnSOD, p27 and Bim were examined using western blot. No synergistic upregulation of of mnSOD or p27 was observed and no change in Bim expression was seen with any of the treatments (data not shown), suggesting that FOXO3a must be eliciting this increase in pro-apoptotic potency through its other downstream targets (supplemental figure 8.1).

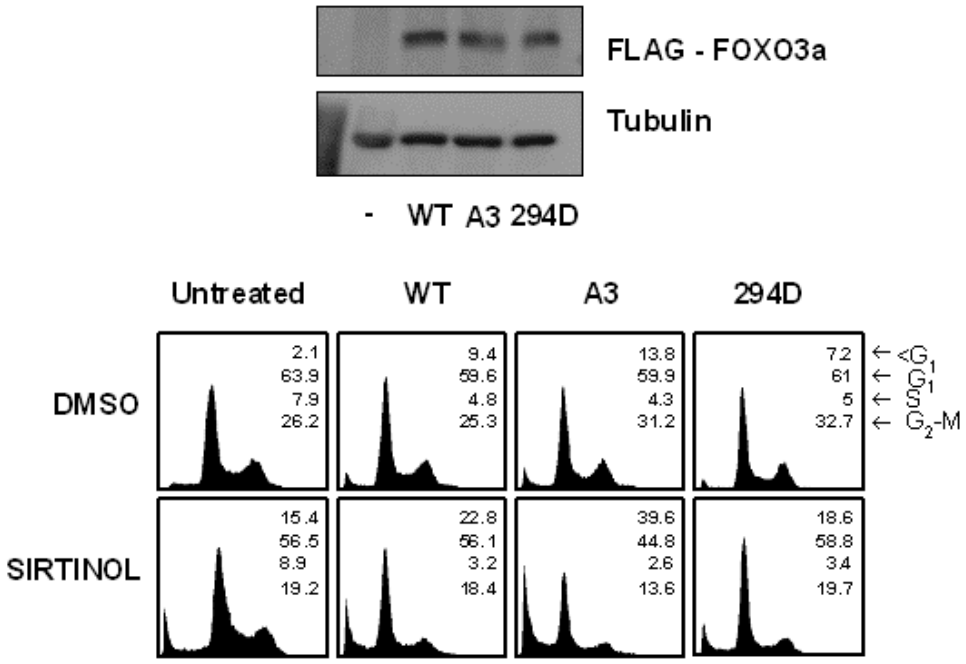


Figure 6.4 Sirtinol treatment combines with overexpression of FOXO3a to induce apoptosis MCF7 cells were transfected with FOXO3a (WT/A3/294D) for 24h and treated with 50μM of sirtinol for a further 24h. Protein expression was elucidated using western blot analysis. Cell cycle analysis of the same samples was performed after propidium iodide staining.

6.2.3 Increasing cellular expression of FOXO3a increases the proapoptotic potency of Lapatinib

Previously we and others have shown FOXO3a to be important in the proapoptotic potency of the tyrosine kinase inhibitors gefitinib and lapatinib [291-293]. Also Lapatinib indirectly activates FOXO3a while not activating other cell mechanisms such as JNK, meaning it is an optimal model for looking at the role of active FOXO3a in cancer treatment [293]. To confirm this SkBr3 cells were treated with 0-10 μM of Lapatinb for 24 hours, protein expression was examined using western blot (Figure 6.6). Lapatinib treatment resulted in a dose-dependent decrease in the AKT phosphorylation. Interestingly, dephosphorylation and activation of FOXO3a was observed even at the lowest concentration, 0.1 μM (Figure 6.6). Moreover, a dose-dependent increase in the cellular expression of FOXO3a was observed, suggesting that one of the mechanisms of action of lapatinib is to increase the cellular expression of FOXO3a to increase its cytotoxicity (Figure 6.6). To access whether sirtinol treatment could complement lapatinib treatment by increasing the expression of FOXO3a, SkBr3 cells were treated with 1μM lapatinib and 50 μM of sirtinol and in combination for 0, 8, 16 and 24 hours. Protein expression was confirmed by western blot (Figure 6.7).

Lapatinib treatment caused the dephosphorylation of EGFR, HER2 and AKT, and subsequently FOXO3a, resulting in its activation (Figure 6.7). Sirtinol treatment stabilized FOXO3a expression but did not result in a change of AKT phoshorylation even though treatment did result in the dephosphorylation of EGFR and HER2 (albeit much slower when compared with lapatinib treatment) (Figure 6.7). Combination treatment resulted in the stabilization of FOXO3a and its activation. Mimicking the observation seen with the FOXO3a:ER inducible system. This increase in activated FOXO3a resulted in the cleavage of proapoptotic marker caspase-3 more readily when compared to single treatments of lapatinib and sirtinol, suggesting the cells are more readily undergoing apoptosis (Figure 6.7). To confirm this increase in activity the caspase 3-7

activity Glo-assay (Figure 6.8) was treated with 1μM lapatinib and 50 μM of sirtinol and in combination for 0, 2, 4, 6 and 8 hours. Combination of lapatinb and sirtinol treatment caused a 4-fold increase in cleaved caspase-3 before any increase is observed with single treatment of each compound, reiterating that these cells are undergoing apoptosis more readily and that combinatorial treatment is more cytotoxic than single treatment. Cell viability was also performed to confirm that the effects seen were not due to large differences in the cell populations (Figure 6.8). Of course, fewer cells are present in the combination treated cells after 8 hours but this is because they are detaching from the 96-well plate and undergoing apoptosis. In the SRB growth assay, dead or dying cells are lost in the steps of the assay; meaning only viable cells are stained and recorded. This is not the case for the caspase-3/7 assay where all cells are retained.

