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NOVEL THERAPEUTIC TARGETS IN NSCLC RESISTANCE TO ERLOTINIB

By

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A thesis submitted for the degree of Doctor of Philosophy

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Statement of originality

I declare that with the exception of where it is clearly indicated and acknowledged within the text this thesis is a result of my own work. In addition parts of chapters 4 and 5 have been published with myself as one of the first authors in a research article entitled “Decreased glutathione biosynthesis contributes to EGFR T790M-driven erlotinib resistance in non-small cell lung cancer.” This article can be found at the end of this thesis in chapter 10.

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Abstract

Approximately 10% of non-small cell lung cancers (NSCLCs) have an activating mutation within the kinase domain of epidermal growth factor receptor (EGFR). Competitive tyrosine kinase inhibitors (TKIs), such as Erlotinib and Gefitinib, have been developed to therapeutically exploit this. These compounds have shown excellent clinical responses in approximately 77% of patients with EGFR-mutant driven lung cancer. However, despite positive initial response, patients develop resistance, and this is often attributed (50% of cases) to the T790M secondary mutation in the ATP binding pocket of EGFR. This mutation increases the receptors affinity for ATP which competes out reversible first-generation inhibitors such as erlotinib and gefitinib. This thesis aims to better understand the underlying mechanisms involved in the resistance to this compound.

Changes in metabolism have frequently been associated with tumour progression and drug resistance in many cancer types. Here, to identify novel resistance mechanisms, ¹H-NMR metabolic profiling was performed on EGFR-driven NSCLC cells with and without, the additional T790M-EGFR resistance mutation. This study identified both glutathione (GSH) and phosphatidylcholine metabolism as two key pathways perturbed in erlotinib-resistant cells.

Interestingly, ¹H-NMR revealed lower levels of both oxidised and reduced forms of GSH in erlotinib-resistant cell lines and this was verified by colorimetric assays. This change correlated with reduced mRNA expression of GSH synthesizing enzymes in resistant cell lines. Increasing cellular GSH levels by siRNA-mediated targeting of GSH degrading enzymes sensitised erlotinib-resistant cells to erlotinib treatment. In addition, the use of GSH synthesizing enzyme inhibitors such as Buthionine Sulfoximine (BSO), which targets the gamma-glutamylcysteine synthetase (GCS, GSH synthesising enzyme) and degrading enzyme inhibitors such as Ethacrynic acid (EA) (inhibits glutathione S-transferase) increased and decreased resistance to erlotinib, respectively. EA also showed promising results *in vivo* as BALB/c nude mice, injected subcutaneously with PC9ER cells, showed increased sensitivity to erlotinib treatment when co-administered with EA. Furthermore, we demonstrated that mutant EGFR signalling can modulate the transcription of GSH enzymes likely via changes in the regulation of the transcription factor, Nuclear Factor (Erythroid-Derived 2)-Like 2 (NRF2). Targeting NRF2 using siRNAs decreased GSH abundance and increased erlotinib resistance. However, at present we do not understand how raised GSH levels might cause resistance to erlotinib in lung cancer cells expressing activated T790M-EGFR.

To discover other differences between erlotinib-resistant and sensitive cells, we employed reverse phase protein array (RPPA) and, following DAVID bio-informatics analysis, identified changes in both beta-oxidation and ataxia telangiectasia mutated kinase (ATM)/Ataxia Telangiectasia and Rad3-related kinase (ATR) cell cycle checkpoint proteins. Changes in these signalling molecules were confirmed by Western blotting. Yet, only targeting the ATR pathway affected the resistance to erlotinib in PC9ER cell lines. Indeed, inhibition of WEE1 with the compound MK1775 may hold promise as a treatment for patients with erlotinib-resistant NSCLC.

In short, this work has identified GSH degrading enzymes and WEE1 as novel targets for reversing erlotinib resistance in EGFR-driven NSCLC. However, further investigation is needed to understand the mechanisms involved.

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Abbreviations

1H-NMR	Proton Nuclear Magnetic Resonance
4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1
ACC	Acetyl-CoA Carboxylase
ACHE	Acetylcholinesterase
ADAM17	ADAM metallopeptidase domain 17
AHR	aryl hydrocarbon receptor
ALK	Anaplastic lymphoma kinase
AMP	Adenosine monophosphate
AMPK	Adenosine Monophosphate-activated Protein Kinase
ANOVA	Analysis Of Variance
AP-2	activator protein-2
APS	Ammonium Persulphate
ARE	Antioxidant Response Element
ARNT	AHR nuclear translocator
ARTN	Artemin
ATM	Ataxia Telangiectasia Mutated kinase
ATP	Adenosine Triphosphate
ATR	Ataxia Telangiectasia and Rad3-related kinase
BASCs	bronchioalveolar stem cells
Bcl-2	B-cell CLL/lymphoma 2
BSA	Bovine Serum Albumin
BSO	Buthionine Sulfoximine
CAT	Carnitine Acetyltransferases
CBL	Casitas B-Lineage Lymphoma Proto-Oncogene
CCD	Coiled-Coil Domain Containing 6
CCT	CTP:Phosphocholine Cytidylyltransferase
CDK1	Cyclin-dependent kinase 1
CDKs	Cyclin Dependent Kinases
CDP	Cytidine Diphosphate-Choline
CDP-E	CDP-Ethanolamine
ChAT	Choline Acetyltransferase
ChIP	Chromatin Immunoprecipitation
CHK	Choline Kinase
ChK1	Checkpoint Kinase 1
ChK2	Checkpoint Kinase 2
CHPT1	Choline Phosphotransferase 1
CIS	Cancer in situ
CK2	casein kinase II
CNC-bZIP	Cap 'N' Collar-Basic Leucine Zipper Proteins
CNNTB	Catenin-Beta
Co-IP	Co-Immunoprecipitation
CPT	Choline Phosphotransferase

CPT1	Carnitine Palmitoyltransferase I
CREB	cyclic AMP response element binding protein
CRM1	Chromosomal Maintenance 1
Cul3	Cullin-3
CV-ANOVA	Cross-validated analysis of variance
D3T	3H-1,2-dithiole-3-thione
DAG	Diacylglycerol
DAPK	Death-Associated Protein Kinase
DAPT	N-[N-(3,5-difluorophenacetyl)-L-alanyl]-(S)-phenylglycine t-butyl ester
DDR2	Discoidin domain receptor tyrosine kinase 2
DMEM	Modified Eagle's medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide
DPP3	Dipeptidyl peptidase 3
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
EA	Ethacrynic Acid
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediamine Tetra Acetic Acid
EGF	Epidermal Growth Factor
EGFRm	activated mutant EGFR
EK	Ethanolamine Kinase
EMBL	European Molecular Biology Laboratory
EMSA	electrophoretic mobility shift assay
EMT	Epithelial-Mesenchymal Transition
EpRE	Electrophile Responsive
EPT	Ethanolamine:1,2-diacylglycerol Ethanolamine-Phosphotransferase
ERK	Extracellular signal-Regulated Kinase
ET	CTP:phosphoethanolamine cytidyltransferase
FAD	Flavin Adenine Dinucleotide (Reduced)
FADH ₂	Flavin Adenine Dinucleotide (Oxidised)
FAK1	Focal Adhesion Kinase 1
FCS	Foetal Calf Serum
FDA	Food and Drug Administration
FDR	false discovery rates
FGF	Fibroblast Growth Factor
FGF2	Fibroblast Growth Factor 2
FGFR1	Fibroblast growth factor receptor 1
FRA1	Fos-related antigen 1
Gab-1	GRB2-associated-binding protein 1
GAP	RAS GTPase-activating protein
GC	gas chromatography
GCL	Glutathione Cysteine Ligase
GCLc	Glutamate-Cysteine Ligase Catalytic Subunit

GCLm	Glutamate-Cysteine Ligase Modifier Subunit
GDNF	glial cell line-derived neurotrophic factor
GDPD	Glycerophosphodiester Phosphodiesterase
GFAP	Glial Fibrillary Acidic Protein
GFLs	glial cell line-derived family ligands
GFR α	GDNF family receptor- α
GGT	Gamma-Glutamyl Transferase
GGT1	gamma-glutamyltransferase 1
GPX	Glutathione Peroxidase
Grb2	Growth factor receptor-bound protein 2
GSH	Glutathione
GSK3 β	glycogen synthase kinase 3 β
GSK3 β	Glycogen synthase kinase-3
GSR	Glutathione-Disulphide Reductase
GSS	Glutathione Synthetase
GSSG	Glutathione Disulfide
GST	glutathione-S-transferase
GST	Glutathione S-Transferase
GSTT2	glutathione-S-transferase theta 2
H ₂ O ₂	Hydrogen Peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF-1 α	hypoxia-inducible factor-1 α
HPLC	high-performance liquid chromatography
HSP27	Heat shock protein 27
HSP90	Heat Shock Protein 90
IP ₃	inositol 1,3,5-triphosphate
JAK	Janus kinase
JNK	c-Jun N-terminal kinases
KEAP1	Kelch-Like ECH-Associated Protein 1
KIF5B	kinesin family member 5B gene
LB	Luria-Bertani
LC	liquid chromatography
LC-ESI-MS/MS	liquid chromatography-electrospray ionization-tandem mass spectrometry
LKB1	Liver kinase B1
LTK	Leukocyte Receptor Tyrosine Kinase
Maf	Masculoaponeurotic Fibrosarcoma
MDK	PTN-related factor midkine
MDR 1	Multidrug Resistance Protein 1
MEK 1/2	mitogen-activated protein kinase kinase 1/2
MEKK1	ERK Kinase Kinase-1
MET	Mesenchymal–epidermal transition
MKK4/7	MAP Kinase Kinase-4/7
MO25	mouse protein 25

MRE	metal response
MS	mass spectrometry
mTOR	<u>mechanistic target of rapamycin</u>
MUC-1	Mucin-1
Myt1	Myelin Transcription Factor 1
NADP	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NCAM	neuronal cell adhesion molecules
Nck	non-catalytic region of tyrosine kinase adaptor protein 1
NCOA4	Nuclear Receptor Coactivator 4
NEB	Neuroepithelial bodies
NF1	Neurofibromin 1
NF-κB	nuclear factor κ-light-chain-enhancer of activated B cells
NKX2-1	NK2 homebox 1
NMR	Nuclear Magnetic Resonance
NO	Nitric Oxide
NRF1	Nuclear Respiratory Factor 1
NRF2	Nuclear Ractor (Erythroid-Derived 2)-Like 2
NRTN	neurturin
NSCLC	Non Small Cell Lung Cancer
OPLS-DA	Orthogonal Projections To Latent Structure Discriminant Analysis
p70S6K	Ribosomal Protein S6 Kinase
PAK1	P21 Protein (Cdc42/Rac)-Activated Kinase 1
PALB2	Partner And Localizer Of BRCA2
Par	Pareto Variance
PBS	Phosphate-Buffered Saline
PCYT1	Phosphate Cytidylyltransferase 1
PEMT	Phosphatidylethanolamine N-Methyltransferase
PI3K	Phosphoinositide 3-Kinase
PIK3CA	phosphatidylinositol-3-kinase, catalytic, alpha
PIP ₂	phosphatidylinositol 4,5-diphosphate
PIP ₃	PI-(3,4,5)-triphosphate
PKA	protein kinase A
PKC	protein kinase C
PL	Phospholipases
PLA	Phospholipase A
PLA2G6	Phospholipase A2, Group VI
PLAG215	Phospholipase A2, group XV
PLC	phospholipase C
PLD	Phospholipase D
PSPN	persephin
PTEN	Phosphatase and tensin homolog deleted on chromosome ten
PTN	Pleiotrophin
qPCR	Quantitative Real-Time PCR

Rb	retinoblastoma protein
RIPA	Radioimmunoprecipitation Assay
RNA	Ribonucleic Acid
ROR1	receptor tyrosine kinase-like orphan receptor 1
ROS	Reactive Oxygen Species
RPPA	Reverse Phase Protein Array
RSK	p90 ribosomal S6 kinase
S6K	Ribosomal Protein S6 Kinase
SAPKs	stress-activated protein kinases
SEM	Standard Error Of The Mean
Seq	Sequencing
SH2	Src homology 2
shRNA	short hairpin RNA
siRNA	Small Interfering RNA
SOC	Super Optimal broth Catabolite
SOS	Son of Sevenless
SPC	Surfactant Protein C
SQSTM1	Sequestosome 1
STK11	Serine/Threonine Kinase 11
STRAD	STE20-Related Kinase Adaptor
TAE	Tris-Acetate-EDTA
TAP	Tandem affinity purification
TBST	Tris-Buffered Saline/Tween
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TEMED	Tetramethylethylenediamine
TGF alpha	Transforming growth factor alpha
TITF-1	Thyroid transcription factor 1
TKI	Tyrosine Kinase Inhibitor
TRIM33	tripartite motif-containing 33
TSC2	tuberin
VEGFA	vascular endothelial growth factor A
XREL	xenobiotic response element-like elements
γ-GC	Gamma-Glutamylcysteine

Chapter 1.0 Introduction

1.1 Cancer

Cancer is a group of diseases characterised by abnormal cellular proliferation and the ability to invade and metastasise to local lymph nodes or distant parts of the body. It is often defined by hallmarks which were proposed by Hanahan and Weinberg. They initially identified 6 hallmarks that differentiate cancer from normal cells. These included the ability of cancer cells to evade apoptosis, insensitivity to growth suppressors, deregulated/sustained growth signals, sustained angiogenesis, limitless replicative potential and finally the ability to invade tissue and metastasize (Hanahan and Weinberg, 2000). New hallmarks have now been added to this list that includes dysregulation of cellular energetics, avoiding immune destruction, genomic instability and mutations as well as tumour promoting inflammation (Hanahan and Weinberg, 2011) (**Figure 1.1**).

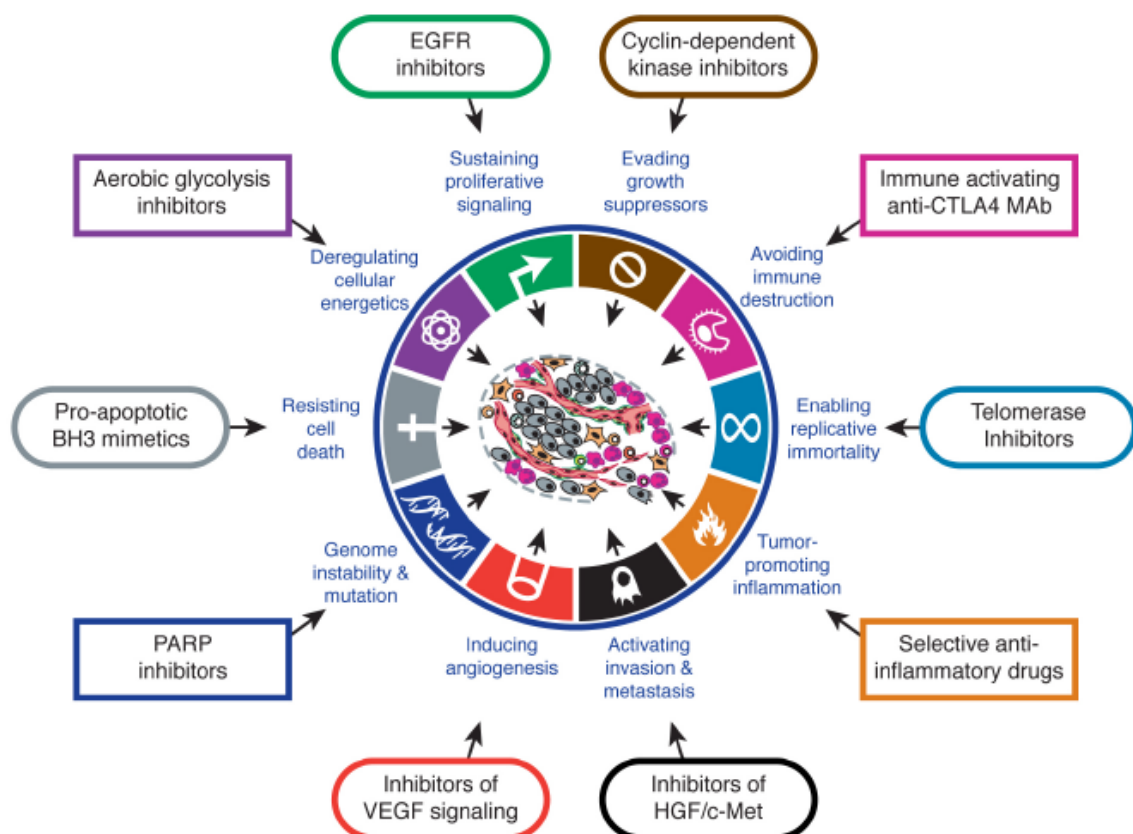


Figure 1.1: Therapeutic Targeting of the Hallmarks of Cancer. Hanahan and Weinberg have identified 10 hallmarks of cancer which may provide therapeutic target strategies for treatment. A number of drug compounds have been developed to interfere with each of the capabilities that are essential for tumour growth and progression (taken from (Hanahan and Weinberg, 2011)).

1.1.1 Stage and grading of cancer

In the clinics severity of cancer is further defined by their stage and grade. The staging system used is dependent on the type of cancer and determines how far the cancer has spread within the body. Grading is used by pathologists to describe how aggressive the tumour is in terms of its microscopic appearance. The majority of staging systems are based upon tumour size, localisation, cell type and its metastatic spread to nearby lymph nodes or distant parts of the body. The TNM staging system is probably the most widely used staging system (Greene; et al., 2013). T refers to the primary tumour; N to the number of lymph nodes colonised and M to the presence of distant metastasis (Rami-Porta et al., 2009, Lababede et al., 1999, Greene; et al., 2013). The tumour size (T) is described as one of the following TX (tumour cannot be measured), T0 (Tumour cannot be found), T1, T2, T3 and T4 the higher the number the larger the primary tumour (Rami-Porta et al., 2009, Lababede et al., 1999). The region of the lymph nodes (N) is described in a similar pattern NX (cannot be measured), N0 (no cancer in lymph nodes) and increase N number by the amount of lymph nodes where cancer is present (Rami-Porta et al., 2009, Lababede et al., 1999). Distant metastasis (M) is either described as MX (metastasis cannot be measured), M0 (cancer has not metastasized to other parts of the body) or M1, (cancer has spread to other parts of the body) (Rami-Porta et al., 2009, Lababede et al., 1999, Greene; et al., 2013). The TNM staging system gives great detail on the stage of cancer, however, combinations are normally then grouped into 5 stages:

Stage 0 – Abnormal cells are present although, they have not spread to nearby tissue (carcinoma in situ (CIS))

Stage I – The cancer is relatively small and has not spread

Stage II – The cancer has grown but has not spread

Stage III – The cancer is large and may have metastasized to surrounding tissues and/or lymph nodes.