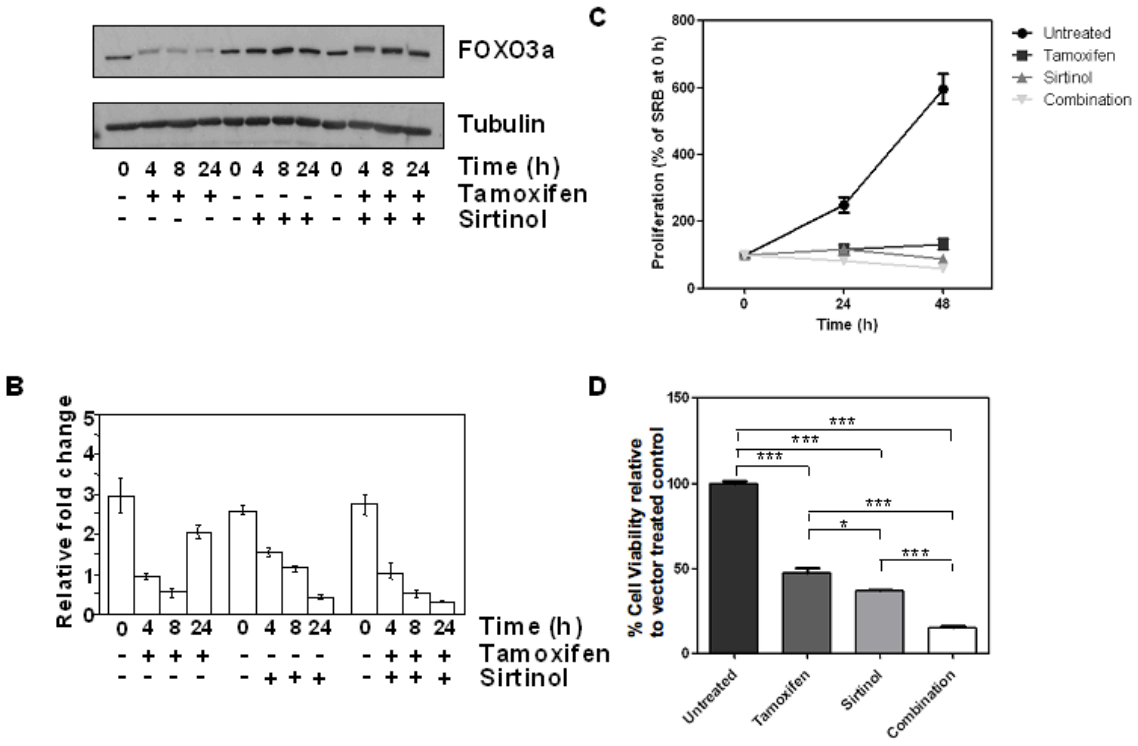


Figure 6.5 Combining FOXO3a activation with sirtinol treatment results in a marked effect on proliferation A DL23 cells expressing a FOXO3a:ER fusion protein we treated with 200nM Tamoxifen, 25μM sirtinol and in combination for 0-48h. Protein expression was determined using western blot. **B** Cells were collected and total RNA was isolated. FOXO3a mRNA levels were analyzed by real-time quantitative PCR normalized with L19 mRNA levels. Experiments were done in triplicate. Mean ± SD. **C** Cell proliferation was determined by SRB assay. **D** DL23 cells were treated with 200nM tamoxifen, 25μM sirtinol and in combination for 24h, cell proliferation was determinded using SRB assay. Results shown are mean ± SD of three independent experiments. Statistical analysis was performed using one way ANOVA (* = $p<0.05$, ** = $p<0.01$ *** = $p<0.001$).

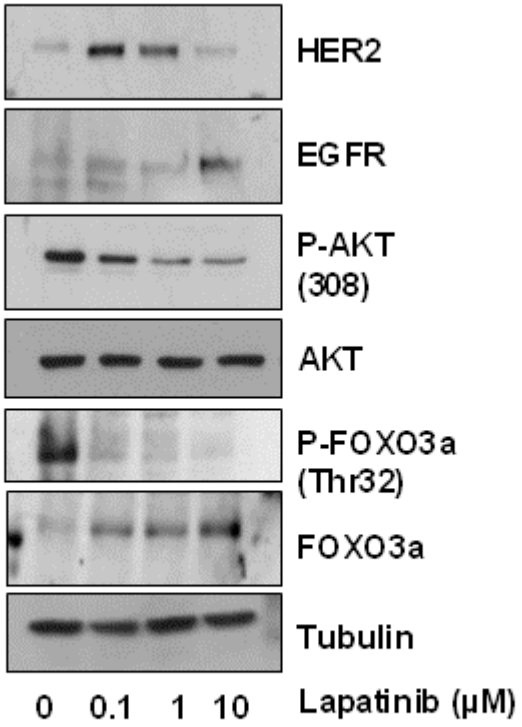


Figure 6.6 Lapatinib activates FOXO3a and increases its expression in a dose-dependent manner SkBr3 cells were treated with 0-10μM of lapatinib for 24 h. Protein expression of total cell lysates was determined using western blot.

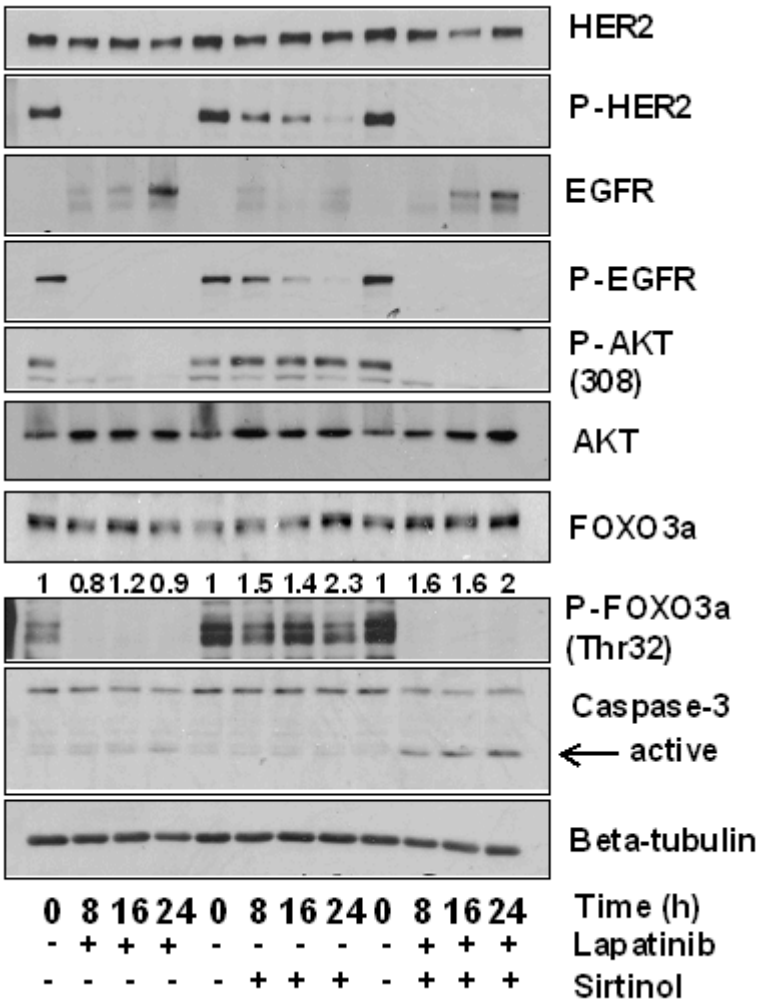


Figure 6.7 Lapatinib and sirtinol treatment synergise SkBr3 cells were treated with 1μM of Lapatinib, 50μM Sirtinol and in combination for 0-24h. Protein expression was determined using western blot.

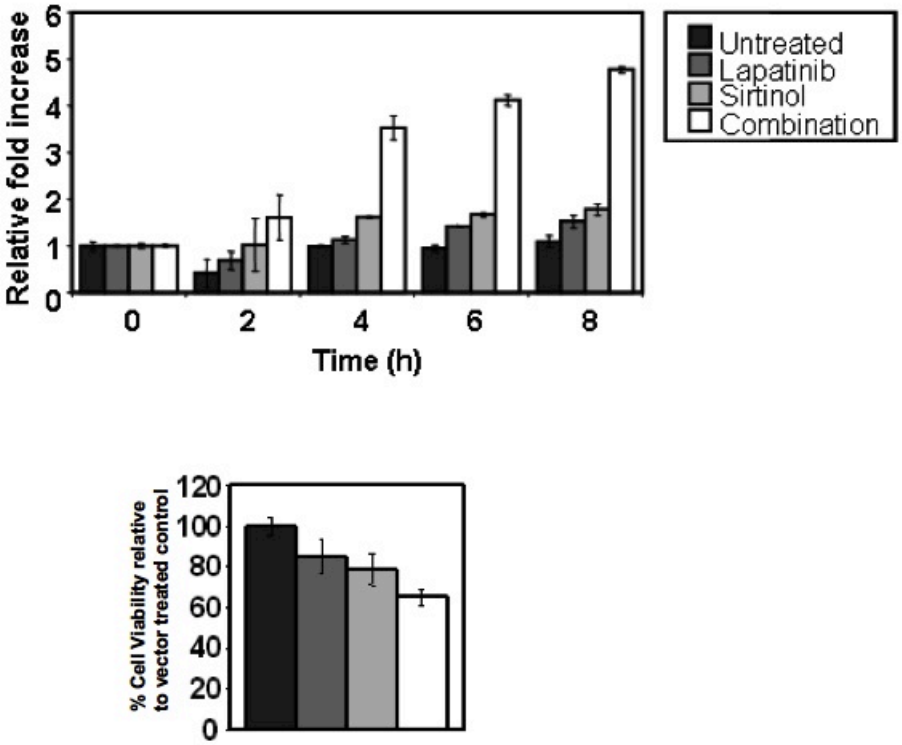


Figure 6.8 Lapatinib and sirtinol treatment synergise to activate caspase 3 Cleaved caspase 3/7 activity assay was performed in SkBr3 cells were treated for 0-8h with 1μM of Lapatinib, 50μM Sirtinol and in combination . Results of two independent experiments in triplicate. Mean ± SD. The proliferation assay SRB was also performed to confirm cell population

6.2.4 Lapatinib treatment synergises with sirtinol treatment

To confirm the effects on cell viability, cell cycle analysis was performed. SkBr3 cells were treated with an increasing concentration of sirtinol (0-50 μM) alone and in combination with 1 μM Lapatinib treatment (Figure 6.9). Cell cycle analysis confirms that combinatorial treatment of lapatinib with sirtinol resulted in a marked increase in the induction of apoptosis compared with single treatment. Cells treated with lapatinib had a sub G1 population of 13.8% compared with the DMSO treated control (5.9%), 50 μM of sirtinol treatment caused a sub G1 population of 10.3%, but when treated in combination this apoptotic population rose in a dose-dependent manner to 31.7% (Figure 6.9). To assess whether this complementation was synergistic, the growth assay SRB was performed. SkBr3 cells were treated for 24 hours with an increasing concentration of lapatinib (0-10 μM) and sirtinol (0-50 μM) and in combination (Figure 6.10A). IC50s were also calculated for lapatinib and sirtinol over a larger concentration range (18.10 μM and 69.91 μM respectively)(Figure 6.10B). From these data, we calculated the degree of synergy using the combination index (CI) algorithm of Chou and Talalay [394]. In general, CI values less than one indicate synergy and above one indicate antagonism. The CI algorithm has been used previously to calculate the degree of synergy between other drug combinations of chemotherapeutic agents [395-398]. Sirtinol was found to synergise with lapatinib, and this was most efficacious at the lower concentrations of lapatinib treatment, the combination index being 0.42, 0.57 and 0.76 for 1, 5 and 10 μM of lapatinib, respectively (Figure 6.10C). It was also noted that EX527 cotreatment with lapatinib was not synergistic (Figure 6.11) but antagonistic to the proapoptotic potency of lapatinib. This results bears similarity to the experiment where EX527 was treated in combination with paclitaxel (Figure 3.10), where it protected MCF-7s from paclitaxel treatment. Highlighting the fact that dual inhibitors, like sirtinol, that inhibit more than one sirtuin family member are likely to provide benefit in combination.

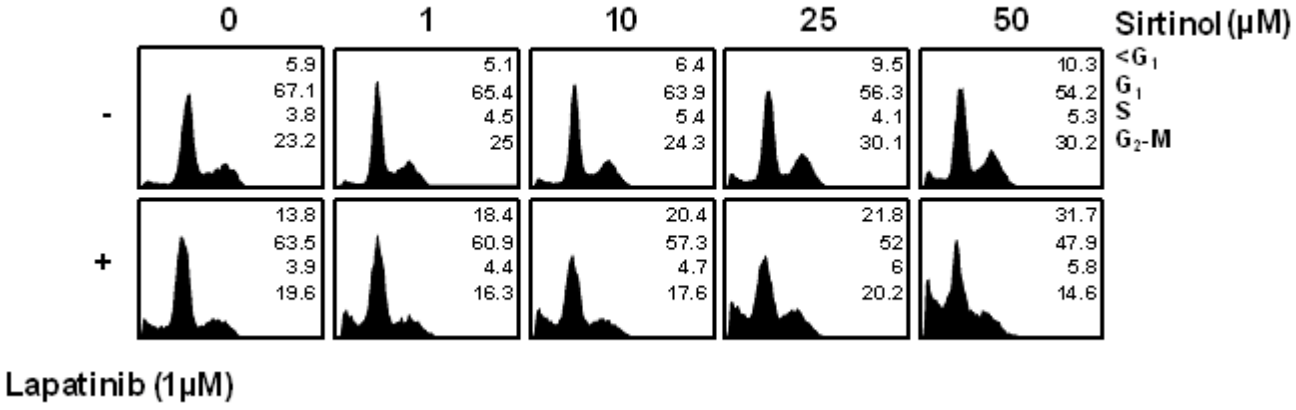
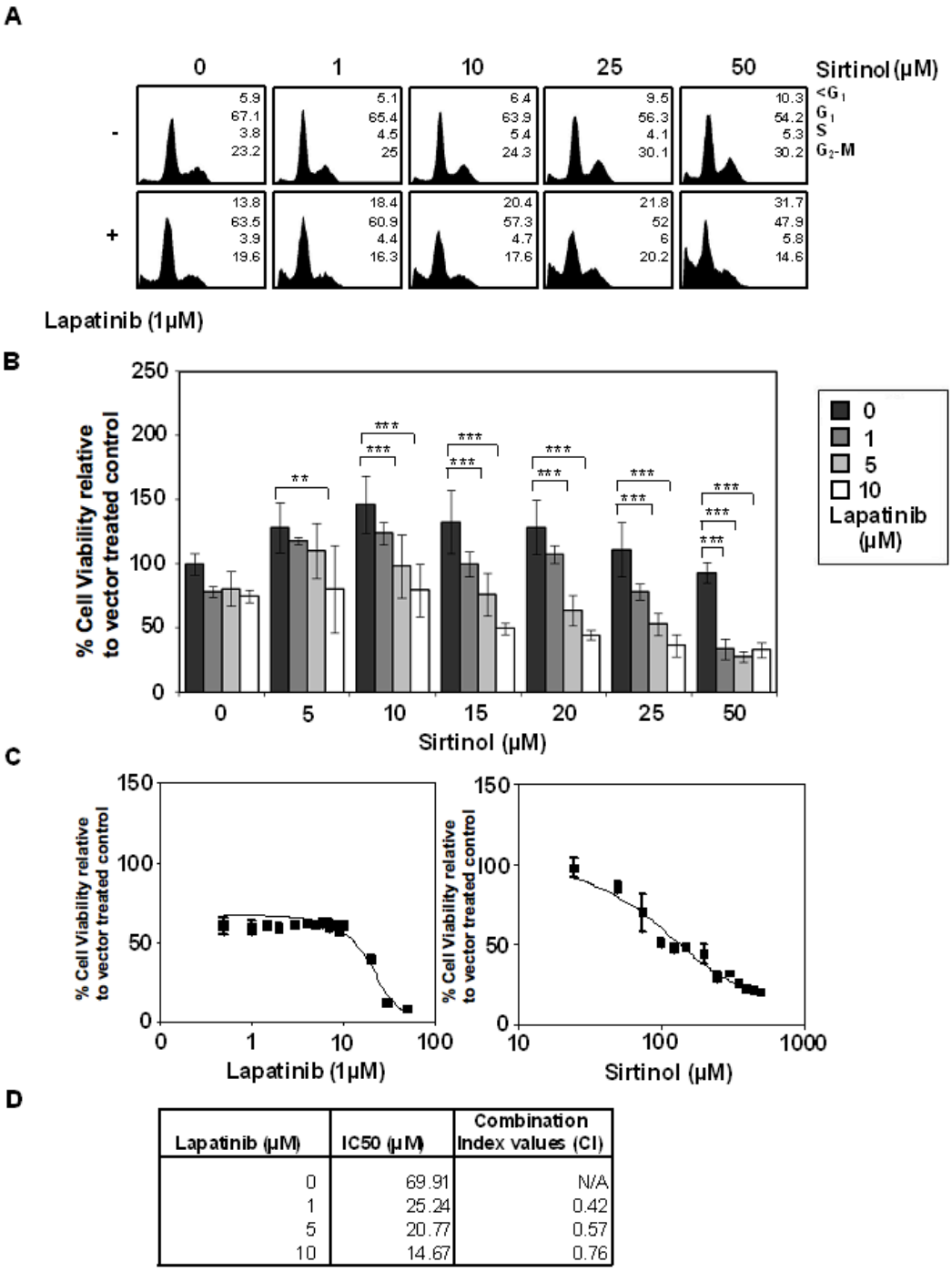


Figure 6.9 Sirtinol synergises with Lapatinib SkBr3 cells were treated with increasing concentrations of Sirtinol alone or in combination with lapatinib (1μM) for 24 hours. Cell cycle analysis was performed after propidium iodide staining.