Stage IV – The cancer has metastasized to distant parts of the body

Cancer grade unlike stage is not dependent on localisation but rather histology of the cancer tissue. It is dependent on if the cells are well differentiated, undifferentiated or poorly differentiated. As with staging, the grading system can also differ dependent on the cancer type, however, the most commonly used is outlined below (Greene; et al., 2013):

GX: Grade cannot be assessed (undetermined grade)

G1: Well differentiated (low grade)

G2: Moderately differentiated (intermediate grade)

G3: Poorly differentiated (high grade)

G4: Undifferentiated (high grade)

1.1.2 Cancer incidence and statistics

Cancer is the leading cause of death worldwide (WHO, 2014b). The WHO estimated in 2012 that there was 8.2 million cancer-related deaths and 14 million new cases (WHO, 2014b). This number is expected to rise, possibly in response to population growth and an aging population. The WHO proposed that by 2030 there will be in excess of 20 million new cases diagnosed and 13 million cancer-related deaths (WHO, 2014b).

Cancers are commonly sub-divided on the basis of the tissue/cells in which they originate from within the body. This results in 5 broad categories: (1) Carcinomas, which arise from epithelial tissues such as the skin or the lining of the gut or covering layer of internal organs. An example of an epithelial cancer is basal cell carcinoma (WHO, 2014b); (2) Sarcomas, which originate from supporting tissues such as bone, cartilage, fat, muscle and connective tissue e.g. osteosarcoma (WHO, 2014b); (3) Leukaemia, which originate from blood forming tissue e.g. acute lymphocytic leukaemia (WHO, 2014b); (4) Lymphomas or myelomas, which originate from cells involved in the immune system e.g. Hodgkin lymphoma (WHO, 2014b); (5) Central nervous system (CNS) cancers, which begin in the brain or spinal cord e.g. Brain stem glioma (WHO, 2014b). There are over 200 different types of cancer, the most common of which are shown in **Figure 1.2**. Lung cancer is an example of an epithelial-derived cancer and is the commonest cancer killer. When separated into sexes it is more prevalent in men than women (WHO, 2014b).

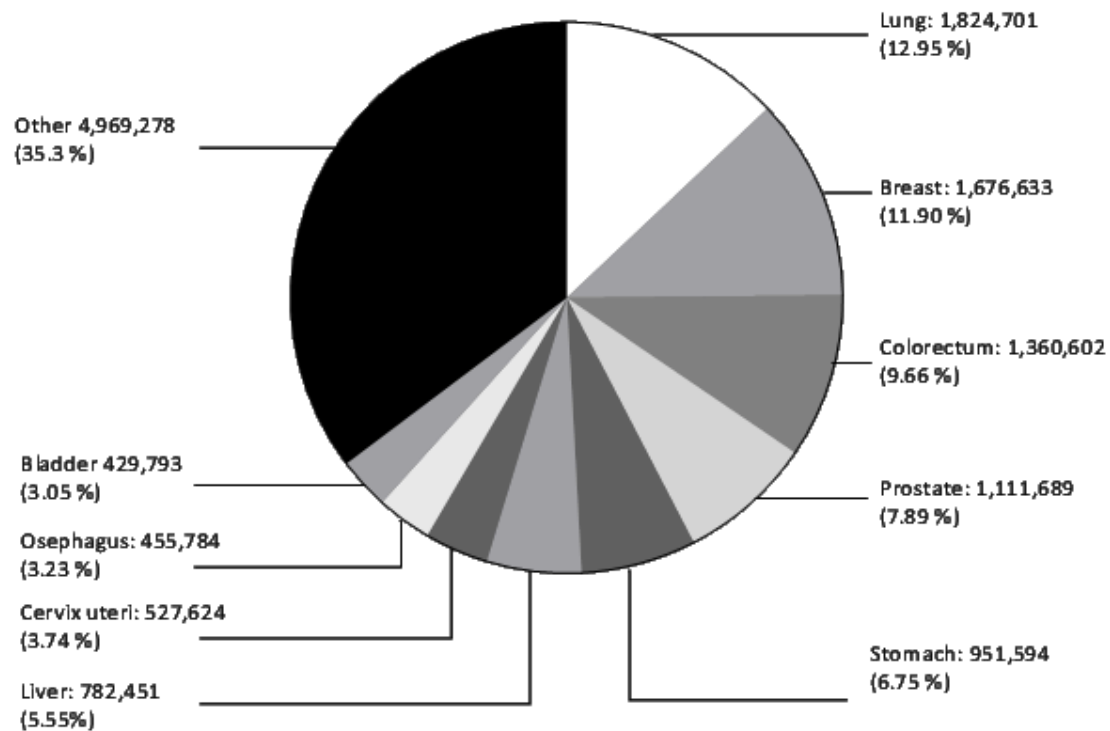


Figure 1.2: Estimated number of cancer cases. The incidence of cancer types worldwide in both man and women in 2012. Numerical Data was obtained from the world health organisation (WHO, 2014b).

1.1.3 Economic impact

While discussing cancer statistics it is important to discuss the economic burden associated with cancer. The annual economic cost attributed to cancer has been calculated to be 1.16 Trillion USD per year (WHO, 2014b). This figure is accounting for prevention and treatment, as well as the annual economic value of disability-adjusted life years (DALYs). However, this fails to account for the long-term costs to families and caregivers, in addition to the value of human suffering, which are likely to be significantly higher than the value predicted by DALYs. Alternative statistics such as a value of statistical life (VSL) approach, which tries to account for these additional costs, estimates the total cost to be nearer to 2.5 trillion USD (WHO, 2014b). These estimates highlight the need for investments in cancer control and prevention due to the economic potential return that could be achieved through access to prevention, early detection and better targeted treatments.

1.2 Lung Cancer

Lung cancer is the most prominent cause of cancer related deaths worldwide (WHO, 2014a). In the UK, there were around 35,900 lung cancer related deaths in 2014, which accounted for 22% of all cancer deaths in the UK (Cancer Research UK, 2015). Lung cancer is subcategorised into

two main sub-types small cell lung cancers (SCLC) and non-small cell cancer (NSCLC). SCLCs are fast growing, metastasize quickly and are rare in non-smokers (Jackman and Johnson, 2005). NSCLC are more common in smokers and they account for 85-90 % of lung cancer cases (Molina et al., 2008, Jemal et al., 2011). NSCLCs are sub-categorised into Adenocarcinoma, Squamous cell carcinoma and large cell carcinoma. The survival of lung cancer patients varies depending on the type, stage at diagnosis and treatment available. Survival of lung cancer patients is poor, in England and Wales only 5 % of people who are diagnosed with lung cancer survive their disease for 10 years or more (Cancer Research UK, 2015). However, survival is increased in patients who get diagnosed under the age of 40 (Cancer Research UK, 2015). The poor survival rate associated with lung cancer is commonly attributed to the early dissemination of the disease as well as the development of resistance to drug treatments (Woodhouse et al., 1997). Indeed, at the time of diagnosis ~70% of NSCLC patients have locally advanced or metastatic disease (Yang et al., 2005a).

1.2.1 Aetiology

Major determinants in lung cancer incidence include smoking and to a lesser extent radon and asbestos exposure (commonly associated with mesothelioma). It is estimated that tobacco smoking accounts for 86 % of lung cancers this is by direct (active smoking, 83 %) and indirect exposure (passive-smokers, 3 %) (Parkin, 2011). The prevalence of smoking has started to decline in recent years, which has led to lung cancer becoming more frequent in former smokers (Molina et al., 2008, Yang et al., 2005b) and also in life-long non-smokers.

1.2.2 Origin of lung cancer cell lineages

The origin of the different lung cancers varies between subtypes. Indeed, adenocarcinomas are typically localised to the lower airway, the bronchioles or alveoli while squamous cell carcinomas are within the upper airways and SCLC is in the mid-level bronchi (Sutherland and Berns, 2010, Hanna and Onaitis, 2013). The localisation of specific subtypes to certain areas of the lung led to the reasoning that they arise from cell types specific to those regions. In order to understand this, we must first discuss the structure and cell types which are localised within each region of the pulmonary system. The nose and throat are the location of the pulmonary airways which lead to the pharynx and trachea. The trachea is comprised of basal, goblet (mucosal), clara and ciliated cells (Breeze and Turk, 1984). As the airways continue the epithelium-variant clara cells and pulmonary neuroendocrine cells cluster to form neuroepithelial bodies (NEB) (Breeze and Turk, 1984). The airways then divide into bronchi

which are further subdivided into more bronchi and bronchioles, which are predominantly covered in clara and ciliated cells (Breeze and Turk, 1984). The Bronchioles end with alveolar sacs where gas exchange occurs within the alveoli clusters (Breeze and Turk, 1984). In order to facilitate this function alveoli contain specialised alveolar cells that form extremely thin (~0.5 micrometres) walls (Breeze and Turk, 1984). Epithelial alveolar cells can be subdivided into alveolar type-I cells (type I pneumocytes) and the more abundant alveolar type-II cells (type II pneumocytes) (Breeze and Turk, 1984). The alveoli are surrounded by a network of capillaries which aid gas exchange (Breeze and Turk, 1984).

The basal layer of the bronchial epithelium, give rise to SCLC. These cells contain neuronal cell adhesion molecules (NCAM) and neurosecretory granules which led to the suggestion that they may arise from neuroendocrine cells that make up NEBs (Neal and Hoskin, 2009, Onganer et al., 2005). In contrast, adenocarcinoma cells are derived from bronchioalveolar stem cells (BASCs), characterised by the presence of the Clara cell marker CC10 and the alveolar type-II marker, Surfactant Protein C (SPC) (Sutherland and Berns, 2010). Squamous cell carcinoma are preferentially localised to the upper trachea in which tracheal basal cells are localised (Sutherland and Berns, 2010). Keratin 5 and 14, markers of tracheal basal cells, are expressed in squamous cell carcinoma, implying that these may be the cells from which originates this cancer type (Sutherland and Berns, 2010). **Figure 1.3** shows a simplified diagram summarising the origins of these 3 lung cancer types.

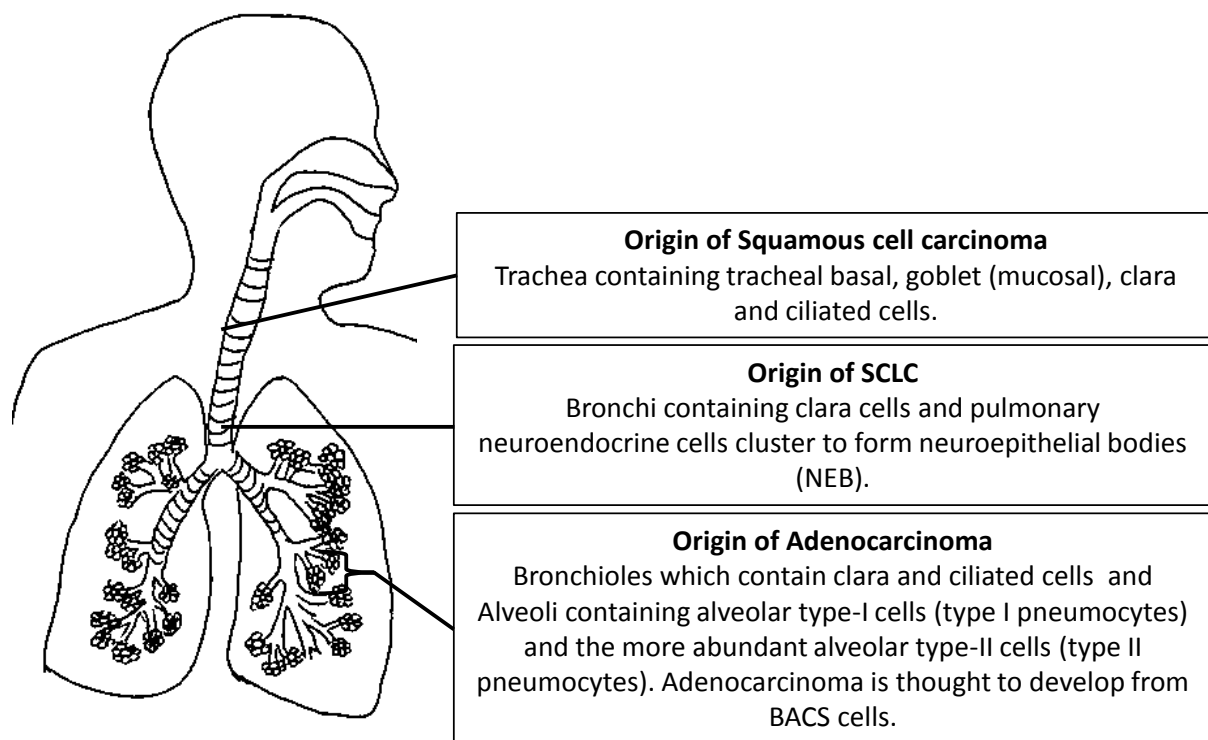


Figure 1.3 Origin of lung cancer. Squamous cell carcinoma and SCLC originate in the upper airways. Squamous cell carcinoma believed to develop from tracheal basal cells, whereas, SCLC are thought to be from neuroendocrine cells. Adenocarcinomas originate in the lower airways and are thought to develop from bronchioalveolar stem cells (BASCs).

1.2.3 Presentation, diagnosis and standard treatments of lung cancer

Lung cancer can present in many ways and often the symptoms are not specific but common to other chest conditions. This generally results in a delayed diagnosis. A simple way to consider symptoms for any cancer including lung cancer is to divide them into symptoms caused by the primary cancer, by the metastases and the generalised features of malignancy. The primary tumour growing in the lung typically causes cough and, because of obstruction of airways, may trigger chest infections. As the tumour grows it may press on nerves causing local chest pain or a hoarse voice and if it erodes into blood vessels the patient may start to cough up blood and become short of breath. Central chest tumours may press on the oesophagus and cause difficulty with swallowing. Symptoms from metastases depend on their location. For example metastases to the bones causes bone pain and those to the brain might result in headaches and epileptic convulsions. Generalised features of cancer include loss of appetite, energy, weight and development of fevers (Hopwood and Stephens, 1995).

Lung cancer diagnosis requires for nearly all patients a biopsy to enable histopathological examination. There are broadly two types of lung cancer defined by the histological appearance, the commonest being non-small cell (NSCLC) and the remainder being small cell lung cancer (SCLC). NSCLC can be further subdivided into adenocarcinomas, squamous carcinomas and large cell carcinomas but from a therapy perspective they are either treated as squamous or non-squamous (adeno) carcinomas.

The treatment of NSCLC generally can involve surgery, chemotherapy and/or radiotherapy. Surgery has been shown to be the most effective treatment for NSCLC with the greatest chance of achieving long-term cure (Myrdal et al., 2001). However, surgery is only an option for patients with earlier stage disease. As the vast majority of patients are diagnosed at later stages they are reliant on non-surgical therapies. The therapy given is also dependent on the type of lung cancer. SCLC for example, is nearly always metastatic from the outset and so rarely amenable to surgical cure. Therefore, cisplatin or carboplatin combined with etoposide with or without additional radiotherapy is the current standard of care for SCLC patients (Stevenson, 2016b). NSCLC patients presenting with advanced/non-operable disease also require radiotherapy and/or combination chemotherapy. Patients with locally advanced inoperable disease are often treated initially with radical radiotherapy with or without additional chemotherapy, whilst those with metastatic disease receive combination agents chemotherapy with or without additional radiotherapy. The type of chemotherapy varies, with adenocarcinomas receiving pemetrexed in

combination with cisplatin or carboplatin, whilst patients with squamous carcinomas receive gemcitabine with platinum agent. More recently, several new molecular changes have been identified in NSCLC that drive the malignant phenotype and for which new targeted therapies exist. These will be discussed in sections 1.2.4 below (Blais and Hirsh, 2014, Stevenson, 2016a).

1.2.4 Genetic aberration in lung cancer

A number of genetic changes have been found within lung cancer cells. The risk of Lung cancer and susceptibility is increased by the presence of germ-line mutations in genes such as p53 (Hwang et al., 2003), retinoblastoma (Sanders et al., 1989) and EGFR (Bell et al., 2005). The prevalence of the most common gene mutations in lung cancer is outlined in **Table 1.1** and a number of those will be discussed within this section.

Table 1.1 Common genetic and epigenetic transformations associated with different subtypes of lung cancer. Percentage of cases for each mutation or amplification event is shown. *Mutations have been identified within patient samples but incidence is unknown. Adapted from (Herbst et al., 2008) including data from (Hammerman PS, 2012, Jin et al., 2010, Yokomizo et al., 1998, Bergethon et al., 2012, Shan et al., 2015, Miao et al., 2014, Kohno et al., 2012, Dabir et al., 2014, Takeuchi et al., 2012b, Abdelraouf et al., 2015, Ding et al., 2008).