Lapatinib (μM)	IC50 (μM)	Combination Index values (CI)
0	69.91	N/A
1	25.24	0.42
5	20.77	0.57
10	14.67	0.76

Figure 6.10 Sirtinol synergises with Lapatinib **A** SkBr3 cells were treated with 0-10μM Lapatinb alone or in combination with 0-50μM of Sirtinol for 24 h. Statistical analysis was performed using one way ANOVA (* = $p<0.05$, ** = $p<0.01$ *** = $p<0.001$). **B** SkBr3 cells were treated with 0-100 μM of lapatinib and 0-500 μM of sirtinol for 24 h. Cell proliferation was determined by sulforhodamine B assay. Results shown are mean ± SD of three independent experiments. IC50s were calculated using graphpad (lapatinib = 18.10 μM, sirtinol = 69.91) **C** combination index analysis of lapatinib and sirtinol treatment.

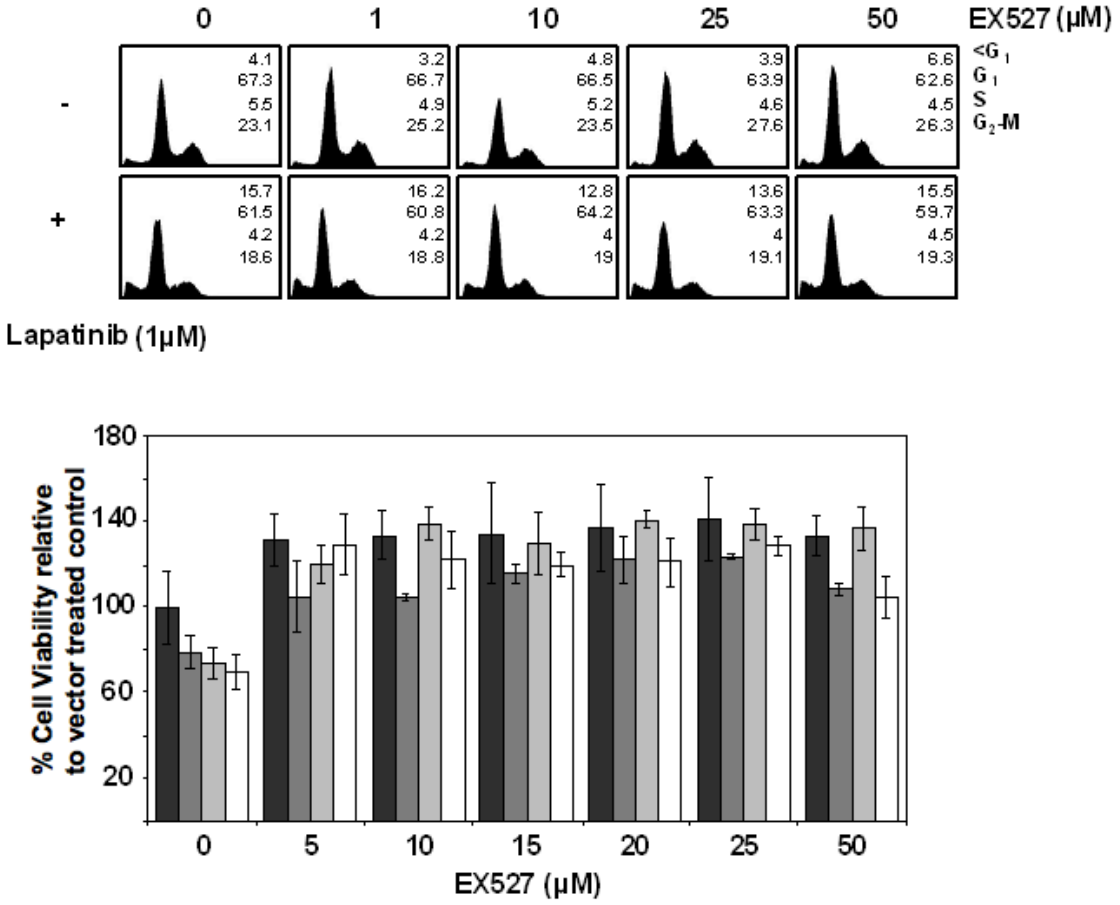


Figure 6.11 EX527 does not synergise with Lapatinib **A** SkBr3 cells were treated with increasing concentrations of EX527 alone or in combination with lapatinib (1μM) for 24 hours. Cell cycle analysis was performed after propidium iodide staining. **B** SkBr3 cells were treated with 0-10μM Lapatinb alone or in combination with 0-50μM of EX527 for 24 h. Cell proliferation was determined by sulforhodamine B assay. Results shown are mean ± SD of three independent experiments.

To assess whether this synergism was dependent on the presence of FOXO family members, NIH 3T3 WT and FOXO-/- were treated with DMSO, Lapatinib (10 μM), sirtinol (40 μM) and in combination for 72 hours, and cell viability was accessed using SRB (Figure 6.12A). Lapatinib treatment resulted in a decrease in cell viability from 100% (DMSO control) to 84.7%, sirtinol treatment resulted in a decrease of viability to 84.4%, but combination resulted in a statistically significant ($p<0.001$) decrease of cell viability to 46.2%, when compared to single treatments and the DMSO controls. In the FOXO-/- no statistically significant effect on cell viability was observed under any treatment, highlighting the role of FOXO proteins in the efficacy of lapatinib and sirtinol treatments and the efficacy of combination treatment (Figure 6.12A).

Interestingly, when NIH 3T3 wildtype cells were treated with 0-10 μM of lapatinib alone or in combination with 0-100 μM of sirtinol, there was an additive effect seen at lower concentrations of sirtinol 0-40 μM but not at higher concentrations. (Figure 6.12B) This correlates with the concentrations of sirtinol treatment that are dependent on the presence of FOXO proteins to elicit its cytotoxic effect (Figure 6.1), reiterating the importance of FOXO3a in the synergism between lapatinib and sirtinol. Above 50 μM of sirtinol treatment, other proteins such as p53 are activated (see figure from chapter 1) and become the main factor in controlling cell fate. Because of this fact I was unable to distinguish the synergistic effect of combination treatment in NIH 3T3 cells. But it is interesting to note that NIH 3T3 cells do not overexpress EGFR or HER2, the cellular targets of lapatinib, meaning sirtinol could increase its efficacy in patients who do not display HER2 overexpression or EGFR hyperactivation, meaning combining lapatinib with sirtuin inhibitors could display benefit in a wider subset of patients.

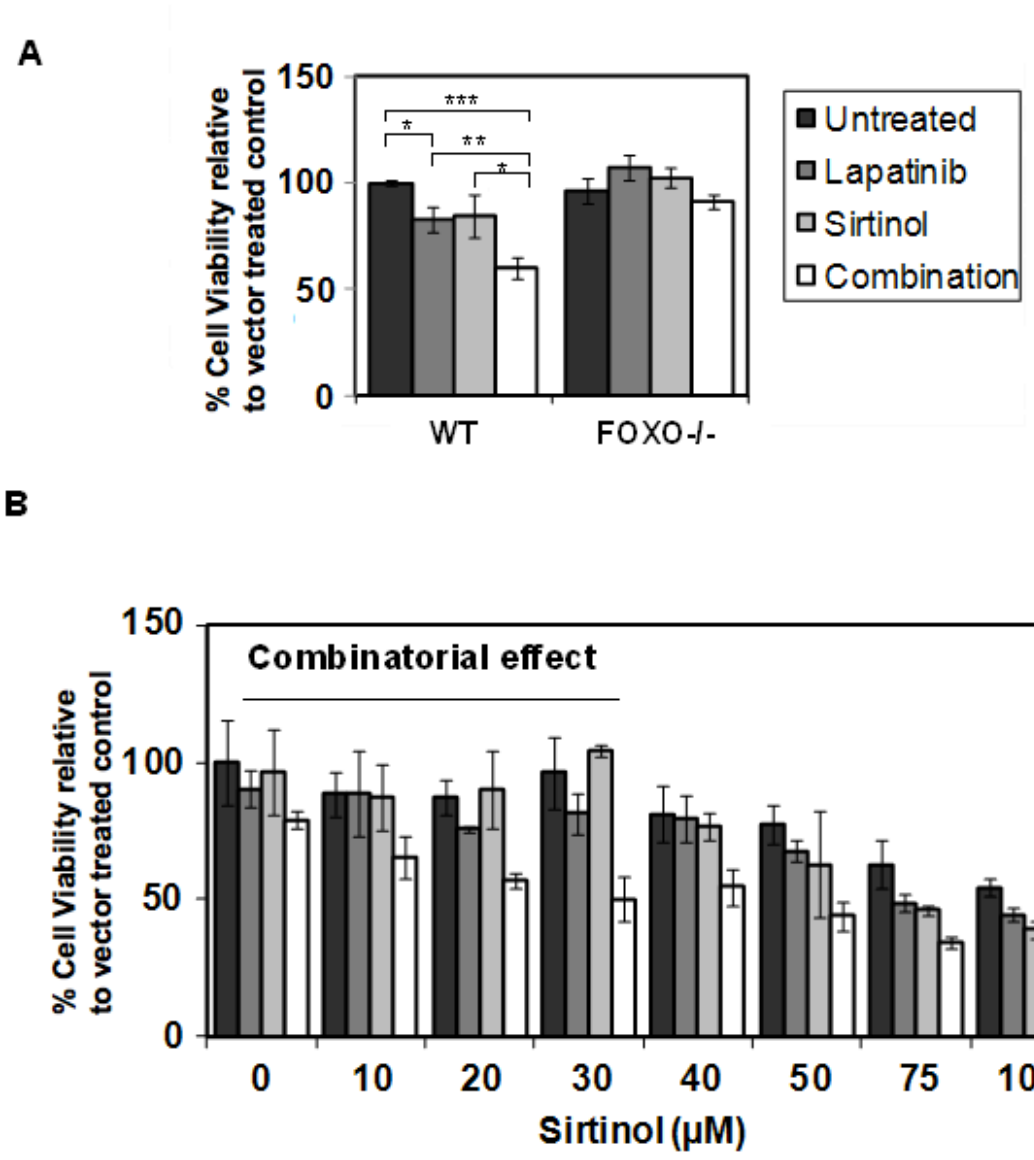


Figure 6.12 Loss of FOXO reduces the potency of Lapatinib A NIH 3T3 cells WT and FOXO null MEFs were treated for 72 hours with 1μM Lapatinib, 30μM of Sirtinol and in combination. **B** NIH 3T3 WT cells were treated for 72 h with 0-10 μM of lapatinib and 0-100μM of sirtinol and in combination. Cell proliferation was determined by sulforhodamine B assay. Results shown are mean ± SD of three independent experiments. Statistical analysis was performed using one way ANOVA (* = $p<0.05$, ** = $p<0.01$ *** = $p<0.001$).

6.3 DISCUSSION

We and others have shown previously the transcription factor FOXO3a to be important in the proapoptotic potency of the tyrosine kinase inhibitors gefitinib and lapatinib [291-293] We have also shown that sirtinol elicits its induction of apoptosis in a p53 dependent manner [316]. Here we show that the cytotoxic effect of sirtinol, at low concentrations, is dependent on the presence of FOXO family members. Examining this further the effect of sirtinol treatment on FOXO3a was found to cause its stabilization, which was independent of mRNA expression, meaning sirtinol treatment resulted in the acetylation of FOXO3a and its subsequent stabilization.

We then addressed whether the stabilizing effect of sirtinol treatment could complement FOXO3a activation. Overexpression of the constitutively active (non-phosphorylatable) FOXO3a caused a marked increase in sub G1 cells, compared to WT FOXO3a and the 294D mutant (phospho-mimic), which could not be stabilized. This observation suggests that the FOXO3a 294D mutant did not cause an increase in apoptotic cells when treated with sirtinol because it could not be stabilized, meaning the proapoptotic function of sirtinol was due to the stabilization and activation of FOXO3a.

The efficacy of targeted therapies such as lapatinib, Gefitinib and Herceptin [102, 291-293] are directly associated with the expression of FOXO3a. To address the hypothesis of whether sirtinol cotreatment could increase the proapoptotic potency of lapatinib, we took the ER negative, HER2 overexpressing cell lines SkBr3 and treated them with single treatment of laptinib (1μM 0-24 h) sirtinol (50μM 0-24 h) and in combination. Lapatinib treatment resulted in the dephosphorylation of EGFR, HER2 and AKT, leading to the dephosphorylation and subsequent activation of FOXO3a. Sirtinol treatment increases the cellular expression of FOXO3a (2.3 times higher than the control), this was independent of FOXO3a desphosphorylation of Thr32, suggesting the stabilization of FOXO3a

is independent of PI3K signalling cascade. Sirtinol treatment did lead to the dephosphorylation of EGFR and HER2, albeit, much slower than is observed with lapatinib treatment. Others have shown that SAHA treatment results in destabilization of EGFR mRNA resulting in loss of expression [399], we did not observe a decrease in protein expression but it does suggest that sirtinol and possibly other class III inhibitors have multiple cytotoxic mechanisms, like class I and II HDAC inhibitors.

Statistical analysis showed that sirtinol synergised with lapatinib treatment and that this synergy was more potent with lower concentrations of lapatinib. This reiterates the hypothesis that the cellular availability of the intracellular effector, FOXO3a is the factor limiting efficacy of lapatinib treatment, meaning combination treatment with sirtuin inhibitors, namely sirtinol, would reduce the amount of lapatinib needed, increasing efficacy but reducing toxicity. Also performing a dose-titration of lapatinib treatment in SkBr3 cells (0-10 μM for 24 h), shows a dose dependent increase in FOXO3a expression, but dephosphorylation of FOXO3a (Thr32) was observed at 0.1 μM of lapatinib treatment suggesting sirtinol treatment is mimicking one of the mechanisms of action of lapatinib, meaning less is needed when treated in combination with sirtinol. Moreover, SkBr3 cells contain mutant p53, highlighting the fact that the proapoptotic potency achieved with combinatorial treatment of lapatinib and sirtinol is not a result of p53 induced apoptosis. Also the concentrations used are below that which are seen to induce the acetylation of p53 [316], and the effect of sirtinol at these concentrations are dependent on the presence of FOXO family members. We also tested the ability of EX527 to synergise with lapatinib treatment, conversely it was antagonistic of lapatinib treatment, suggesting that a dual inhibitor like sirtinol elicits its effect by stabilising its downstream targets (p53 and FOXO3a), something EX527 is unable to do because it is inefficient at inhibiting multiple sirtuin proteins.