Histological Subtype	Non Small-Cell Lung Cancer		Small-Cell Lung Cancer
	Squamous-Cell Carcinoma	Adenocarcinoma	
Precursor Genetic Change	p53 mutation	KRAS mutation, EGFR mutation	Overexpression of c-Met
Cancer			
KRAS mutation	Very rare	10-30%	Very rare
BRAF mutation	3%	2%	Very rare
EGFR			
Kinase domain mutation	Very rare	10-40%	Very rare
Amplification	30%	15%	Very rare
Variant III mutation	5%	Very rare	Very rare
FGFR1 Amplification	20%	Very rare	~50%
HER2			
Kinase domain mutation	Very rare	4%	Very rare
Amplification	2%	6%	Unknown
ALK Fusion	Very rare	7%	Unknown
MET			
Mutation	12%	14%	13%
Amplification	2-21%	2-20%	Unknown
TITF-1 amplification	15%	15%	Very rare
p53 mutation	60-70%	50-70%	75%
LKB1 mutation	19%	34%	Very rare
PIK3CA			
Mutation	2%	2%	Very rare
Amplification	33%	6%	4%
PTEN mutation	10.2%	1.7%	10%
ROS1 rearrangement	4%-2%	1.2-2.6	Unknown
NRAS mutation/amplification	1 %	Rare	Very rare
DDR2 mutation	4%	Rare (0.5%)	4.6 %
RET rearrangement	Very rare	1-2%	Unknown*

1.2.4.1 P53 mutations

The tumour suppressor protein p53 is a hub for a number of signalling pathways including the control of cell cycle and apoptosis (Vousden and Prives, 2009). It is nearly always mutated in SCLC and frequently so in NSCLC primary tumours and cell lines (Brauch et al., 1987, Chiba et al., 1990, Nigro et al., 1989). Due to the large number of mutations identified, a database was established to show known mutations in various tumour types and cell lineages (Hollstein et al., 1994, Hainaut et al., 1997). Mutations in p53 resulting in its inactivation or loss of function are the most common of the genetic mutations (Campling and el-Deiry, 2003). Such mutations have been shown to have an increased incidence in smokers, particularly in the case of SCLC and squamous cell carcinoma (Gibbons et al., 2014). A number of studies have implied p53 mutant tumours have a worse prognosis and may be less susceptible to chemotherapy and radiation treatments (Campling and el-Deiry, 2003).

1.2.4.2 RAS subfamily amplification and mutations

RAS subfamily of the small GTPase superfamily, consists of HRas, KRas, and NRas, and are involved in a multitude of signalling pathways relevant to health and disease. Moreover, although the Ras subfamily share conserved sequences, their protein products have distinct roles in development and tumorigenesis (Esteban et al., 2001, Haigis et al., 2008, Quinlan et al., 2008). The principles of activation of Ras proteins are similar. They act as molecular switches that are turned-on upon GTP binding while they are turned off by the hydrolysis of this nucleoside triphosphate into GDP. This process of GDP for GTP exchange is tightly regulated by exchange factors such as Son of Sevenless (SOS) that turn on Ras signalling and GTPase activating proteins (RasGAPs) that hydrolyse GTP to turn off Ras signalling. In lung cancer, mutations in both KRas and to a lesser frequency NRas have been identified (Ding et al., 2008). These leave Ras in a constitutively active state. Despite the close relationship between NRas and KRas the distribution and the type of mutations identified are different. Indeed, the most frequent mutation in NRAS occurs through changes to codon Q61 (80%) causing a glutamine to leucine substitution at position 61 (NRas Q61L) in about 50% of cases (Ohashi et al., 2013). In contrast, KRAS mutations are predominantly G:C>T:A transversions (Ohashi et al., 2013). NRAS mutated cell lines showed sensitisation to MEK inhibition via selumetinib and trametinib treatment leading to inactivation of ERK (Ohashi et al., 2013). The importance of NRAS to lung cancer cell growth was demonstrated by siRNA knockdown resulting in growth inhibition (Ohashi et al., 2013). KRAS, unlike NRAS, is one of the most commonly mutated proteins in lung adenocarcinoma (Riely et al., 2009). The majority (97%) of KRAS mutations are localised to exon

2 and 3 (Brose et al., 2002). KRas mutations are common within lung adenocarcinoma and rare in squamous cell carcinoma (Brose et al., 2002). These mutations commonly result in the constitutive activation of this small GTPase (Riely et al., 2009). While there has been no identification of over-lapping function of mutated KRAS with other known oncogenic mutations, KRAS mutations have been shown to be negative predictive markers for the efficiency of the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) such as erlotinib and gefitinib (Riely and Ladanyi, 2008, Riely et al., 2009).

1.2.4.3 MET amplification and mutation

The proto-oncogene MET is otherwise known as the hepatocyte growth factor receptor. Binding of its ligand, hepatocyte growth factor, results in activation of this receptor tyrosine kinase, which then phosphorylates a series of tyrosine residues within the receptor. These serve as docking sites for a series of second messenger molecules which themselves become targets for tyrosine phosphorylation by the activated receptor (Organ and Tsao, 2011). This results in the activation of a number of signalling pathways including the Ras/ERK, Phosphatidylinositol-4,5-bisphosphate 3 kinase (PI3K)/AKT, or Phospholipase C gamma (PLC gamma)/Protein kinase C (PKC) pathways (Organ and Tsao, 2011). MET amplification has been shown to have increased incidence in patients with, and be mechanistically responsible for, acquired resistance to EGFR TKI treatment (da Cunha Santos et al., 2011). Indeed, MET amplifications have been identified as a mechanism of acquired resistance to treatment with EGFR-TKI (Engelman et al., 2007b). In such settings, the dual inhibition of EGFR and MET led to apoptosis in a preclinical model (Xu et al., 2012). Indeed, MET overcomes inhibition of EGFR by TKIs through heterodimerization with ERBB3, resulting in sustained activation of the PI3K/AKT signalling pathway (Engelman et al., 2007b). In addition to amplifications, a number of mutations in MET have been noted including, point mutations within the kinase domain as well as the Y1003 mutation in the juxta-membrane domain have shown to result in constitutive activation and expression of MET, respectively (Giordano et al., 2000, Kong-Beltran et al., 2006, Peschard et al., 2001).

1.2.4.4 BRAF mutations

The proto-oncogene BRAF, is a serine/threonine protein kinase, which has a role in proliferation and cell survival (Luk et al., 2015). Mutations in BRAF have been identified in a number of malignancies including lung carcinoma (~3%) (Davies et al., 2002), hairy-cell leukaemia (100%) (Tiacchi et al., 2011), melanoma (~40%) (Platz et al., 2008), papillary thyroid carcinoma (30-50%) (Davies et al., 2002) and colorectal carcinoma (~10%) (Davies et al., 2002). In a study by Paik et

al of 697 lung adenocarcinoma patients, 18 patients with BRAF mutations were identified (Paik et al., 2011). Furthermore, this study showed these mutations to occur within the kinase domain, including the V600E (50%), G469A (39%), and D594G (11%) mutations (Paik et al., 2011). The V600E mutation results in the constitutive activation of BRAF, which in turn results in the phosphorylation of MEK (Wan et al., 2004). Activated MEK phosphorylates/activates ERK resulting in activation of a number of transcription factors including c-fos and Elk-1 and subsequent driving of cell cycle progression and cell survival (Torii et al., 2006). In addition to V600E, the G469A and D594G mutations have both been implicated in increased kinase activity (Davies et al., 2002). BRAF inhibition has shown to increase progression free survival of patients harbouring the BRAF V600E mutation in advanced stage melanoma patients (Hauschild et al., 2012). Moreover, the targeting of BRAF has shown promise in a number of cancers including gastrointestinal stromal tumours, papillary thyroid cancers, ovarian cancer, colorectal cancer as well NSCLC (Falchook et al., 2012).

1.2.4.5 Fibroblast growth factor receptor 1 (FGFR1) amplifications

FGFRs are a family of receptor tyrosine kinases (RTKs) consisting of four members denoted 1-4. They differ from other RTKs in that they have a split rather than single tyrosine kinase domain. The FGFR signalling pathway has been shown to be involved in a number of processes including proliferation, anti-apoptosis, stemness, and angiogenesis (Katoh and Nakagama, 2014). Oncogenic development has been shown through dysregulation of FGFR signalling via gene amplification, chromosomal translocation, and point mutation (Ahmad et al., 2012). In lung cancer, amplification of FGFR1 is predominantly found in small cell and squamous cell carcinoma in former and current smokers (Seo et al., 2014, Schultheis et al., 2014). In addition, activating mutations of the receptor is also rarely found. Furthermore, early studies indicated that inhibition of FGFR using specific FGFR TKIs significantly reduced tumour size in a few squamous carcinoma lung cancer patients (Turner and Seckl, 2010).

1.2.4.6 HER2 amplification/mutations

HER2 also known as Neu or ErbB2 is a member of the transmembrane receptor tyrosine kinase EGFR family of receptors which include EGFR (ErbB1), HER3 (ErbB3), and HER4 (ErbB4). Binding of ligand to any of the other EGFR family members results in the formation of homo or heterodimers, the preferred binding partner of which is HER2 (Yarden and Slivkowski, 2001, Graus-Porta et al., 1997). While HER2 is not able to bind directly to ligand, it can potently amplify signalling by increasing the binding affinity of heterodimers to ligands as well as

promoting receptor recycling/stability (Graus-Porta et al., 1997, Pinkas-Kramarski et al., 1996, Wang et al., 1998, Worthylake et al., 1999). Following hetero or homodimerization, C-terminal tyrosine residues are autophosphorylated and cytoplasmic signal transducers recruited. Cytoplasmic signal transducers include PLC- γ 1, the Ras-Raf-MEK-MAPKs and PI3K-Akt-ribosomal S6 kinase 1 (S6K1) pathways, Src, the stress-activated protein kinases (SAPKs), the PAK-JNKK-JNK pathway and the signal transducers as well as activators of transcription (STATs) (Yarden and Sliwkowski, 2001). The variety of downstream mediators explains the ability of activated HER2 to regulate a number of processes including proliferation, differentiation, motility, adhesion, protection from apoptosis, and transformation (Wang et al., 2006b). HER2 has been found to be overexpressed in NSCLC patients and increased copy number for this gene was associated poor prognosis (Hirsch et al., 2002). However, EGFR TKIs have shown a positive response in patients with this molecular profile (Cappuzzo et al., 2005, Hirsch et al., 2002). In addition to amplifications, a number of mutations have been identified within HER2 in NSCLC patients (Shigematsu et al., 2005). The majority of these mutations fall within the kinase domain (Shigematsu et al., 2005). The most commonly identified mutation is G776 (YVMA), an in-frame insertion in exon 20 (Wang et al., 2006b). This mutation results in an increase in HER2 kinase activity as well as enhanced downstream signalling leading to increased cell survival, invasiveness and tumorigenicity (Wang et al., 2006b).

1.2.4.7 Anaplastic lymphoma kinase (ALK) fusion

ALK also known as CD246 (cluster of differentiation 246) belongs to the insulin receptor superfamily. It is comprised of an extracellular domain, single-pass transmembrane region and an intracellular kinase domain (Iwahara et al., 1997). ALK is activated by ligand binding; ligands which bind to ALK include the secreted growth factor pleiotrophin (PTN) and the PTN-related factor midkine (MDK) (Wellstein, 2012). ALK activation has been shown to activate a number of pathways including the MAPK and PI3K pathways, which induce proliferation (Palmer et al., 2009). It has also been shown to activate NF-kappa-B possibly downstream of IRS1 and AKT (Kuo et al., 2007). Activating mutations and translocations in ALK have been identified in a number of cancers including neuroblastoma (George et al., 2008), anaplastic large-cell lymphoma (Kutok and Aster, 2002) and NSCLC (Soda et al., 2007). An ALK fusion protein, EML4-ALK is found in about 4-6% of lung adenocarcinomas. This constitutes a cytoplasmic chimeric protein with constitutive kinase activity (Li et al., 2013). Although the EML4-ALK is fairly uncommon in NSCLC, it is more prevalent in never-smoker adenocarcinoma patients (Li et al., 2013). Additional ALK rearrangements have been identified including fusion with KIF5B and TFG (Takeuchi et al., 2009,

Rikova et al., 2007). Patients with ALK gene rearrangement in their tumours respond very well to therapy with an orally bioavailable targeted agent called crizotinib, which inhibits the ALK kinase (Kwak et al., 2010, Shaw et al., 2013). This small-molecule is now used as first line therapy in such patients (Chuang and Neal, 2015).

1.2.4.8 Thyroid transcription factor 1 (TTF-1) amplification

TTF-1 also known as NK2 homeobox 1 (*NKX2-1*), is a homeodomain-containing transactivating factor with a role in lung development as well as in transactivation of surfactant proteins (SPs) predominantly expressed in Clara cells (Minoo et al., 1999, Ghaffari et al., 1997, Tang et al., 2011). Furthermore, it has been implicated in the etiology of NSCLC (Kwei et al., 2008, Tanaka et al., 2007). The dependency of lung adenocarcinoma on TTF-1 was shown via knockdown by RNA interference resulting in inhibition of growth and apoptosis (Tanaka et al., 2007). Conversely, expression of TTF-1 was associated with good prognosis in stage-I adenocarcinoma patients (Anagnostou et al., 2009). While amplification of TTF-1 was also identified within lung squamous cell carcinomas (Perner et al., 2009), the pertinence of this in the clinical outcome of NSCLC patients is unclear. Indeed, Weir and colleagues showed no difference in survival of adenocarcinoma patients with and without amplification of TTF-1 (Weir et al., 2007). This was disputed by Tang *et al* who showed increased TTF-1 copy number gain was associated with improved prognosis and overall survival (Tang et al., 2011). Furthermore, TTF-1 copy gain showed a strong association with KRAS mutant patients and to a lesser extent in EGFR mutated patients (Tang et al., 2011). Indeed, TTF-1 protein expression was prevalent in lung adenocarcinomas only and furthermore was significantly associated with female, never-smokers and the presence of EGFR mutations (Tang et al., 2011).

1.2.4.9 Liver kinase B1 (LKB1) mutations

LKB1 also known as Serine/threonine kinase 11 (STK11) is the upstream kinase of adenosine monophosphate-activated protein kinase (AMPK) and AMPK-related kinases (Lizcano et al., 2004). As such it has an important role in the regulation of beta oxidation and fatty acid synthesis as well as maintaining cell polarity, thereby inhibiting inappropriate expansion of tumour cells (Jansen et al., 2009). Furthermore, through interaction with P53, LKB1 has been shown to induce apoptosis (Karuman et al., 2001). LKB1, acts within a heterotrimeric complex with the pseudokinase STE20-related adaptor (STRAD alpha/beta) and the scaffolding protein mouse protein 25 (MO25) (Zeqiraj et al., 2009). It is important to note that germ-line mutations in LKB1 result in the development of Peutz–Jeghers syndrome - benign hamartomatous polyps

as well as mucocutaneous pigmentation (Mehenni et al., 1998). The Peutz–Jeghers syndrome confers an increased risk of cancer, most significantly in gastrointestinal tumours, but also lung cancer (Giardiello et al., 1987, van Lier et al., 2010). LKB1 was identified to be bi-allelically and somatically mutated and inactivated in lung adenocarcinoma (Sanchez-Cespedes et al., 2002). LKB1 mutations are predominantly nonsense or frameshift mutations or large intragenic deletions which are thought to result in truncated proteins (Sanchez-Cespedes, 2011). In addition, mutations have also been identified within the kinase domain e.g. A205T in head and neck squamous cell carcinoma, these result in inability of LKB1 to phosphorylate substrates such as AMPK (Mehenni et al., 1998, Ylikorkala et al., 1999, Qiu et al., 2006).

1.2.4.10 Phosphatidylinositol-3-kinase, catalytic, alpha (PIK3CA) mutations and amplifications

Mutations in PIK3CA have thus far been found in the helical or catalytic domains which result in constitutive activation of the PI3K/AKT pathway (Samuels et al., 2004). The majority of mutations occur within exon 9 encoding the helical domain, and exon 20 which encodes the kinase domain of PIK3CA (Samuels et al., 2004). Three mutational hotspots within PIK3CA have been identified: E542K, E545K, and H1047R (Kang et al., 2005). Mutations within these hotspots have shown an increased kinase activity leading to activation of the AKT-signalling pathway (Samuels et al., 2004, Kang et al., 2005). Mutations within PIK3CA have shown to coexist with EGFR/KRAS mutations (Wang et al., 2014). Patients who carry mutations within PIK3CA alone showed significantly worse survival than those with both PIK3CA and EGFR or KRAS mutations (Wang et al., 2014). In addition to mutations, gain in copy number of PIK3CA has also been reported in squamous cell carcinoma, adenocarcinoma and SCLC cases (Wang et al., 2014).

1.2.4.11 Phosphatase and tensin homolog deleted on chromosome ten (PTEN) mutations

The tumour suppressor protein PTEN is a lipid and protein phosphatase (Li et al., 1997). PTEN dephosphorylates PI-(3,4,5)-triphosphate (PIP₃) and thereby negatively regulates the PI3K/AKT/mTOR (mechanistic target of rapamycin) pathway leading to G1 cell cycle arrest (Georgescu, 2010). It has also been implicated in the inhibition of migration via the regulation of FAK (Tamura et al., 1999). In a study by Jin *et al* of 176 NSCLC tumours, PTEN tumours were only noted in patients who had previously smoked and was more frequent in squamous cell carcinoma (10.2%) than adenocarcinoma (1.7%) cases (Jin et al., 2010). No mutational hotspots have thus far been identified.