Previous publications have highlighted that the acetylation of FOXO1 alters its DNA-binding affinity and sensitises it to phosphorylation [400]. These publications only assessed lysine residues within the DNA binding domain, not regions associated with stability or degradation (294, 388 and 425). This study also used a pan sirtuin inhibitor nicotinamide which does not specifically target sirtuins but all NAD dependent proteins within the cell. Moreover, they only observed an increase in FOXO1 acetylation when cells were treated in combination with TSA, the HDAC class I and II inhibitor. Others have also shown that forkhead proteins, namely foxP3, can be stabilized with a combination of HDAC inhibitors [401], we have show for the first time using a single sirtuin inhibitor, sirtinol, can cause the stabilisation of FOXO3a.

Sirtinol treatment also causes the stabilization of p300 (supplemental figure 8.2), which could explain for the increased proapoptotic potency of sirtinol. SIRT2 is known to regulate the autoacetylation of p300 [402], inhibiting more sirtuins results in its own autoactivation and increased interaction with targets such as p53 and FOXO3a, eliciting a proapoptotic response. The HDAC class II inhibitor, Tricostatin A, has been shown previously to increase the interaction of p300 with p53 [403].

This study supports the use of HDAC class III inhibitors as a viable therapy in HER2 overexpressing tumours. p53 mutations are associated with poor clinical outcome and are the most frequent cellular event in carcinogenesis [404]. Sirtuin inhibitors function dependently and independent of p53 mutations. In patients that have mutant p53, sirtinol can be used to increase the efficacy of FOXO3a targeting therapy, as well as in WT p53 containing tumours, having its own cytotoxic effects.

In this study we show that combining sirtinol with lapatinib, increases its pro-apoptotic potency and that this increase in potency is due to the stabilization and activation of FOXO3a. Recently the interest in developing compounds that effect

the translocation of FOXO3a from the cytoplasm to the nucleus and the subsequent antiproliferative effect in cancer cell lines has been highlighted [289]. But these inhibitors would encounter the same issues as already approved therapies. The deregulation of the PI3K pathway traps FOXO3a in the cytoplasm, but resistant cells (Figure 4.2) have been associated with an increase in phosphyated FOXO3a and reduced total FOXO3a. Sirtuin inhibition with sirtinol treatment increases the cellular pool of FOXO3a, independent of AKT phosphorylation, increasing the efficacy therapeutic compounds that indirectly activate FOXO3a. Developing compounds that have a similar effect on FOXO3a would be a more worthy line of research.

6.4 FUTURE WORK

Investigating the exact mechanism of action sirtinol treatment has on FOXO3a, by cloning FOXO3a acetylatable and non-acetylatable mutants (mutating lysine residues to alanines and glutamine). With these tools we would be able to mimic the proapoptotic effect sirtinol treatment has on FOXO3a and investigate which downstream targets are eliciting the activation of apoptosis and cell death.

During the undertaking of the thesis we also investigated the subset of genes that were eliciting the proapoptotic role of FOXO3a but were unable to identify the downstream target(s) responsible. To investigate this further, CHIP-seq could be used to identify the genes that FOXO3a is binding to under differing treatment regimes. Not only would it identify the genes involved but also the promoter regions to which FOXO3a is binding to.

Developing compounds that target both EGFR/HER2 and sirtuins and evaluating its efficacy compared to established targeted therapies, e.g. lapatinib, gefitinib and herceptin. Moreover, evaluating its efficacy in different cell lines (different disease types, lung, breast, ovarian and prostate) and all cell lines that are resistant to established chemotherapeutic regimes such as tamoxifen and aromatase inhibitors.

CHAPTER 7

FINAL DISCUSSION

7.1 Targeting HDACs in cancer

The study of HDACs has increased exponentially over the last decade, mainly due to the discovery of their role in cancer progression, metastasis and the development of resistance, but also due to the development of small molecules that can specifically target HDACs *in vivo* influencing their activity.

The most well defined HDAC inhibitor (HDACi) to date is the class I and II small molecule inhibitor SAHA (Vorinostat), which was the first to be approved by the FDA in 2006 for the treatment of cutaneous T-cell lymphoma (CTCL) [308]. Since the approval of SAHA many HDAC inhibitors have entered clinical development, the most recent success being Romidepsin (Istodax) which was also approved by the FDA for the treatment of CTCL in 2009 [405]. Although clinical trials of many compounds are ongoing, currently no HDAC inhibitor has been approved for the treatment of solid tumours i.e. breast cancer, and no class III HDAC inhibitor has progressed from preclinical research into clinical trials for cancer treatment. The compounds cambinol and tenovin-6 have been the most rigorously tested sirtuin inhibitors to date, both suppressing tumour growth in nude mice xenographed with burkitt lymphoma and human melanoma cells, respectively [311, 312]. Although more recently a milestone has been reached of testing a sirtuin inhibitor in a clinical setting, the potent SIRT1 inhibitor EX527 (SEN0014196) entered into a phase I clinical trial for the treatment of huntington's disease early in 2010. Although this study is testing sirtuin inhibition in a different disease state, it will provide valuable information about the tolerability and toxicity of sirtuin inhibitors in healthy adults, answering concerns on issues such as cardiac toxicity, which has been an issue with HDAC class I and II inhibitors [308], and could yet prove to be a bump in the road for sirtuin inhibitors as a cancer treatment due to the fact that SIRT1,-3 and -7 appear to have cardio-protective roles in blocking stress-induced cardiac hypotrophy and possibly other stress-related diseases [406]. If proved successful, targeting class III HDACs could provide a selective advantage over other family members

because class III inhibitors like sirtinol do not affect the class I and II HDACs, and the established class I and II inhibitors, like TSA, do not affect Sirtiuns [309].

Most if not all previous publications have elucidated the molecular targets of sirtuin inhibitors using combinations of high concentrations of inhibitors, for example, nicotinamide and TSA, or in combination with cytotoxic agents like etoposide [315]. Although these are valid studies and bring valuable information about possible combinatorial therapies that could be used, they skirt around the molecular biology of how to specifically target and produce a therapeutic benefit from this interesting family of proteins.

An aim of this thesis was to elucidate the mechanism of action of known sirtuin inhibitors. With this study I believe it has become apparent that pan sirtuin inhibitors, like nicotinamide, that target all sirtuins and any NAD+ dependent protein in the cell, only work at high concentrations (in the miliolar range) and do not appear to display any benefit. Specifically targeting two or more sirtuins is a better but a discernibly more challenging scenario. One, having to decipher the exact combination of sirtuins to target, and two, whether using a single inhibitor that can specifically target the desired sirtuins and not other family members is achievable, or combining specific single inhibitors of each protein is a better and more worthwhile approach. Here it has been shown that inhibition of SIRT1 and SIRT2 in combination results in the induction of apoptosis, but the cell cycle profile is not the same as observed with sirtinol treatment, indicating that their inhibition does lead to the induction of apoptosis but that other family members should also be inhibited for sirtuin targeted therapies to elicit a more cytotoxic effect and a potent induction of apoptosis in cancer cells.