1.2.4.12 ROS1 rearrangement

The receptor tyrosine kinase ROS1, is part of the insulin receptor family, closely related to ALK and Leukocyte Receptor Tyrosine Kinase (LTK) human RTKs (Robinson et al., 2000). It is a single-pass transmembrane protein consisting of an intracellular C-terminal tyrosine kinase domain and a large extracellular N-terminal domain (Davies and Doebele, 2013). The function of ROS-1 has yet to be fully explained and the ligand which activate ROS-1 are as yet unknown. Due to its structure, it is thought to be involved in cellular attachment (Acquaviva et al., 2009). Interestingly, chromosomal translocation of ROS1 has been identified in lung cancer (Rikova et al., 2007). Indeed, SLC34A2-ROS1 was identified within the HCC78 NSCLC cell line and additionally the CD74-ROS1 fusion was identified in a NSCLC patient sample (Rikova et al., 2007). ROS1 fusion proteins result in constitutive kinase activity and sensitivity to TKIs in vitro (Kohno et al., 2015). ROS1 rearrangement has not been shown to have over-lapping function with other oncogenic mutations such as those in EGFR (Kohno et al., 2015). Indeed, preclinical studies showed that cells containing ROS1 fusion do not respond to EGFR TKIs (Rikova et al., 2007). However, treatment with the ALK and ROS1 inhibitor, crizotinib, has shown a good clinical response in tumours which harbour ROS1 fusions and has been approved by the Food and Drug Administration (FDA) for treatment in this patient group (Mazieres et al., 2015).

1.2.4.13 Discoidin domain receptor tyrosine kinase 2 (DDR2) mutations

DDR2 is a receptor tyrosine kinase, which is activated by ligand binding to extracellular collagen. In tumour cells the function of DDRs is believed to be to interrupt cell-matrix communication and facilitate pro-migratory and pro-invasive cascades (Valiathan et al., 2012). In a study by Hammerman *et al* on samples from 290 squamous cell carcinoma patients, 11 showed mutations in various regions of DDR2, including the kinase domain (Hammerman et al., 2011). Furthermore, DDR2 mutated squamous cell carcinoma cell lines had increased sensitivity to the tyrosine kinase inhibitor dasatinib and sh-RNAs targeting DDR2 resulted in decreased proliferation possibly as a result of cell death (Hammerman et al., 2011). Dasatinib treatment has shown to have positive clinical effects in patient tumours harbouring mutations in DDR2, resulting in partial remission in the clinic (Hammerman et al., 2011). No mutations within DDR2 have been identified in SCLC, however mutations have been noted in adenocarcinoma patients although these are rare (Ding et al., 2008, Yashima et al., 2014).

1.2.4.14 RET fusion proteins

RET is a single-pass transmembrane RTK, composed of a large extracellular domain, a transmembrane region followed by an intracellular kinase domain (Ibanez, 2013). The receptor is activated through binding to glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) such as neurturin (NRTN), artemin (ARTN) and persephin (PSPN). This is facilitated by the GDNF family receptor- α (GFR α) family of co-receptor, which are attached to the cell surface by glycosylphosphatidylinositol linkage (Arighi et al., 2005). Following ligand binding RET forms homodimers, which results in autophosphorylation of intracellular tyrosine residues and recruitment of adapter and signalling proteins causing activation of a number of downstream pathways (Arighi et al., 2005, Ibanez, 2013). RET activates many signalling molecules a number of which bind to its docking sites at phosphotyrosine residues 1062 (pY1062) and pY1096 (Mulligan, 2014). This leads to the activation of the RAS/MAPK and PI3K/AKT pathways as well as the recruitment of the ubiquitin ligase, Casitas B-Lineage Lymphoma Proto-Oncogene (CBL) that is involved in the down-regulation of RET (Scott et al., 2005, Hayashi et al., 2000, Besset et al., 2000). RET chromosomal rearrangement has been noted in NSCLC, more frequently in adenocarcinoma. Chromosomal rearrangement, produces a fusion gene made up of juxtaposition of the C-terminal region of the RET protein with the N-terminal portion of another protein which leads to the constitutive activation of RET (Takeuchi et al., 2012a). A number of RET fusion genes partners have been identified in NSCLC including kinesin family member 5B gene (KIF5B) (Ju et al., 2012), Coiled-Coil Domain Containing 6 (CCDC6) (Chao et al., 2012, Wang et al., 2012), Nuclear Receptor Coactivator 4 (NCOA4) (Wang et al., 2012), ALK (Lipson et al., 2012) and tripartite motif-containing 33 (TRIM33) (Falchook et al., 2016). Interestingly, treatment of NSCLC tumours containing RET fusion proteins have shown positive response to the TKIs cabozantinib (inhibitor of VEGFR and to a lesser extent MET, Kit, Flt-1/3/4, Tie2, AXL, c-Met and RET) and vandetanib (inhibitor of VEGFR2, EGFR and RET) (Drilon et al., 2013, Gautschi et al., 2013).

1.3 EGFR

As EGFR is so important for the work of this thesis, we shall introduce background information on this receptor before describing its role in lung cancer.

1.3.2 EGFR protein

EGFR is a 486 amino-acid tyrosine kinase receptor. It is made of 4 extracellular domains denoted as ligand binding 1 (L1), cysteine rich 1 (CR1), (L2) and (CR2) (Bajaj et al., 1987), and three

intracellular domains separated by a single transmembrane sequence (Ferguson, 2008) (**Figure 1.4**).

The extracellular domain L1 and L2, are predominantly the domains that facilitate ligand binding (Garrett et al., 2002). Whereas, the CR2 domain has shown to be involved in the localisation of EGFR to caveolae/lipid rafts prior to ligand binding (Yamabhai and Anderson, 2002). A number of ligands have been suggested to bind to the EGFR receptor including EGF, transforming growth factor alpha (TGF alpha), amphiregulin or heparin (Khazaie et al., 1993). Binding of ligand results in the formation of homodimers or heterodimers with other members of the EGFR family of receptors HER2 (neu, erbB2), HER3 (erbB3), and HER4 (erbB4) (Tao and Maruyama, 2008, Carpenter, 1987). The intracellular domain consists of 3 subdomains the juxtamembrane domain, a kinase domain, and a C-terminal regulatory domain (Ferguson, 2008) (**Figure 1.4**).

The juxtamembrane domain has been shown to be essential to the activation of the kinase (Thiel and Carpenter, 2007), by stabilisation of the activated asymmetric kinase dimer, through the formation of an antiparallel dimer with juxtamembrane domains of other receptors (Red Brewer et al., 2009, Jura et al., 2009). In addition, it has also been shown to have a role in ligand binding to the extracellular domain (Macdonald-Obermann and Pike, 2009).

The kinase domain of EGFR differs to that of other RTKs in that its conformation is similar to those seen in active kinases (Stamos et al., 2002). This may in part be due to EGFR not requiring activation-loop phosphorylation for activation (Gotoh et al., 1992). The C-terminal regulatory domain is rich in proline residues and contains a number of phosphorylation sites (**Figure 1.5**) (Gajiwala, 2013). These phosphorylation sites specifically recruit proteins involved in a number of signalling pathways (Yarden and Sliwkowski, 2001). Phosphorylation of these sites is not required for enzyme activity, as the receptor can be allosterically activated by asymmetric dimerization of its kinase domain (Zhang et al., 2006b). It is important to note that both the C-terminal and the juxtamembrane domains are both involved in the stabilisation of the kinase domain (Gajiwala, 2013).

In lung cancer, the targeting of EGFR for therapeutic benefit began before the identification of specific activating mutations were known. Indeed, in unselected populations, the EGFR inhibitor, gefitinib, showed a response rate of 10-20% (Fukuoka et al., 2003, Kris et al., 2003). This led a number of studies to research why a particular subset of patients showed a positive initial response to such treatments.

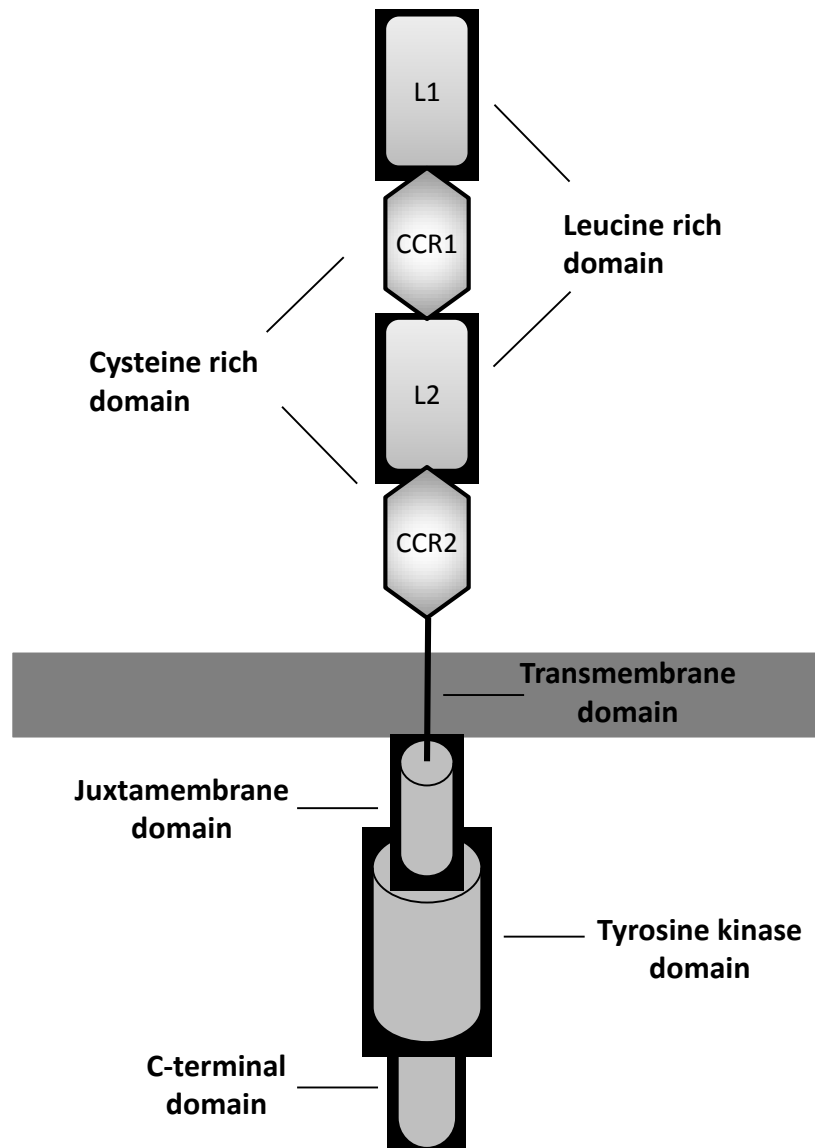


Figure 1.4: The structure of EGFR. EGFR extracellular domain composed of 2 Leucine rich domains L1 and L2 and 2 cysteine rich domains CCR1 and CCR2. A single pass transmembrane domain separates the intracellular domain from the extracellular domain consisting of 3 subdomains the juxtamembrane domain, a tyrosine kinase domain, and a C-terminal regulatory domain.

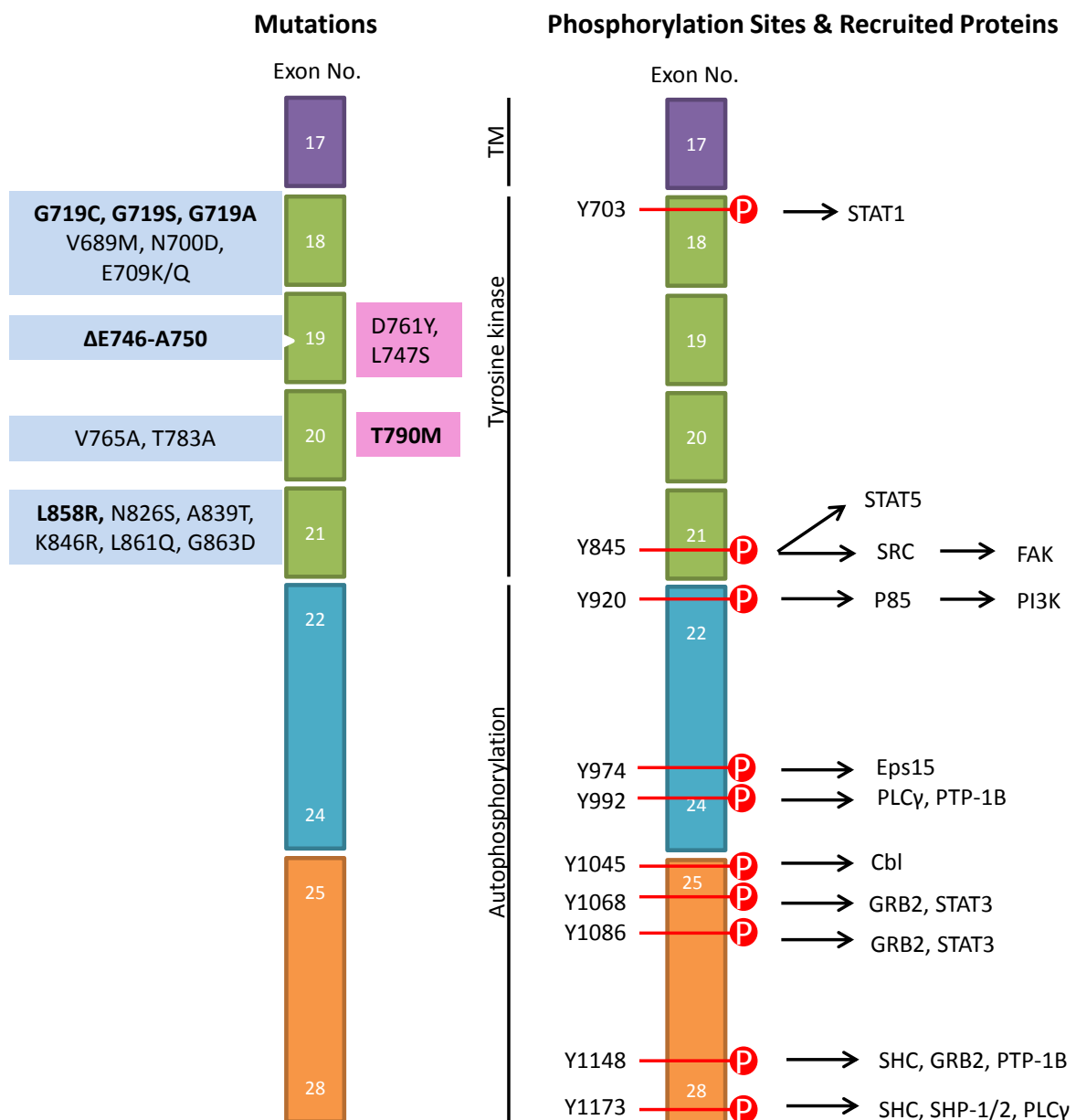


Figure 1.5 A representation of EGFR showing the distribution of exons and mutations within the transmembrane domain (TM) and the intracellular domain comprised of tyrosine kinase and autophosphorylation regions of the C-terminal domain. **Left panel:** The most common mutations are located within the tyrosine kinase domain. Mutations associated with sensitivity and resistance to Gefitinib/Erlotinib are indicated in pale blue and in pale pink, respectively. The most prevalent mutations are in bold. **Right panel:** EGFR phosphorylation sites and the pathways they activate.

1.3.1 EGFR mutations in NSCLC

Somatic mutations in the EGFR kinase domain were simultaneously reported by two groups in 2004 (Paez et al., 2004, Lynch et al., 2004). These studies showed that the activating mutations within the EGFR have clinical implications as they increased sensitivity to first generation EGFR TKIs (see section 1.4 below) such as Gefitinib (Lynch et al., 2004, Paez et al., 2004). Since these initial studies a number of EGFR mutations have been identified (**Figure 1.5**). The 4 most common activating mutations in EGFR identified include point mutations in exon 18 (G719X, G719S, G719A), deletions in exon 19, insertions in exon 20, and point mutations in exon 21 (L858R and L861Q) (Sharma et al., 2007). The two most frequently identified mutations are those in exon 19 of which there are over 20 different variants, and the L858R mutation, whereby the 858 codon in exon 21 is converted from leucine to arginine (Sharma et al., 2007). These mutations increase the autophosphorylation activity of EGFR on intracellular tyrosine residues (de Gunst et al., 2007). The mutated EGFRs show increased sensitivity to first generation TKIs, possibly as a result of an increase in drug affinity for the receptor (Mulloy et al., 2007)

Despite the increase in sensitivity of mutant EGFR to these targeted agents, resistance inevitably develops and is commonly attributed to additional mutations within the receptor (Sharma et al., 2007). The most common additional mutation is in exon 20 involving the substitution mutation of threonine to methionine at codon 790 (Oxnard et al., 2011, Maemondo et al., 2010, Kobayashi et al., 2005). This insertion of the bulky methionine has been suggested to reduce the ability of TKIs such as Erlotinib and Gefitinib to bind to the ATP binding pocket, resulting in an increase in ATP binding (Yun et al., 2008). However, this has been disputed, predominantly due to the fact that the receptor remains sensitive to structurally similar irreversible inhibitors indicating that drug binding to the T790M mutated receptor may not be reduced. Other secondary mutations have also been identified, although far less common than the T790M. These include T854A in exon 21 (Bean et al., 2008) and L747S and D761Y in exon 19 (Costa et al., 2008, Balak et al., 2006). Each of these mutations renders patients resistant to first line TKI treatment through distinct mechanisms of action. The T854A mutation may affect drug sensitivity in a similar manner as the T790M mutation. Indeed, first-generation EGFR TKIs contact with residue T854 and its change to alanine may increase the size of the binding pocket, negatively influencing Erlotinib binding (Lin and Bivona, 2012). The mechanisms of resistance for L747S and D761Y are less well known, although, it is believed that the L747S mutation induces

the active conformation of the receptor and the D761Y is thought to affect the receptor's catalytic cleft (Lin and Bivona, 2012).

1.3.2 Mechanism of EGFR activation and regulation

Ligand binding to the EGFR via its extracellular domain results in an increase in receptor dimerization, accompanied by increased intracellular tyrosine kinase activation (Yarden and Schlessinger, 1987). EGFR catalyses the transfer of γ -phosphate from ATP to the C-terminal domain of EGFR in a trans manner, as well as to tyrosine residues on exogenous substrates (Honegger et al., 1989). Following auto-phosphorylation adapter/signalling molecules identify the phosphorylated tyrosine residues within the C-terminus, resulting in the activation of a number of signalling pathways (Hackel et al., 1999). The activation of EGFR has also been proposed as the mechanism by which this receptor migrates from caveolae rich/lipid raft to the bulk of the membrane component (Mineo et al., 1999). EGFR expression can then be regulated through receptor internalisation via clathrin-coated pits (Wiley et al., 1991).

EGFR protein interactions have been the focus of many studies and thus far over 100 different interacting proteins have been identified (Morandell et al., 2008). A basic overview of EGFR signalling pathways is seen in **Figure 1.6**. A number of these pathways are outlined below:

A number of Src homology 2 (SH2) domain containing proteins interact with EGFR including phospholipase C- γ 1, phosphatidylinositol-3-kinase, growth factor receptor-binding protein 2 (Grb2) and Src. The role of the SH2 domain is vitally important for EGFR signal through their ability to bind to phosphorylated tyrosine residue. Indeed, with the SH2 domain representing one of the largest classes of phosphor-tyrosine recognition domains (Pawson et al., 2001, Huang et al., 2008). SH2 domains interact with phosphor-tyrosine residues in a sequence specific manner (Pawson, 1995). Through this action it is able to regulating intra- and intermolecular protein—protein interactions.

Activation of the RAS/RAF/MEK/ERK pathway by EGFR has shown to regulate cellular proliferation and survival. Upon EGFR activation, a complex of adapter proteins, Grb2 and Son of Sevenless (SOS), bind directly or through association with Shc to docking sites on EGFR (Lowenstein et al., 1992, Batzer et al., 1994). This results in the conformational modification of SOS, which is able to recruit and activate RAS (Freedman et al., 2006). RAS-GTP activates RAF-1, which phosphorylates mitogen-activated protein kinase kinase 1/2 (MEK1/2), ultimately leading to the activation of transcription factors involved in cell proliferation (Hallberg et al., 1994,

Liebmann, 2001, Hill and Treisman, 1995). Those transcription factors include MYC, ELK and Fos (Zhang and Liu, 2002).

The activation of the PI3K/AKT pathway facilitates cell growth, resistance to apoptosis, cell invasion and migration (Vivanco and Sawyers, 2002, Shaw and Cantley, 2006). The dimeric enzyme PI3K consists of the p85 regulatory subunit and the p110 catalytic subunit (Vivanco and Sawyers, 2002). The most efficient mechanism for EGFR-dependent activation of PI3K involves dimerization with HER3, due to the multiple docking sites for the p85 subunit available on that isoform (Carpenter et al., 1993, Yarden and Sliwkowski, 2001). The p85 subunit is responsible for binding to docking sites on EGFR, while p110 generates PIP₃, a second messenger involved in the phosphorylation and activation of AKT (Vivanco and Sawyers, 2002). AKT is a serine/threonine-specific protein kinase, which regulates a number of downstream pathways involved in cell survival, cell cycle, regulation of metabolism as well as angiogenesis. AKT regulates cell survival through direct and indirect interaction with, and phosphorylation of, the pro-apoptotic BCL2 family member, BAD (Datta et al., 1997). In addition, AKT has been shown to inactivate glycogen synthase kinase 3 (GSK3)-beta, which leads to increased cyclin D1. This increase inhibits the Forkhead family transcription factors and the tumour suppressor tuberin (TSC2), resulting in reduction of p27Kip1 and subsequent progression into the G1 phase of the cell cycle (Liang and Slingerland, 2003). AKT's role in metabolism is mediated through its interaction with GSK-3 and its role in the translocation of the glucose transporter 4 (GLUT4) (Cross et al., 1995a, Cong et al., 1997). Regulation of angiogenesis occurs through the ability of AKT to increase the secretion of VEGF through hypoxia-inducible factor 1 (HIF-1)-dependent and independent mechanisms (Karar and Maity, 2011).

Interaction of EGFR with Phospholipase C γ results in the hydrolysis of phosphatidylinositol 4,5-diphosphate (PIP₂) producing secondary messengers inositol 1,3,5-triphosphate (IP₃), a soluble molecule capable of diffusing from the membrane, and diacylglycerol (DAG), a co-factor in activation of protein kinase C (PKC) (Patterson et al., 2005, Chattopadhyay et al., 1999). PKC can phosphorylate I κ B kinase (IKK), which results in the activation of the transcription factor NF- κ B (Lallena et al., 1999). Through this action it is able to regulate a number of pathways including innate and adaptive immunity, inflammation and cellular stress responses.

EGFR interacts and activates STAT proteins both directly through the SH2 domain and indirectly through Src (Quesnelle et al., 2007, Silvennoinen et al., 1993). Following activation, STATs dimerize and translocate to the nucleus, activating the transcription of target genes including B-

cell CLL/lymphoma 2 (BCL2) (Bhattacharya et al., 2005), cyclin D1 and D2 (Leslie et al., 2006, Martino et al., 2001) and vascular endothelial growth factor A (VEGFA) (Wei et al., 2003). Through this pathway STATs regulate cell proliferation, differentiation and survival (Quesnelle et al., 2007). Increased activity of upstream STAT regulators such as EGFR and HER-2 promotes the persistent activation of STAT3, which has been implicated in oncogenic development as well as tumour progression (Bromberg, 2002)

SRC is a tyrosine kinase which plays a role in multiple signalling pathways. It is involved in the regulation of cell proliferation, migration, adhesion, angiogenesis, as well as immune function (Yeaman, 2004, Summy and Gallick, 2006). SRC is able to regulate these pathways in part through the activation of a number of proteins including FAK, PI3K and STATs (Summy and Gallick, 2006, Yeaman, 2004). SRC has multiple roles in EGFR signalling in addition to acting as a signal transducer and enhancer of EGFR activation (Leu and Maa, 2003). It has also been suggested to have a role in resistance to EGFR-targeting therapies through independent activation and association with alternative signalling pathways (Ochi et al., 2014, Filosto et al., 2013).

Casitas B-lineage Lymphoma (c-CBL) associates with the SH3 domain of Grb2, which has been implicated in endocytosis of EGFR (Huang and Sorkin, 2005). In addition, c-CBL interacts with the non-catalytic region of tyrosine kinase adaptor protein 1 (Nck), resulting in activation of P21 Protein (Cdc42/Rac)-Activated Kinase 1 (PAK1)(Lu et al., 1997). PAK1 in turn activates c-Jun Kinases (JNKs) through MAP/ERK Kinase Kinase-1 (MEKK1) and MAP Kinase Kinase-4/7 (MKK4/7)(Su et al., 1997). Activated JNKs enter the nucleus and phosphorylate transcription factors such as c-Jun and c-Fos (Smeal et al., 1991). EGFR also activates the oncogene VAV of the Rho family GTPases, which, in addition to its ability to turn on RHO and RAC leading to cytoskeletal rearrangement, also activates the aforementioned pathway (Bustelo, 2000, Miranti et al., 1998).

EGFR activation turns on Focal Adhesion Kinase 1 (FAK1), which is involved in cell motility (Mitra et al., 2005). Furthermore, several proteins that regulate cytoskeletal organisation are also downstream targets of EGFR including Paxillin, Caveolin, E-Cadherin and, indirectly, Catenin-Beta (CNNTB) through the phosphorylation and activation of Mucin-1 (MUC-1)(Li et al., 2001, Sen et al., 2010, Jawhari et al., 1999, Couet et al., 1997).

One factor which largely influences the ability of EGFR to activate downstream signalling molecules is its localisation. EGFR activation of a number of signalling pathways including MAPK and PI3K, occur predominantly at the plasma membrane. Therefore, receptor internalisation would reduce activation of these pathways (Sousa et al., 2012, Brankatschk et al., 2012).

However, internalisation of the activated EGFR has been shown to be required for optimal activation of a subset of signal transducers (Vieira et al., 1996, Teis et al., 2006). Following activation of EGFR it is rapidly internalised and then sorted through the early endosome and is then recycled or subject to lysosomal degradation (Wiley et al., 1991). However, more recently it has been established that the fate of activated EGFR is not limited to EGFR degradation and recycling. Indeed, EGFR has also been shown to traffic to both the nucleus and the mitochondria (Demory et al., 2009, Wang and Hung, 2009). In the nucleus EGFR has been shown to associate with proliferating cell nuclear antigen (PCNA) and DNA dependent protein kinase, which facilitates EGFR function via the promotion of DNA replication and repair (Wang et al., 2006a, Bandyopadhyay et al., 1998, Dittmann et al., 2005). In addition, nuclear EGFR has also been shown to have a role in resistance to multiple cancer therapies such as cetuximab (Li et al., 2009), gefitinib (Huang et al., 2011) and cisplatin (Liccardi et al., 2011). Thereby highlighting the significance of EGFR localisation on downstream signalling.

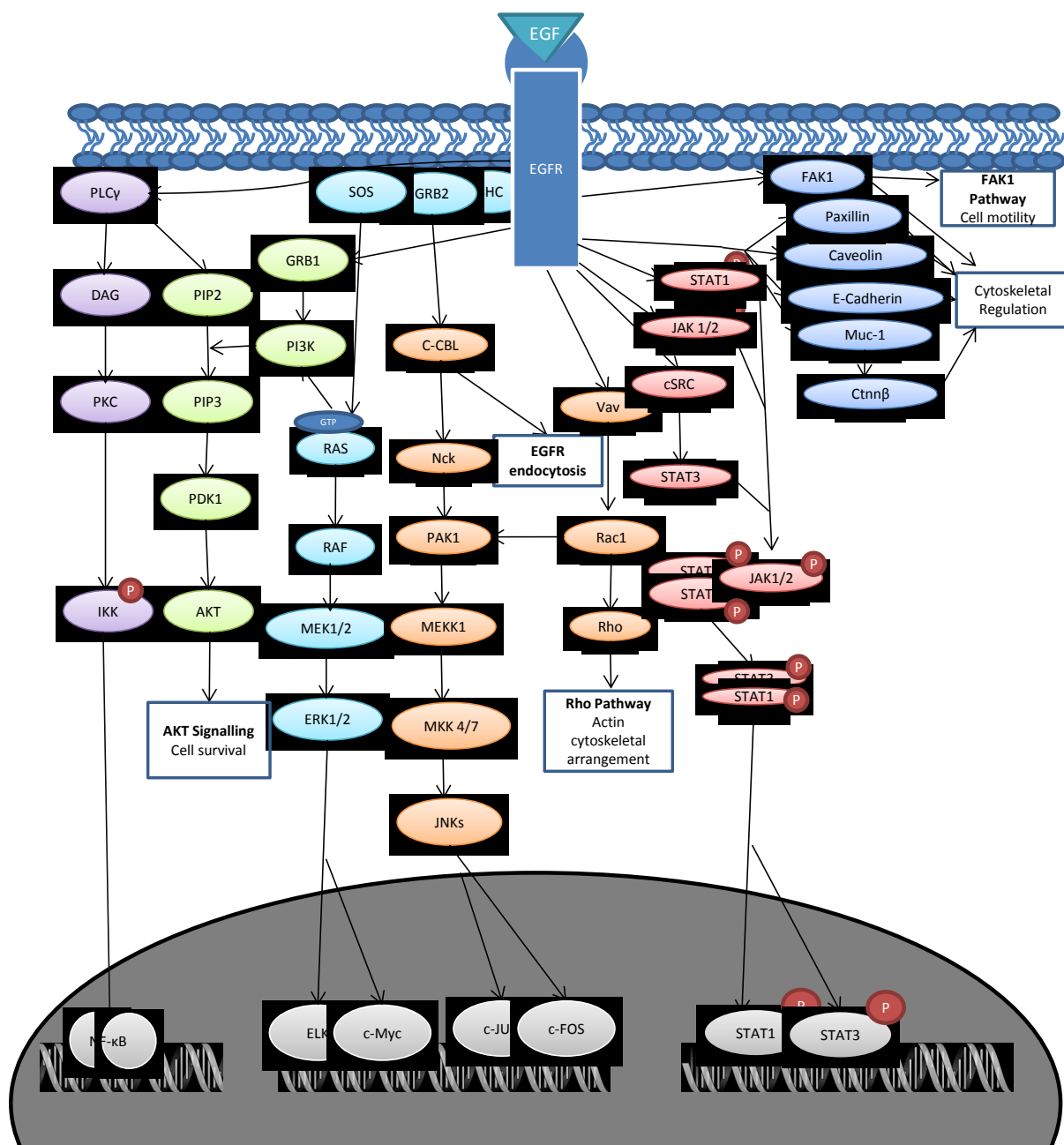


Figure 1.6 EGFR signalling pathway. A schematic figure demonstrating multiple different signalling pathways activated by the EGFR resulting in several different cellular effects such as cell proliferation, survival and migration.

1.4 Tyrosine kinase inhibition of EGFR

A number of TKIs have been developed against EGFR for the treatment of NSCLC. First generation inhibitors are still used in the clinic today. However, there has been a vast amount of research to try and improve these therapies, which has led to the development of second, third and fourth generation inhibitors each of which will be discussed.

1.4.1 First generation EGFR inhibitors

Gefitinib is an orally administered low-molecular-weight anilinoquinazoline. It inhibits the receptors tyrosine kinase activity by outcompeting ATP for the ATP-binding domain/pocket of EGFR (Tiseo et al., 2010). This binding results in the inhibition of autophosphorylation of the receptor, thereby, impeding downstream signal transduction (Tiseo et al., 2010). Indeed, gefitinib treatment has shown to result in the inhibition of proliferation, possibly through the increase in G1 cell cycle arrest following up-regulation of cell cycle inhibitors such as p27 and down-regulation of transcription factors such as c-FOS (Di Gennaro et al., 2003). Gefitinib has also been shown to induce autophagy and apoptosis through the blockage of the PI3K/AKT/mTOR pathway (Zhao et al., 2016).

Treatment of an unselected patient population with gefitinib resulted in a response rate of 10-20% (Lynch et al., 2004, Paez et al., 2004). It was the first cancer therapy to be given accelerated approval by the FDA for the treatment of patients with locally advanced or metastatic NSCLC that did not respond to either platinum-based or docetaxel chemotherapies (Cohen et al., 2003). However, approval was later retracted due to the failure to show clinical benefit by extended life and to a number of adverse effects associated with treatment (FDA, 2009). Indeed, a number of cases reported adverse hepatic and pulmonary events associated with gefitinib treatment (Li et al., 2009, Inomata et al., 2004). The mechanism behind these associated toxicities is relatively unknown. However, a study by Li *et al* suggested that this possibly occurred via a GSH-related mechanism. They demonstrated that gefitinib can be bioactivated in hepatic, intestinal, and pulmonary microsomes, forming in a reactive metabolite that reacts with GSH to form gefitinib-GSH adducts (Li et al., 2009, Paez et al., 2004). As a consequence of its limited efficiency and toxicity, gefitinib was then only indicated for patients who had previously shown a benefit from its use.

Erlotinib, like gefitinib is a first generation TKI. It shares a similar structure as both have a 4-anilino-quinazoline pharmacophore base (**Figure 1.7**). Due to the similarities in structure it is unsurprising that they have similar pharmacokinetic and toxicity profiles in patients. However,

differences in their clinical effectiveness have been identified (Wu et al., 2011). The mechanism behind this may be a result of differential binding of these compounds to the catalytic site of the mutant active EGFR. Indeed, identified mutations cluster around the catalytic cleft and differing mutations have been shown to alter sensitivity to inhibitors to varying degrees (Yun et al., 2007). Clinical trials comparing gefitinib with erlotinib showed erlotinib to increase progression-free survival and be associated with reduced adverse hepatic and pulmonary events (Urata et al., 2016). However, patients inevitably progress following acquired resistance to these drugs with the most prominent mechanism being the appearance of the secondary T790M mutation (Godin-Heymann et al., 2008).

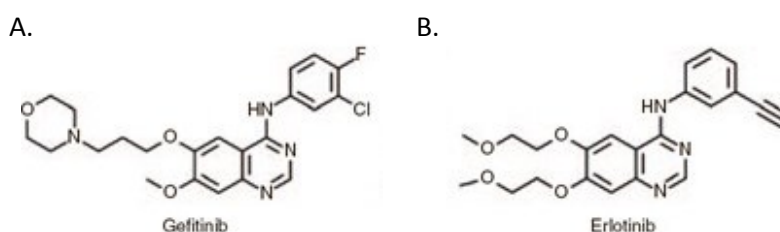


Figure 1.7 Chemical structures of erlotinib and gefitinib. The molecular mass of erlotinib and gefitinib are 429.9 and 446.9 respectively. Figure adapted from (Gonzalez de Castro et al., 2013)

1.4.2 Second generation EGFR inhibitors

Second generation inhibitors were developed in the hope of overcoming the T790M-mediated resistance. These inhibitors were irreversible EGFR TKIs that were proposed to bind well to the T790M mutant receptor. The three discussed in this thesis are afatinib (BIBW2992), dacomitinib (PF0299804) and Neratinib (HKI-272) (Ou, 2012, Rabindran et al., 2004). These are inhibitors of both EGFR and human epidermal receptor 2 (HER2) (Li et al., 2008, Rabindran et al., 2004), with dacomitinib also reported to inhibit HER4 (Gonzales et al., 2008).