I believe we have shown successfully that the sole targeting of SIRT1 would not provide benefit or increased efficacy of known chemotherapeutic compounds, in the contrary, it would reduce their proapoptotic potencies and possibly lead to the

development of mechanisms of resistance. This conundrum could be responsible for the current misunderstanding of the role of sirtuins in cancer cell biology.

Most sirtuin literature is dominated by the question whether SIRT1 is an oncogene or tumour suppressor [407-409]. SIRT1 has been shown to be upregulated in caloric restriction, extending lifespan and reducing the risk of cancer development [236, 238, 410, 411]. But conceptually, if you consider cancer, as an entity in itself, a logical step in its development would be to upregulate SIRT1 (and other sirtuins) to protect itself from DNA damage from genotoxic agents, regulate metabolism and oxidative stress, in an environment that is constantly combating the cancer. SIRT1 may suppress tumour formation but when a tumour has already developed, increased SIRT1 expression may help it to survive. Recently Boily *et al* [412] showed that sirT1 null mice were not prone to cancer formation compared to WT mice, at odds with Wang *et al* who showed that SIRT1 was responsible for chromosomal stability and that SIRT1+/- p53+/- mice were more cancer prone than SIRT1 +/+ p53+/- mice. [413] It is interesting to note that resveratrol was championed as a possible antitumour compound through its action of increasing SIRT1 activity in BRCA1-associated breast cancer [414], but Boily *et al* showed that the antitumour function of resveratrol was in part independent of the presence of SIRT1 [412]. Moreover, recent publications have shown that resveratrol and its derivatives are not direct activators of SIRT1 [330, 344, 415, 416]. This finding buoys our own suspicions of the *in vitro* assays not being the best determinants of compound specificity and ability to influence sirtuin protein activity *in vivo* [316]. They should be used in combination with a range of techniques to make sure they concur with *in vitro* and *in vivo* data. These findings do not detract from the fact that resveratrol does exhibit antitumour activity and has displayed benefit [300], but the involvement and importance of SIRT1 and other sirtuins in eliciting its antitumour effect needs to be more clearly defined.

Misunderstandings or misconceptions of the underlying molecular biology of sirtuins and their roles in various processes have produced effectively two sides of thought, under the banners of tumour suppressor and oncogene, respectively. I believe further research is needed to clarify the roles of sirtuins in cancer cell biology but increasingly evidence is pointing towards their roles in tumour development.

It is important to remember that SIRT2, SIRT3 and SIRT7 have also been shown to be upregulated in tumours [300, 307], and that compounds that inhibit more than one sirtuin, like sirtinol, cambinol and tenovin-6 appear to be more efficacious than single inhibitors like EX527. We confirmed within our own study with siRNA targeting SIRT1, SIRT2 and in combination, that both need to be inhibited to induce apoptosis, suggesting that there is a certain amount of compensation that can occur when only one sirtuin has been targeted. Both SIRT1 and SIRT2 are known to regulate the activity of p53 [274, 417] and it is easy to imagine a scenario where SIRT1s inhibition results in an increase in SIRT2 activity or interaction with p53, deacetylating p53, albeit less efficiently than SIRT1, but sufficient enough to cause a different outcome in cell fate, for example cell cycle arrest rather than apoptosis. It is possible mechanisms like this that make understanding the role of sirtuins in cancer cell biology difficult. The overexpression of one specific sirtuin may be sufficient to protect cancer cells from chemotoxic agents but the inhibition of two or more is required to sensitize cells to cytotoxic compounds. Moreover, the regulation of non-histone proteins that are integral to cell cycle regulation and proliferation, like p53 and FOXO are key cellular targets of sirtuins and their inhibition with HDAC inhibitors results in their stabilization and activation. Interestingly, tenovins were initially identified using a cell-based screen analyzing p53 activation [312, 316]. Also, within our own study inhibition of SIRT1 with EX527 causes the acetylation of a different subset of genes and does not influence the acetylation of p53 on lysine 382, a marker for p53 activation and induction of apoptosis [418]. Moreover, the cytostatic action of EX527 is not dependent upon the presence of wildtype,

mutated or the expression of p53, but is dependent on the presence of FOXO proteins, supporting the importance of the forkhead transcription factors in eliciting the function of sirtuin targeted compounds, be it cytostatic or cytotoxic.

7.2 Sirtuins and chemotherapeutic resistance

I have shown that SIRT1 is important in the development of resistance to cisplatin and that SIRT1 overexpression suppresses the activation of FOXO3a. SIRT1 has been shown to be upregulated in prostate cancer, suppressing the activation of FOXO1. Treatment with SIRT1 targeted siRNA, sirtinol and nicotinamide resulted in the reactivation of FOXO1 and induction of a growth arrest [419].

Sharma *et al* strengthen the knowledge of how cancer cells can develop resistance that do not involve genetic alterations i.e. mutations, but a small percentage of the cell population become 'drug tolerant persisters' due to changes in chromatin structure [420, 421]. Prolonged exposure to cytotoxic agents, such as cisplatin and the targeted therapy gefitinib, led to the development of permanently resistant cells. Also, their results showed that a decrease in histone acetylation on lysine 16 (H4K16) was involved in the development of resistance. H4K16 is known to be one of the main intracellular targets regulated by SIRT1 [246, 422] and loss of H4K16 acetylation was shown by Fraga *et al* to be a 'hallmark of cancer development' and suggested SIRT1 to be the primary culprit for histone acetylation dysregulation in cancer [296]. Moreover, these resistant cells could be resensitised to chemotoxic agents by combining them with HDAC inhibitors. Extrapolating this to our findings, SIRT1 could be the first step in the development of resistance and increasing migratory potential and metastasis, strengthening the importance of developing HDAC targeted therapies.

7.3 Combination therapy

It is becoming apparent that combinatorial treatment with targeted therapies can increase their efficacy. Combined treatment of trastuzumab (Herceptin) and lapatinib has been shown to increase patient free survival in HER2 overexpressing women with metastatic breast cancer, compared to lapatinib treatment alone, despite having disease progression on trastuzumab treatment previously [423]. Lapatinib has also been found to increase progression-free survival in patients with advanced HER2-postive tumours when given in combination with the antimetabolite chemotherapy drug capecitabine (Xeloda) compared with capecitabine alone, in patients who had already failed previous treatment regimes with anthracyclines, taxanes and trastuzumab [349].

We and others have shown the importance of FOXO3a in eliciting the proapoptotic potency of the targeted therapies, such as lapatinib and gefitinib [291-293]. Within this study we forward this knowledge and understanding by showing that loss of the FOXO family members is sufficient to confer resistance to lapatinib and that the cellular expression of FOXO3a is decreased in lapatinib resistant SkBr3 cell lines. More importantly, reinstating FOXO3a expression in FOXO-/- MEFs was sufficient to resensitise them to lapatinib treatment, strengthening the importance of FOXO3a in mediating the response to lapatinib and the therapeutic importance of compounds that can increase the cellular expression of FOXO3a, like sirtinol.