1.4.2.1 Afatinib

Afatinib is an anilinoquinazoline based irreversible inhibitor of EGFR and HER2. It was designed to bind covalently to the cysteine 773 of EGFR and the cysteine 805 of HER2 (Li et al., 2008). Pre-clinical assays by Li *et al* demonstrated an increase in the efficiency of afatinib over first generation TKIs (Li et al., 2008). These promising results led to afatinib entering clinical trials. The LUX-LUNG 2 trials, was a Phase II single-arm study, which assessed afatinib as first- or second-line therapy for NSCLC patients whose tumours harboured EGFR activating mutations within exons 18–21 (Yang et al., 2012). The results of this trial showed promise with 61% of

patients having an objective response (1.6 % complete responses, 59.4% partial responses) (Yang et al., 2012). The LUX-LUNG 3 trial compared the efficacy of chemotherapy with afatinib, whereby the latter prolonged progression-free survival in advanced lung adenocarcinoma with EGFR mutations (Sequist et al., 2013). Furthermore, treatment with afatinib resulted in improved quality of life and a delay in lung cancer-related symptoms (Sequist et al., 2013). This finding was confirmed by the LUX-LUNG 6 study, which compared afatinib with cisplatin/gemcitabine and again showed an improvement in progression-free survival (Wu et al., 2014). The LUX-LUNG 7 study was a direct comparison of afatinib and gefitinib-based treatment (Park et al., 2016). Progression-free survival appeared relatively unchanged between these two therapies. However, time to treatment failure was significantly longer in the afatinib treated group at 13.7 months versus 11.5 months for gefitinib. Finally, the LUX-LUNG-8 trial, compared afatinib to erlotinib as second-line treatment of patients with advanced squamous cell carcinoma (Soria et al., 2015). The objective response, tumour shrinkage and adverse event profiles showed no difference between erlotinib and afatinib treated patients. However, there was an increase in progression-free survival and overall survival in the afatinib-treated group (Soria et al., 2015). Despite these positive data disease progression inevitably followed in nearly every patient. Studies by Wu *et al* have identified the T790M mutation in lung adenocarcinoma that had acquired resistance to afatinib (Wu et al., 2016). Thus, afatinib may not be an adequate treatment to overcome T790M-mediated resistance.

1.4.2.2 Dacomitinib

Dacomitinib is a small-molecule, irreversible tyrosine kinase inhibitor of EGFR, ERBB2 (HER2) and HER4. Pharmacokinetic studies demonstrated its high bioavailability and half-life (Gonzales et al., 2008). It inhibits EGFR through binding cysteine-797 at the ATP-binding site, leading to suppression of EGFR tyrosine kinase activity (Gonzales et al., 2008). Preclinical studies, both *in vivo* and *in vitro*, indicated dacomitinib to be a potent inhibitor of EGFR barring activating mutations as well as the EGFR T790M resistance mutation (Engelman et al., 2007a). The Archer 1009 study compared dacomitinib to erlotinib treatment in 878 unselected previously treated NSCLC patients with advanced stage NSCLC (Ramalingam et al., 2014). Results showed that dacomitinib treatment did not enhance progression-free survival when compared to erlotinib and was associated with an increase in adverse events. The Phase 3 double-blind, randomised, placebo-controlled, BR.26 trial studied patients with advanced or metastatic NSCLC that had previously been treated with three rounds of chemotherapy and either gefitinib or erlotinib (Ellis et al., 2014). This study concluded that Dacomitinib did not increase overall survival in this

treatment group. There are still a number of ongoing clinical trials including a comparison with Gefitinib as a first line treatment of advanced NSCLC.

1.4.2.3 Neratinib

Neratinib is an irreversible TKI, which targets cysteine residues in the ATP binding pocket of HER2 (Wissner et al., 2003). This site is conserved in EGFR, HER2, and HER4 and therefore, neratinib is a pan-HER inhibitor. Treatment with neratinib arrests cells at the G1/S phase transition of the cell cycle and thereby decreases cellular proliferation (Rabindran et al., 2004). *In vitro* studies showed that neratinib was three to four-fold more effective in suppressing proliferation than gefitinib in cell lines containing both EGFR activating mutations and the T790M resistant mutation (Kwak et al., 2005). A Phase I clinical trials in pre-treated NSCLC patients suggested the maximum tolerated dose to be 320 mg/day (Wong et al., 2009). However, the Phase II clinical trial imposed a low dose limit due to development of grade 3 diarrhoea in more than 50% of patients (Sequist et al., 2010). This study showed that neratinib had low bioavailability, possibly as a result of the low dose limitation (Sequist et al., 2010). Despite poor clinical results in NSCLC trials there is currently an ongoing open-label phase 2 study of neratinib in patients with solid tumours containing somatic EGFR, HER2 and HER3 mutations or EGFR gene amplifications.

1.4.3 Third generation EGFR inhibitors

Despite the initial hopes for second generation inhibitors none have shown to efficiently target the T790M in the clinic. A number of third generation inhibitors have been developed including AZD9291 (osimertinib, mereletinib), CO-1686 (rociletinib), HM61713 (BI 1482694), ASP8273, EGF816, and PF-0674777. Unlike first and second generation inhibitors third generation inhibitors have an increase in potency for the T790M mutation over wild-type (wt) EGFR.

1.4.3.1 WZ4002

The covalent pyrimidine EGFR inhibitor, WZ4002, was originally identified as one of three compounds (WZ4002, WZ3146 and WZ8040) which showed an increase in potency from a screening of irreversible kinase inhibitor library specifically targeting the T790M-mutant EGFR (Zhou et al., 2009). Treatment of NSCLC cell lines resulted in inhibition of EGFR, AKT and ERK1/2 phosphorylation (Zhou et al., 2009). The first published crystal structure of EGFR T790M mutant bound to the WZ4002 showed that the compound binds to the ATP binding pocket of EGFR through a covalent bond with cysteine 797 (Zhang et al., 2006b) (**figure 1.8**). The anilinopyrimidine core of WZ4002 forms a bidentate hydrogen bonding interaction with the

hinge residue Met 793 of EGFR (Zhang et al., 2006b). This results in a hydrophobic interaction between 5-Cl substituent on the pyrimidine ring of WZ4002 and the side chain of the gatekeeper residue Met 790 (Zhang et al., 2006b). This interaction is suggested to explain the potency of WZ4002 for the T790M-mutated EGFR. Despite these initial positive results WZ4002 did not progress into clinical trials due to legal issues between the Dana-Farber Cancer Institute and Novartis regarding the rights to develop WZ4002.

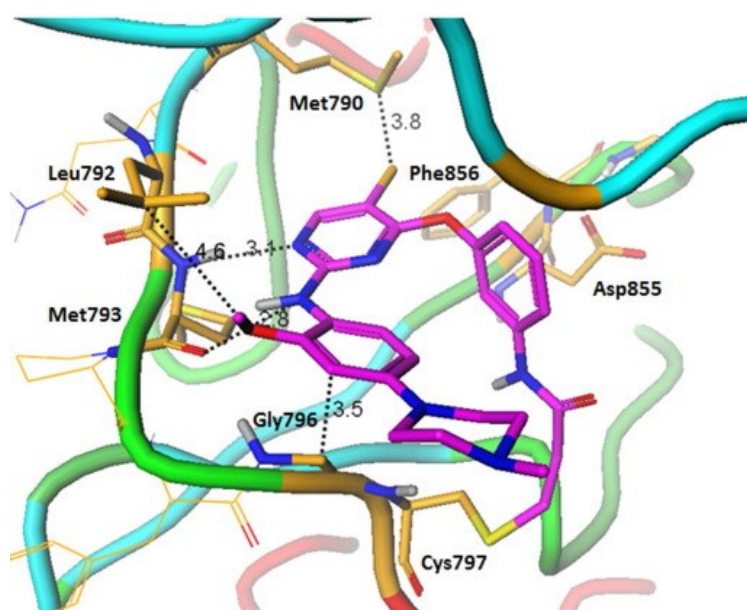


Figure 1.8 Co-crystal structure of EGFR T790M bound with WZ4002

(Taken from (Zhang et al., 2006b)).

1.4.3.2 CO-1686 (rociletinib)

The 2, 4-disubstituted pyrimidine compound, CO-1686, is a novel irreversible mutant-selective inhibitor for the EGFR exon deletion, the L858R and the T790M mutants (Walter et al., 2013a). Indeed, pre-clinical trials showed CO-1686 to have little activity against wild-type EGFR (Walter et al., 2013a). CO-1686 covalently modifies cysteine 797 in the ATP binding pocket of EGFR, inducing apoptosis *in vitro* and anti-tumour activity *in vivo* (Walter et al., 2013a). EGFR-mutated NSCLC patients who had acquired resistance to EGFR inhibitors were enrolled in a phase I/II CO-1686 study (Sequist et al., 2015). This study demonstrated CO-1686 to be active in NSCLC patients with T790M-mutated EGFR. Indeed, a 59 % response rate was noted in patients with T790M-positive tumours (Sequist et al., 2015). However, an update presented at the JP Morgan Healthcare Conference (13th January 2016) showed a considerably smaller confirmed response

rate of 34 %. The most frequent reason for lack of response was due to progression, principally due to brain metastasis and the absence of tumour shrinkage of greater than 30 %. There are currently a number of ongoing trials for CO-1686 shown in **table 1.2**. Despite CO-1686 showing positive results in the early stages of clinical trials, acquired resistance has already been noted (Sequist et al., 2015). Indeed, Piotrowska and colleagues showed that progression following treatment with CO-1686 of T790M-positive EGFR-mutant lung cancers resulted in the emergence of T790-wild-type EGFR populations (Piotrowska et al., 2015). Therefore, T790-wild-type clones become the dominant source of resistance. Moreover, the FDA has recently declined approval of this drug because of poor response rates and toxicity profile, so further clinical development has stopped.

Table 1.2 Ongoing clinical trials for CO-1686 in NSCLC (updated using (ClinicalTrials.gov, 2016c))

Trial name	ClinicalTrials.gov Identifier:	Status
A phase I/II, multicenter, open-label study of EGFRmut-TKI EGF816, administered orally in adult patients with EGFRmut solid malignancies	NCT02108964	This study is currently recruiting participants
A phase 1b/2 study of safety and efficacy of Rociletinib in combination with MPDL3280A in patients with advanced or metastatic EGFR-mutant NSCLC	NCT02630186	This study is ongoing, but not recruiting participants.
TIGER-1: Safety and efficacy study of rociletinib (CO-1686) or erlotinib in patients with EGFR-mutant/metastatic NSCLC who have not had any previous EGFR directed therapy	NCT02186301	This study is ongoing, but not recruiting participants.
TIGER-2: A phase 2, open-label, multicenter, safety and efficacy study of oral CO-1686 as 2nd line EGFR-directed TKI in patients with mutant EGFR non-small Cell Lung Cancer (NSCLC)	NCT02147990	This study is ongoing, but not recruiting participants.
TIGER-3: Open label, multicenter study of Rociletinib (CO-1686) mono therapy versus single-agent cytotoxic chemotherapy in patients with mutant EGFR NSCLC who have failed at least one previous EGFR-directed TKI and platinum-doublet chemotherapy	NCT02322281	This study has closed early and Clovis have stopped clinical development of this molecule.
Study to evaluate safety, pharmacokinetics, and efficacy of Rociletinib (CO-1686) in previously treated mutant epidermal growth factor receptor (EGFR) in non-small cell lung cancer (NSCLC) patients	NCT01526928	This study is ongoing, but not recruiting participants

1.4.3.3 HM61713 (BI 1482694)

HM61713, an irreversible EGFR tyrosine kinase inhibitor which binds to a cysteine residue close to the kinase domain (Lee et al., 2014). *In vitro* and *in vivo* studies showed HM61713 to be a potent inhibitor of growth with limited toxicity (Lee et al., 2014). A phase I/II study of patients with advanced EGFR mutation-driven NSCLC who had previously failed on EGFR TKIs is still ongoing but so far showed an objective clinical response rate of 58.8 % (Park K et al., 2015). They identified a number of dose limitation toxicities involving GI symptoms and elevation of liver and pancreatic enzymes such as aspartate aminotransferase, alanine aminotransferase, amylase, and lipase (Park K et al., 2015). A further update presented at ESMO Asia 2015 showed an additional increase to the objective response rate to 62% in T790M-positive patient (Lee et al., 2015). This led to HM61713 being granted Breakthrough Therapy Designation by the FDA (Stockwell, 2015). However, resistance mechanisms to HM61713 have already been identified including the acquisition of the EGFR C797S mutation (Song et al., 2016)

1.4.3.4 AZD9291 (Osimertinib)

AZD9291 is a potent irreversible EGFR tyrosine kinase inhibitor that is selective for the T790M mutation (Cross et al., 2014). AZD9291 is structurally and pharmacologically distinct from other third generation TKIs such as CO-1686 (Cross et al., 2014). It is a mono-anilino-pyrimidine the structure of which is depicted in **Figure 1.9 A**. The pyrimidine core of AD9291 binds to the hinge region of EGFR (Met-973) by the formation of 2 hydrogen bonds, while its acrylamide group forms a covalent bond with Cys-797 (**Figure 1.9 B**). There are a number of differences which make AZD9291 distinct from TKIs such as CO-1686 including the electrophilic functionality which resides within the pyrimidine C-2 substituent ring, the C-linked and heterocyclic of pyrimidine 4-substituent, and the absence of substitution of pyrimidine 5-position (Cross et al., 2014). Cross and colleagues have shown that AZD9291 potently inhibits both active as well as T790M-mutant EGFRs *in vitro* and *in vivo* (Cross et al., 2014).

There have been a number of clinical trials for AZD9291 in NSCLC, some of which are still ongoing (Gil-Bazo and Rolfo, 2016, Janne et al., 2015, Yang et al., 2016). However, initial results showed a response rate of 59% and duration of response to be 12.4 months (Sequist et al., 2015). In November 2015 it was FDA approved for the treatment of patients with metastatic EGFR T790M mutation-positive NSCLC, who have shown progression following treatment with EGFR TKI therapy (FDA, 2015, Greig, 2016). The drug has also now been approved for use in Europe and recently by NICE in the UK. However, alternative escape mechanisms have already

emerged, including loss of the T790M mutation, activation of the HER2 or MET pathways as well as FGFR1 amplification (Kim et al., 2015, Thress et al., 2015, Planchard et al., 2015). In addition Thress and colleagues identified an additional C797S mutation within EGFR which appears to mediate resistance (Thress et al., 2015). The C797S mutation has also been identified in patients resistant to HM61713 (BI 1482694) treatment (Song et al., 2016). Therefore, despite these promising results resistance to EGFR targeted treatment remains an issue. This highlights the need for further research into resistance mechanisms.

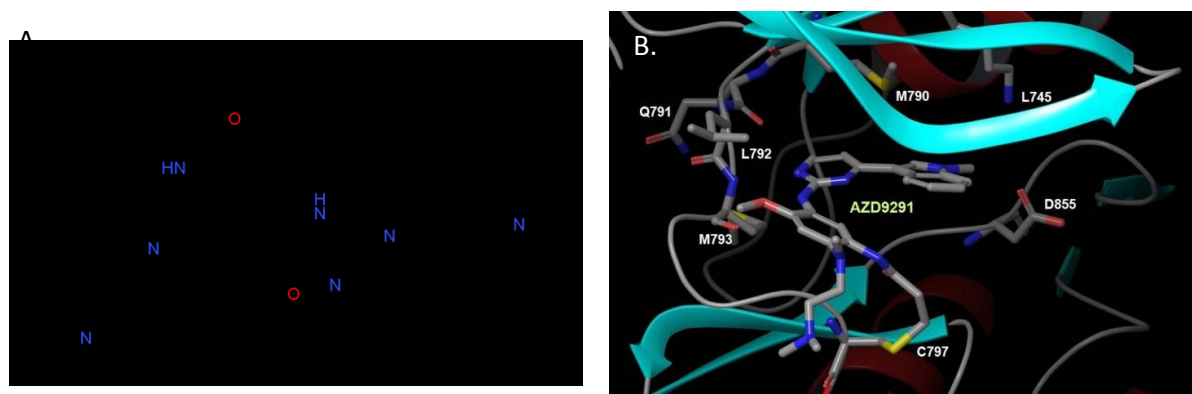


Figure 1.9 AZD9291 chemical structure and structural model showing binding of AZD9291 to EGFR T790M (Taken from (Cross et al., 2014)).

1.4.3.5 ASP8273

ASP8273 is a small molecule irreversible TKI inhibitor, whose structure has not been disclosed (Cheng et al., 2016). ASP8273 has been shown, *in vitro*, to inhibit both the active as well as T790M EGFR mutants with higher potency than the wild-type EGFR (Sakagami et al., 2014). This was shown to be through covalent binding of ASP8273 to the cysteine 797 residue in the kinase domain of EGFR (L858R/T790M) (Sakagami et al., 2014). Interestingly, ASP8273 also showed activity in cell lines which had resistance to AZD9291 and CO-1686 (Konagai et al., 2015). Furthermore, in mouse xenograft models ASP8273 induced tumour regression at 10, 30 and 100mg/kg (Konagai et al., 2015). A phase I/II study of patients in which erlotinib treatment had previously failed showed 25% (3/12) patients carrying the T790M mutation had a partial or complete response to ASP8273 treatment (Yu et al., 2015). There are a number of clinical trials ongoing for ASP8273 shown in **table 1.3**.

Table 1.3 Ongoing clinical trials for ASP8273 (updated using (ClinicalTrials.gov, 2016a))

Trial name	ClinicalTrials.gov Identifier	Status
A study of ASP8273 in epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor-naïve patients with non-small cell lung cancer harbouring EGFR mutations	NCT02500927	Active, not recruiting
An open study of ASP8273 in patients with non-small-cell lung cancer (NSCLC) who have epidermal growth factor receptor (EGFR) mutations	NCT02192697	Active, not recruiting
A study to investigate the absorption, metabolism and excretion of [14C]ASP8273 in subjects with solid tumours	NCT02674555	This study is not yet open for participant recruitment
A Dose Escalation Study of ASP8273 in Subjects With Non-Small-Cell Lung Cancer (NSCLC) Who Have Epidermal Growth Factor Receptor (EGFR) Mutations	NCT02113813	Recruiting
A study of ASP8273 vs. erlotinib or gefitinib in first-line treatment of patients with stage IIIB/IV non-small cell lung cancer tumours with EGFR activating mutations	NCT02588261	Recruiting
A phase I/II, multicenter, open-label study of EGFRmut-TKI EGF816, administered orally in adult patients with EGFRmut solid malignancies	NCT02108964	Recruiting

1.4.4 Fourth-generation selective inhibitor overcoming EGFR C797S

The presence of the additional C797S mutation following treatment with third generation inhibitors has led to the development of fourth generation inhibitors. EAI045 is a fourth-generation selective inhibitor, which overcomes EGFR C797S mediated resistance (Jia et al., 2016). Unlike previous EGFR TKIs which target the ATP-binding sites of the kinase, EAI045 is an allosteric inhibitor (Jia et al., 2016). Crystal structure analysis revealed that it binds to an allosteric site on EGFR created by the displacement of the regulatory C-helix in the inactive conformation of the kinase (Jia et al., 2016). Dimerization-defective/independent mutants were shown to be more sensitive to EAI045 treatment. The combined treatment with cetuximab which is known to block EGFR dimerization, showed a reduction in tumour growth in both L858R/T790M/C797S, and L858R/T790M —mutant-driven lung cancer mouse models (Jia et al., 2016).

Overall, this data highlights the profound effort made by academic and pharmaceutical industries to develop second, third and fourth generation TKIs that bind directly to the T790M-mutated EGFR. Some of these treatments have shown promise and were even incorporated into clinical use. However, the eventual development of resistance to these treatments remains an issue. Furthermore, the presence of secondary mutations within these resistant tumours and the re-occurrence of the T790 wild-type receptor create further challenges. Therefore, a better understanding of changes which occur as a result of the T790M mutation may hold the key to better treatment opportunities for these patients.

1.5 Signalling changes in response to EGFR mutations

The mutated EGFR are catalytically active and tumorigenic and their activation profiles are different from that of the ligand activated wild-type receptors. The phosphorylation status of the mutated receptor exceeds that of unstimulated wild type counterpart. Indeed, the basal EGFR activation induces differential phosphorylation of tyrosine residues (Carey et al., 2006). These induce activation of the MAPK/ERK1/2 and PI3K/AKT pro-survival pathways among others that will be discussed in this section.

An RPPA study of 84 NSCLC cell lines was carried out by Ummanni *et al.* Although the primary aim of this study was not to identify signalling changes associated with EGFR TKIs sensitivity, they have included a small amount of data in this area (Ummanni et al., 2014). They identified that increased phosphorylation, rather than expression, of EGFR was indicative of drug sensitivity. Furthermore, they showed that reduction in proteins involved in the

PI3K/AKT/p70S6K signalling pathway was associated with sensitivity to erlotinib. This pathway was also highlighted by transcriptional analysis of 57 patients with EGFR mutant NSCLC from the EURTAC trial (Karachaliou et al., 2015). Indeed, patients with down-regulation of mTOR upon treatment with Erlotinib demonstrated increased progression-free survival (Karachaliou et al., 2015).

MET

MET expression is increased in EGFR-mutant NSCLC cells (Xu et al., 2010). This is believed to be through the EGFR-mediated activation of hypoxia-inducible factor-1 α (HIF-1 α) (Xu et al., 2010). Furthermore, amplification of MET has been shown to mediate resistance to targeted compounds such as gefitinib (Xu et al., 2010) by driving ERBB3 (HER3)-dependent activation of PI3K (Xu et al., 2010). MET functions through HIF-1 α to induce invasiveness of EGFR-driven NSCLC cells (Xu et al., 2010). Therefore, dual treatment with EGFR and MET inhibitors may prevent tumour metastasis beyond what can be achieved with EGFR TKIs alone.

Nuclear factor (erythroid-derived 2)-like 2 (NRF2)

A study by Yamadori et al identified the NRF2-Kelch-like ECH-associated protein 1(KEAP1) regulatory pathway to play a key role in exacerbating sensitivity to erlotinib (Yamadori et al., 2012). NRF2, is a key regulator of the antioxidant response and therefore has a protective role and that is commonly recognised to modulate drug sensitivity (Wang et al., 2008). The study by Yamadori and colleagues identified that the PC9 NSCLC cell line, which contains the EGFR activating mutation in exon 19, had a constitutively activated NRF2 (Yamadori et al., 2012). Furthermore, they show that induction of cellular stress or the inhibition of KEAP1 reduced the sensitivity to TKI treatment (Yamadori et al., 2012). This highlights the possible role of the NRF2/KEAP1 pathway in the regulation of sensitivity to TKIs such as erlotinib.

Wnt signalling

The Wnt signalling pathway has been implicated in EGFR signalling through its ability to transactivate EGFR (Chen et al., 2001, Hu and Li, 2010, Ji et al., 2009, Sharma et al., 2002). A positive correlation was found in patient tumour samples between EGFR activating mutations and the nuclear accumulation of β -catenin (Suzuki et al., 2007). Furthermore, a whole genome short hairpin RNA (shRNA) screen revealed that the Wnt-tankyrase- β -catenin pathway played a role in resistance to EGFR TKIs (Casas-Selves et al., 2012). Indeed, the inhibition of the activators of the Wnt signalling pathway poly-ADP-ribosylating enzymes tankyrase 1 and 2, was shown to increase sensitivity to EGFR inhibitors (Casas-Selves et al., 2012). This may be explained by the

fact that tankyrase 1 and 2 mediate poly-ADP-ribosylation of AXIN1 and AXIN2 targeting them for degradation resulting in the activation of Wnt signalling. AXIN along with glycogen synthase kinase 3 β (GSK3 β), casein kinase I (CKI) and adenomatous polyposis coli (APC) form the β -catenin destruction complex. Inhibition of tankyrase 1 and 2 results in negatively regulated WNT signalling and its associated downstream pathways such as the transcription of cyclin D1, C-MYC, C-MET etc. (Huang et al., 2009).

Neurofibromin 1 (NF1)

The NF1 gene encodes a RAS GTPase-activating protein (GAP), a protein which binds to G proteins to stimulate GTPase activity. A study demonstrated that lung adenocarcinoma patients who had acquired resistance to EGFR TKIs exhibited low NF1 expression (de Bruin et al., 2014). Indeed, silencing of NF1 *in vitro* using siRNAs increased the resistance of cells to EGFR TKIs (de Bruin et al., 2014). Low levels of NF1 correlated with the inability of erlotinib to fully inhibit the RAS-ERK signalling pathway, thereby suggesting that EGFR-mutant lung adenocarcinoma patients, who are resistant to EGFR TKIs, may benefit from combination therapy with MEK targeted agents (de Bruin et al., 2014).

Death-Associated Protein Kinase (DAPK)

In a methylation-specific array DAPK was found to be hypermethylated in cells which were resistant to erlotinib compared to their sensitive counterparts (Ogawa et al., 2012). Furthermore, stable transfection of DAPK re-sensitised these cells to erlotinib treatment (Ogawa et al., 2012). Although, this was not formally demonstrated, the role of DAPK in regulating apoptosis in addition to reciprocal regulation between DAPK and the EGFR down-stream transducer ERK were proposed as possible mechanisms of DAPK-mediated resistance to EGFR TKIs (Chen et al., 2005). Indeed, the death domain of DAPK interacts with ERK to promote its retention in the cytoplasm (Chen et al., 2005), preventing the transcriptional activities of this kinase.

Fibroblast Growth Factor (FGF)

A study by Terai and colleagues identified overexpression of FGF2 and fibroblast growth factor receptor 1 (FGFR1) to be associated with resistance to gefitinib (Terai et al., 2013). Furthermore, they showed that the FGF2/FGFR1 pathway promoted proliferation of gefitinib-resistant cells (Terai et al., 2013). Similarly, the levels of FGFR2 and FGFR3 have been shown to rapidly increase in response to gefitinib treatment in both wild type and mutated EGFR, mediating a cellular protective function (Ware et al., 2010).

ADAM metallopeptidase domain 17 (ADAM17)

Zhou and colleagues suggested that the up-regulation of ADAM17, a protein involved in the cleavage of membrane bound proteins, membrane-anchored cytokines, cell adhesion molecules, receptors, ligands, and enzymes, played a role in resistance to EGFR TKIs (Zhou et al., 2006). Indeed, the ADAM inhibitor, INCB3619, enhanced gefitinib-mediated inhibition of EGFR signalling in NSCLC (Zhou et al., 2006). ADAM17, through shedding various substrates such as ligands of EGFR, is able to regulate various cellular functions including cell proliferation and migration (Huang et al., 2014). A possible resistance mechanism is the activation of alternative EGFR family members and downstream pathways through release of its ligands. Indeed, an anti-ADAM17 inhibitory antibody was shown to suppress proliferation and motility in both the presence and absence of gefitinib. Treatment with this antibody led to the decrease in both the endogenous and the bradykinin-stimulated shedding of HER ligands, which was accompanied by a reduction in phosphorylation of HER receptors and downstream signalling pathways (Huang et al., 2014).

Janus kinase (JAK)

JAK2 has been implicated as a resistance mechanism independent of the EGFR T790M mutation and amplification of MET (Harada et al., 2012). Indeed, inhibition of JAK2 with the compound TG101348 resulted in enhanced cytotoxicity of erlotinib (Zhang et al., 2015). The combination was shown to induce apoptosis and downregulation of EGFR, BCL-XL and survivin expression as well as phosphorylation of EGFR and STAT3 (Zhang et al., 2015). A further study by Ochi and colleagues in xenograft models demonstrated that dual inhibition of JAK and EGFR with pacritinib inhibitor and erlotinib respectively, resulted in increased tumour shrinkage when compared to treatment with either drug alone (Ochi et al., 2016).

Receptor tyrosine kinase-like orphan receptor 1 (ROR1)

Induction of NK2 homeobox 1 (NKX2-1) has been shown to induce expression of ROR1, which results in an increase in pro-survival signalling (Yamaguchi et al., 2012). Knockdown of ROR1 inhibits proliferation of lung adenocarcinoma cell lines, including those carrying EGFR-resistance mutations (Yamaguchi et al., 2012). Indeed, high levels of ROR1 have been shown to be associated with a decrease in progression-free survival in patients carrying the T790M mutation, while ROR1 inhibitors enhanced the efficiency of erlotinib treatment (Karachaliou et al., 2014).

Epithelial-Mesenchymal Transition (EMT)

Lastly, Increased epithelial-mesenchymal transition (EMT) as a result of overexpression of the single-pass transmembrane receptor Notch-1, has been implicated in resistance as well as cancer cell metastasis (Xie et al., 2012). EGFR TKI-resistant cell lines showed an upregulation of Notch-1 (Xie et al., 2013). Conversely, the silencing of Notch-1 in gefitinib-resistant cell lines resulted in re-sensitisation to Gefitinib treatment, which was associated with impairment of cell invasion and anchorage-independent growth (Xie et al., 2013). Furthermore, *in vivo* studies showed that treatment of resistant lung cancer xenografts with the notch inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]- (S)-phenylglycine t-butyl ester (DAPT) decreased proliferation and increased apoptosis, resulting in tumour growth retardation (Xie et al., 2013).

In short, a number of signalling pathways have been shown to be altered in response to mutations in EGFR. However, as yet, these findings have mostly not been translated to the clinic.

1.6 Metabolic changes in response to EGFR mutations

Metabolic changes have long been shown to be associated with cancer development. Indeed, Hanahan and Weinberg highlighted abnormal metabolic pathways as a hallmark of cancer back in 2011 (Hanahan and Weinberg, 2011). Furthermore, in addition to their role in cancer development, changes in metabolism have also been implicated in drug resistance (Rahman and Hasan, 2015).

A number of metabolic characteristics have been associated with sensitivity to chemotherapeutic agents and radiation (Harper et al., 2002). Indeed, cells which are sensitive to these treatments are associated with the following traits: (1) Higher mitochondrial membrane potential, (2) an increase in proton transfer against the proton gradient of the mitochondria, (3) production of ATP primarily through glucose metabolism, (4) higher levels of reactive oxygen species (ROS) in stressed conditions, resulting in increased DNA damage and susceptibility to apoptosis (Harper et al., 2002). In contrast, parameters associated with resistance include: (1) Lower mitochondrial membrane potential, (2) reduction in transfer of protons against the proton gradient of the mitochondria (proton leak), (3) higher use of beta-oxidation and increased cytosolic glycolysis, (4) lower levels of ROS production under stressed conditions, with associated reduced DNA damage and decreased susceptibility to apoptosis (Harper et al., 2002).

Furthermore, metabolic changes have also been linked to resistance to targeted therapies (Locasale, 2012). Indeed, studies into resistance to lapatinib, an inhibitor of EGFR and HER2 receptor autophosphorylation, showed that resistance was not a result of abrogation of the RAS, PI3K and mTOR pathways (Komurov et al., 2012). Instead, resistant cells showed an

increased expression of a number of metabolic pathways including glucagon signalling, glucose uptake and gluconeogenesis (Komurov et al., 2012). This suggested an increase in the glucose deprivation response network, which correlated with high clinical relapse rates in HER2-positive breast cancer patients (Komurov et al., 2012). Furthermore, targeting of the glucose deprivation response networks could modulate the response to lapatinib treatment. This study highlights the role of metabolism in resistance to targeted therapies and suggests that novel combination therapies may hold the key to overcoming acquired resistance.

There have been a number of studies which have discussed EGFR mutations driving alteration in metabolic signatures (Makinoshima et al., 2015, Serizawa et al., 2014, Xie et al., 2016). Indeed, Serizawa and colleagues utilised capillary electrophoresis time-of-flight mass-spectrometry (MS) analysis to compare the profiles of the erlotinib-sensitive cell line PC9 and its resistant counterpart PC9ER (Serizawa et al., 2014). This study identified an increased representation of enzymes involved in the metabolism of the antioxidant molecule glutathione (GSH) in PC9ER cells. The levels of glutathione-S-transferase theta 2 (GSTT2), gamma-glutamyltransferase 1 (GGT1) and GGT5 were all increased in the resistant cell line PC9ER (Serizawa et al., 2014). GSTT2 is a member of the glutathione-S-transferase (GST) family of proteins, which catalyse the conjugation of reduced GSH to a variety of compounds. GGT1 and GGT5 are both members of the gamma-glutamyltransferase family of enzyme, which cleaves the gamma-glutamyl bond of GSH resulting in the release of the dipeptide, cysteinyl-glycine and glutamate. This was an interesting insight into metabolic changes that could occur in response to erlotinib, particularly since increase in GSH levels, rather than its degradation, is traditionally associated with drug resistance (Zhang et al., 1998).

1.6.1 The role of GSH in drug resistance

GSH is a cellular detoxification antioxidant molecule. It is known to react with a number of molecules within the cell, including reactive oxygen species (ROS) and drug compounds (Masella et al., 2005, Mates, 2000). GSH has been suggested to be involved in drug resistance through modulation of a number of pathways or via its action as an antioxidant, reducing oxidative stress within the cell. The antioxidant function of GSH is particularly involved in resistance to alkylating agents and redox cycling drugs (Colvin et al., 1993). Indeed, reduction in GSH resulted in increased sensitivity to these agents (Colvin et al., 1993).

GSH has been shown to directly conjugate with xenobiotic compounds (**Figure 1.10 A**). A number of erlotinib-GSH and gefitinib-GSH conjugates have been identified (Li et al., 2010b, Li et

al., 2009). These conjugates have been suggested to result in toxicities associated with treatment. The mercapturic acid pathway is involved in detoxification of glutathione-conjugate and extracellular export of drug compounds (**Figure 1.10 B**), resulting formation of mercapturic acid (Meister, 1988). Hence, GSH and the mercapturic acid pathway is involved in resistance to a number of drug compounds, although some drugs may become activated through this pathway resulting in cytotoxicity (Commandeur et al., 1995).

As such, GSH metabolic enzymes such as GSTs have an important role in drug resistance to a number of compounds including chemotherapy agents, insecticides and herbicides (Tew, 1994, McLellan and Wolf, 1999, Cummins et al., 2013, Enayati et al., 2005). High levels of GST have been shown in various cancer types (Tew, 1994). It has been proposed that GST mediates resistance through two mechanisms: the detoxification of drugs as well as the sequestration of JNKs (Adler et al., 1999). Indeed, JNK has a key role in the activation of apoptosis via both phosphorylation of pro- and anti-apoptotic proteins and transactivation of transcription factors such as c-Jun (Dhanasekaran and Reddy, 2008).