It is becoming more apparent that the non-histone targets of sirtuins play an important role in the regulation of cell cycle progression, offering something different to class I and II HDACs. I believe the future of combinatorial therapy lies within the grasp of sirtuins. The development of compounds like CUDC-101 which combine recent advancements in targeted therapies, with potent HDAC class I and II inhibitors, in my opinion, pave the way for the development of novel therapies [424]. CUDC-101 is effectively a receptor tyrosine kinase inhibitor

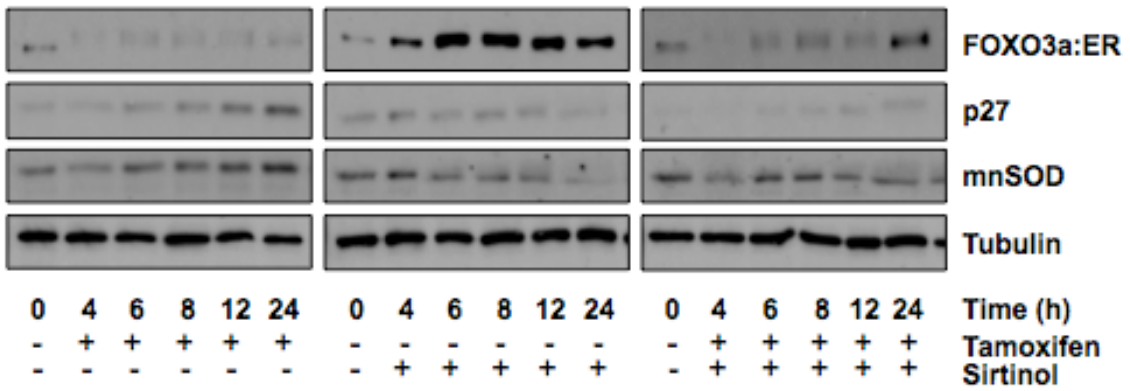
fused to a HDAC class I and II inhibitor which displays potent inhibition of EGFR, HER2 and HDAC (2.4, 15.7 and 4.4nM, respectively) and is more toxic in cancer cell lines that monotreatment of erlotinib, vorinostat, lapatinib and in combination [424]. Cai *et al.* have proven it is possible to combine HDAC inhibitors and targeted therapies and still maintain their specific potencies. More recently, CUDC-101 has been shown to inhibit proliferation in 54 human cancer cell lines (Breast, Pancreatic, Liver, CTCL, Ovarian and more) and be more effective in reducing tumour size in a range of tumour types in xenograph models, when compared with vorinostat, erlotinib and lapatinib treatment [425]. Because of the non-histone targets of sirtuins, namely FOXO3a, and the dependence of sirtuin inhibitors like sirtinol on FOXO3a to elicit its cytotoxic activity. A similarly designed compound with a sirtuin inhibitor instead of a class I and II inhibitor would hypothetically be able to deliver even more therapeutic benefit that CUDC-101, if sirtuin inhibitors can be shown not to be indirectly toxic to animal models and humans. Sirtinol treatment alone results in the stabilization of both FOXO3a and p53, increasing their cellular expressions causing the activation of proapoptotic pathways [316]. Sirtinol also synergises with lapatinib treatment in a FOXO3a dependent mechanism to cause a marked increase in the induction of apoptosis in SkBr3 cells. Interestingly, one of the mechanisms of action of lapatinib is to cause a dose-dependent increase in FOXO3a expression, yet only the lowest concencentration of lapatinib (0.1μM) is required to inhibit the PI3K signaling cascade and activate FOXO3a. Essentially I believe lapatinib and sirtinol synergise because sirtinol treatment mimics one of the mechanisms of action of lapatinib i.e. stabilizing FOXO3a. This means less lapatinib is needed in combination treatment compared to lapatinib treatment alone, possibly reducing adverse off-target side-effects. To confirm whether sirtinol and lapatinib could be combined as one structure, synergy would have to be assessed using isobologram analysis, where synergy is calculated using set molar ratios, for example L1:S1, L1:S10 etc (L = lapatinib and S- sirtinol).

Conceptually, it is interesting to consider the recent research that has shown EGFR/HER2 targeted therapies resensitise both HER2 overexpressing and HER2 negative tumours to endocrine therapy [11, 426]. Xia *et al* showed quite eloquently that lapatinib treatment resulted in an increase in ER activity which was due to the activation of FOXO3a [291]. In this setting, lapatinib treatment is shifting HER2 overexpressing tumours back to dependence on ER signaling and ER mediated proliferation, promoting tumour survival, and that this shift can be exploited therapeutically using endocrine therapy. But essentially what is occurring is that FOXO3a is not efficiently activating proapoptotic mechanisms inducing apoptosis and cell death, but targets genes that promote survival. SIRT1 has been shown to mediate forkhead activity by deacetylating FOXO3a resulting in the activation of genes involved in destressing and survival, such as mnSOD and catalase [223, 265], and we have shown that SIRT1 is upregulated during the development of resistance to cisplatin. In HER2 overexpressing tumours, SIRT1 expression is protecting cancer cells from the cytotoxic effects of lapatinib by limiting the repertoire of genes that FOXO3a can activate. Inhibiting sirtuins with chemical inhibitors would not only have their own cytotoxic function but reduce the ability of tumours to upregulate prosurvival mechanism by re-enabling FOXO3a to activate transcriptional targets involved with a proapoptotic response, increasing the efficacy of targeted therapies. Moreover, exploiting these mechanisms of resistance with endocrine therapy is only viable when tumours shift their dependency back to ER driven proliferation. Recently Turner *et al* have shown that the fibroblast growth factor receptor 1 (FGFR1) is amplified in ~10% breast cancer cases and is associated with poorer prognosis and resistance to endocrine therapy [13, 427]. If tumours revert to or develop FGFR1-dependent signaling after lapatinib treatment, then the combination of endocrine agents will not have an increased cytotoxic effect, allowing tumours to persist. This possible scenario reiterates that increasing the efficacy of targeted therapies by modulating FOXO3a expression and activity with HDAC inhibitors could prove to be a more successful endeavor, reducing the likelihood of the development of resistance, killing tumours outright.

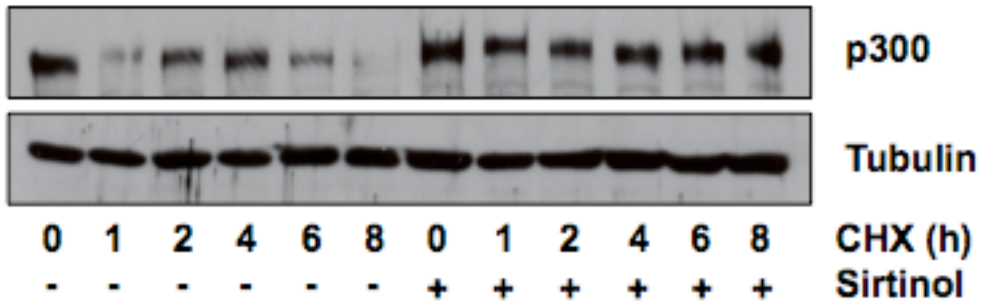
I believe that the research undertaken in this thesis does shed light on how breast cancer cells develop resistance and the importance sirtuins and FOXO family members play in this dynamic process. Furthermore, I believed we have shown that this can be exploited therapeutically with small molecule inhibitors. Further study is required to explore these research themes, but I believe that it is a step – albeit a small one – in improving cancer therapies, leading to the ultimate goal of successfully, responsibly and sustainably improving lives.

CHAPTER 8

SUPPLEMENTAL DATA



Supplemental Figure 8.1: FOXO3a activation and sirtinol treatment in combination do not increase the expression of p27, mnSOD or Bim DL23 cells expressing a FOXO3a:ER fusion protein we treated with 200nM Tamoxifen, 25μM sirtinol and in combination for 0-24h. Protein expression was determined using western blot.



Supplemental Figure 8.2 Sirtinol treatment stabilizes p300 MCF-7 cells were treated for 0-8h with CHX and in combination with 25μM sirtinol or the DMSO vector control. Protein expression was examined using western blot.

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