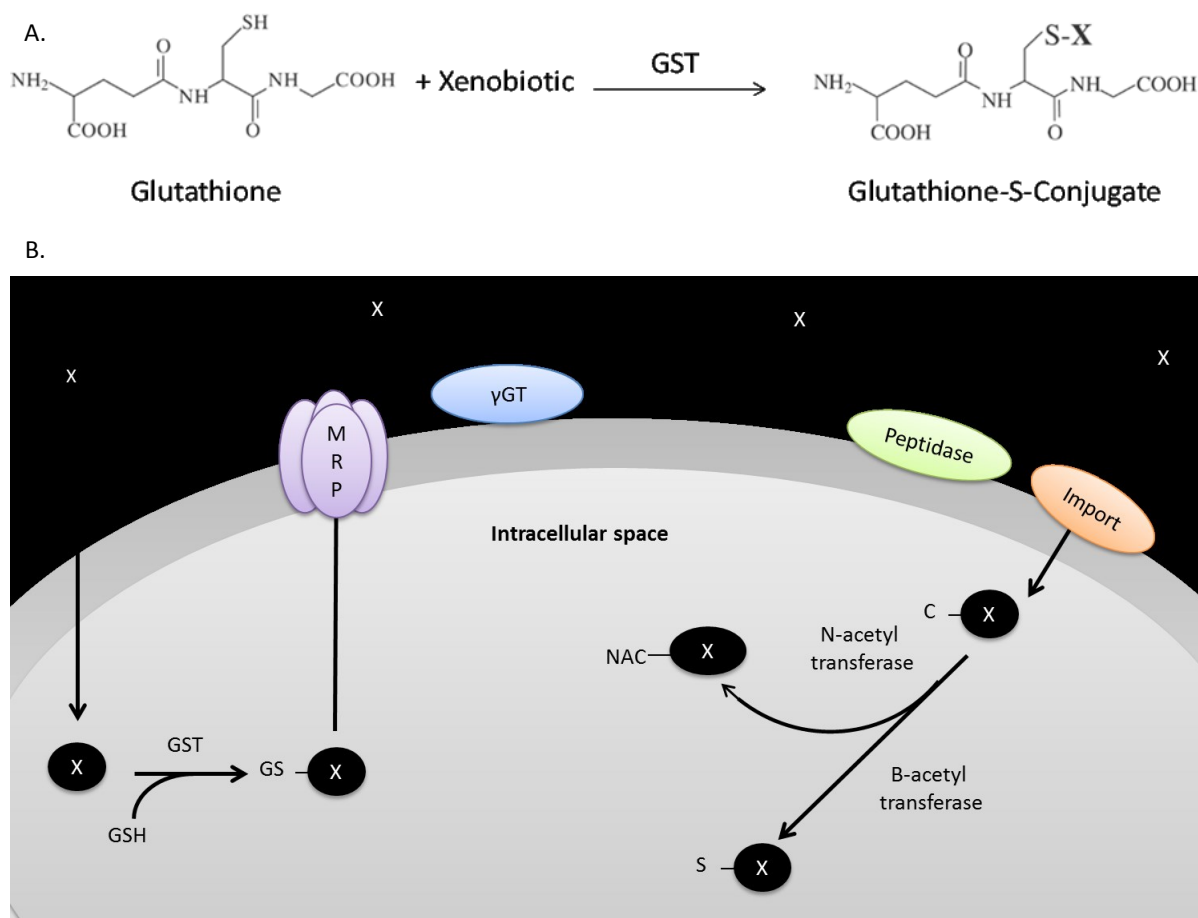


Figure 1.10 GSH conjugates with xenobiotic via GST mediated mechanism and simplified schematic of detoxification via the mercapturic synthesis pathway. (A) The sulfhydryl group of GSH interacts with a number of xenobiotic molecules in a reaction which is catalysed by GST. **(B)** Xenobiotics (including drug compounds) are represented as **X**. Xenobiotic bind to GSH, which renders them more hydrophilic. This reaction can be catalysed via GSH although, this could also be a spontaneous reaction (Armstrong, 1997). The newly formed GSH-conjugate is then pumped out of the cell by members of the multidrug resistance-associated protein family (**MRP**) (Borst et al., 2000). The cell surface bound gamma-glutamyl transferase (**γGT**) catalyses removal of γ-glutamyl group of GSH. Dipeptidases/aminopeptidases (**Peptidase**) then remove the glycyl group, resulting in the formation of cysteine S-conjugates. The cysteine S-conjugates re-enter the cell via a number of transporters including organic anion transport polypeptides and cystine/cysteine importers (**Import**). Once inside the cell two different mechanisms have been reported. The acetylation by N-acetyl transferases action which results in the formation of mercapturic acid versions of the xenobiotics which are more polar and water soluble, hence generally less toxic and can be excreted out of the body. Alternatively cysteine S-conjugate β-lyase which results in the production of highly reactive thiol (Cooper et al., 2011). Figure adapted from (Ramsay and Dilda, 2014).

1.6.1.1 Mechanism of modulation of glutathione levels

GSH is vital to the antioxidant response and its levels are tightly regulated within the cell. GSH is synthesised in two ATP-dependent steps, initially L-glutamate and cysteine are combined to form gamma-glutamylcysteine in a reaction which is catalysed by glutamate cysteine ligase (GCL) (Meister, 1988). This being a rate limiting step, expression of GCL is important in the regulation of this pathway. The second step in this reaction is catalysed by gamma-glutamylcysteine, whereby, glycine is added to the C-terminus of the gamma-glutamylcysteine (Meister, 1988). The efficiency of this step is dependent on the availability of precursor molecules such as cysteine, glycine and glutamate and the activity of enzymes which catalyse the reactions for GSH synthesis (Meister, 1988).

As GCL is the rate limiting enzyme of GSH synthesis, there have been a number of studies into its transcriptional regulation. The promoter region of GCL was found to contain activator protein-2 (AP-2), metal response (MRE), and antioxidant response (ARE)/electrophile responsive (EpRE) elements (Lu, 2009). The MRE element is a cis-acting transcription factor that is responsible for metal-responsive control and is found within genes which are involved in metal metabolism and homeostasis (Giedroc et al., 2001). This may be in response to induction of oxidative stress due to increase metal levels (Woods and Ellis, 1995). ARE is a cis-acting transcriptional enhancer, which activates the transcription of a number of anti-oxidant genes within the cell (Rushmore et al., 1990). An ARE sequence has also been identified within promoter region of GSS, the second enzyme in the GSH synthesis reaction (Lee et al., 2005). Furthermore, overexpression of the transcription factors, Nuclear respiratory factor 1 (NRF1) and Nuclear factor (erythroid-derived 2)-like 2 (NRF2) significantly increased promoter activity of GSH metabolic enzymes (Lee et al., 2005).

NRF1 and NRF2 are Cap 'n' collar-based leucine zipper proteins (CNC-bZIP), which can trans-activate target genes (Ohtsuji et al., 2008). They contain the following domain structures: the conserved Neh2 domain (an N terminal regulatory domain), the transactivation domain and the C-terminal bZIP domain (Ohtsuji et al., 2008). The C-terminal bZIP domain allows NRF1 and NRF2 to heterodimerize with members of the small Avian Musculoaponeurotic Fibrosarcoma (MAF) protein family that is required for their DNA binding (Jaiswal, 2004). NRF2 has shown to be more potent than NRF1 in the transactivation of antioxidant genes (Ma, 2013, Ohtsuji et al., 2008, Lee et al., 2005). Due to the importance of NRF2 in antioxidant response its activity is tightly regulated. Kelch-like ECH-associated protein 1 (KEAP1) has been identified as an NRF2

interactive partner (Itoh et al., 1999a, Dhakshinamoorthy and Jaiswal, 2001). Further characterisation revealed that homodimerization of KEAP1 is required for binding to NRF2 (Tong et al., 2006a) (**Figure 1.11A**). The mechanism by which KEAP1 regulates NRF2 was demonstrated through expression of NRF2 alone or in combination with KEAP1. Expression of NRF2 alone resulted in its accumulation in the nucleus, while co-expression with KEAP1 prevented this and the presence of a panel of electrophiles restored NRF2 nuclear localisation (Itoh et al., 2010a). This suggested that KEAP1 sequesters NRF2 in the cytoplasm until oxidative or electrophilic insult frees it to enter the nucleus. Under oxidative stress the DLG motif of NRF2 is thought to dissociate from KEAP1 due to a conformational change as a result of electrophilic modifications of cysteine residues within KEAP1 (Bryan et al., 2013) (**Figure 1.11B**). This NRF2, now solely bound to KEAP1 by the ETGE motif, cannot be degraded and sequesters KEAP1. Newly formed NRF2 molecules can then translocate to the nucleus where it heterodimerizes with small MAF proteins to bind the ARE of target genes (Bryan et al., 2013, Itoh et al., 1997). The expression levels and function of NRF2 are regulated both transcriptionally and post-transcriptionally. Some of these mechanisms will be discussed below.

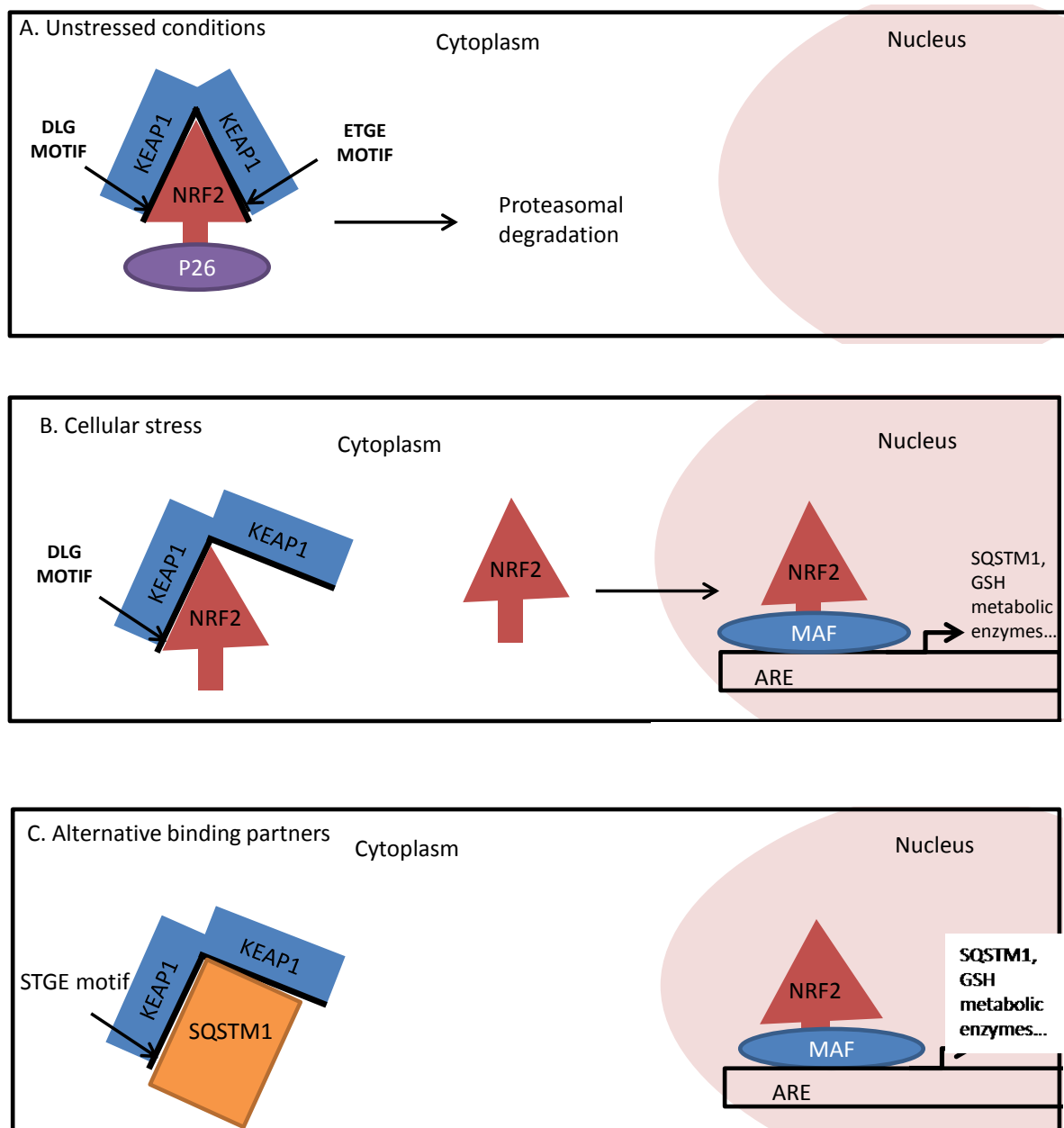


Figure 1.11 Schematic representation of KEAP1 regulation of NRF2. (A) KEAP1 binds to NRF2 via both the ETGE and DLG motif and targets NRF2 for ubiquitination and proteasomal degradation. (B) Oxidative stress allows NRF2 to dissociate from KEAP1 ETGE motif, thereby avoiding proteasomal degradation. Newly formed NRF2 is then able to translocate to the nucleus to form heterodimers with MAF proteins and transcribe ARE containing genes. (C) Alternative KEAP1 binding partners such as SQSTM1, bind to KEAP1 via the STGE motif displacing NRF2. SQSTM1 contains an ARE sequence and therefore is also transcribed by NRF2 thereby creating a positive feedback loop.

1.6.1.1.2 Additional KEAP1 binding partners

A number of studies have identified additional KEAP1 binding partners (**summarised in Figure 1.12**) through a variety of approaches including affinity purifications and mass-spectrometry. Furthermore, as some of these interactions have been found to occur through a conserved STGE motif, these additional KEAP1 binding partners may have a role in NRF2 regulation. A number of these will be discussed within this thesis.

Liquid chromatography-electrospray ionization-tandem mass-spectrometry (LC-ESI-MS/MS) identified SQSTM1 as a KEAP1 binding partner, and this was validated by co-immunoprecipitation (Co-IP) experiments (Copple et al., 2010). SQSTM1 is a scaffolding/adaptor protein involved in multiple signalling pathways (Moscat et al., 2007, Seibenhener et al., 2007). In addition, it plays a role in sequestering damaged/misfolded proteins into aggregates and the shuttling of polyubiquitinated proteins for both proteasomal and lysosomal degradation (Moscat et al., 2007, Seibenhener et al., 2007). The silencing of SQSTM1 increases the levels of KEAP1 and decreases those of NRF2 (Copple et al., 2010). This resulted in the decrease of the basal mRNA and protein levels of a number of ARE-containing genes (Copple et al., 2010). SQSTM1 regulates KEAP1 by localising it into aggregates, preventing the degradation of NRF2 due to its inability to interact with the E3 ubiquitin ligase complex (Lau et al., 2010). Komatsu and colleagues identified the consensus sequence that enables SQSTM1 to bind to KEAP1 as (D/N)X(E/S)(T/S)GE (Komatsu et al., 2010). Furthermore, they showed that increased SQSTM1 results in increased NRF2 due to SQSTM1 outcompeting NRF2 for KEAP1 binding sites (Komatsu et al., 2010) (**Figure 5.11C**). Interestingly, SQSTM1 is transcribed in an NRF2-dependent manner as its promoter region contains an ARE, thereby forming a positive feedback loop (Ishii et al., 2000, Jain et al., 2010) (**Figure 5.11C**).

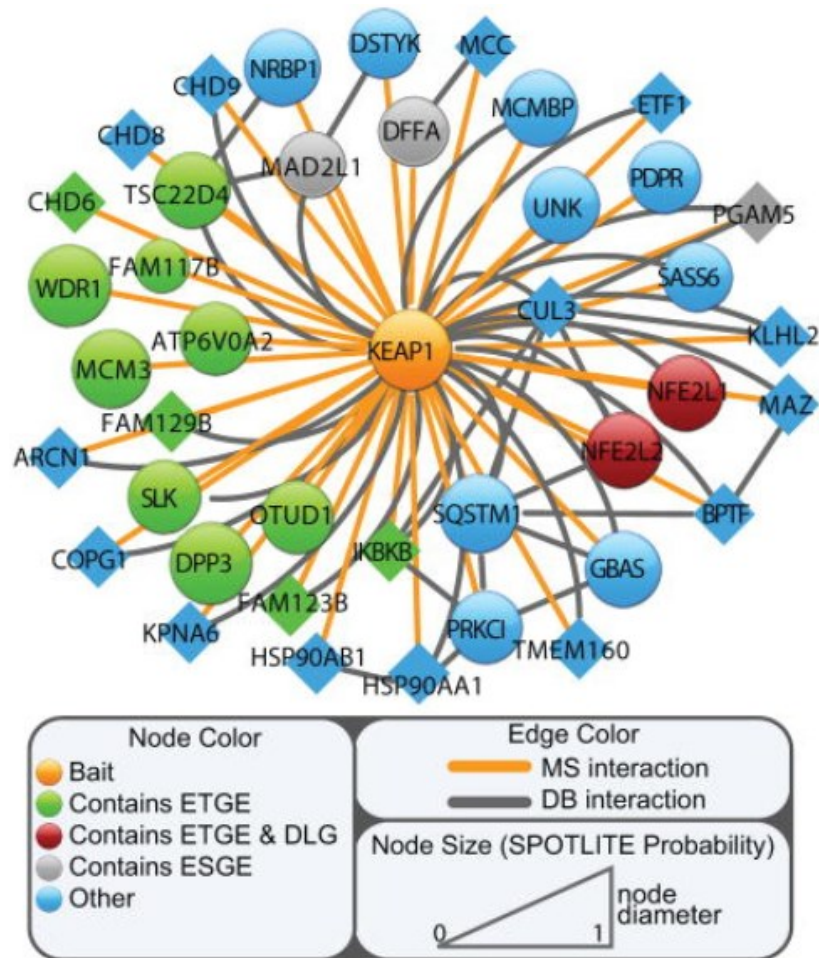


Figure 1.12 Representation of KEAP1 protein interaction network identified through affinity purification and mass-spectrometry. The colour and size of each of the nodes represent their probabilistic scoring approach, sequence and source of data. Circular nodes sized upon their probability scoring using SPOTLITE analysis software (10% false discovery rates; FDR) (Goldfarb et al., 2014). Triangular nodes represent borderline SPOTLITE scores observed across multiple affinity purification and mass-spectrometry (APMS) runs. Taken from (Hast et al., 2013).

KEAP1 has many other interacting partners amongst which is the Partner and localizer of BRCA2 (PALB2), a nuclear protein involved in the intranuclear localization, stability, as well as DNA repair function of BRCA2 (Ma et al., 2012). This interaction was shown to be direct via an extended ETGE (LDEETGE) KEAP1 binding motif (Ma et al., 2012). Although, the majority of KEAP1 is cytoplasmic, evidence suggests KEAP1 to have a nuclear role, via blocking NRF2 access to ARE, facilitating its export out of the nucleus for its degradation (Sun et al., 2007). PALB2 is able to inhibit this interaction by outcompeting NRF2 for KEAP1 binding sites (Ma et al., 2012)

Mass-spectrometry-based proteomics has identified another binding partner of KEAP1, namely the Wilms Tumour Gene on X Chromosome (WTX) protein (Major et al., 2007). This tumour suppressor protein is also known to negatively regulate WNT/beta-catenin signalling via interaction with ubiquitin ligase adaptor family containing β -transducin repeat promoting ubiquitination and degradation of β -catenin (Camp et al., 2012). The interaction of WTX with KEAP1 is direct and achieved through its ETGE motif (Camp et al., 2012). Furthermore, WTX was shown to compete with NRF2 for KEAP1 binding sites and inhibition of WTX resulted in ubiquitination and degradation of NRF2 following cytotoxic insult (Camp et al., 2012).

Dipeptidyl peptidase 3 (DPP3) was identified by Hast and colleagues to be a further interacting partner of KEAP1 through proteomic analysis (Hast et al., 2013). DPP3 is a zinc-dependent hydrolase that cleaves dipeptides from the N-termini of a number of proteins and as such plays a role in a number of physiological processes (Kumar et al., 2016). Hast and colleagues showed a direct interaction of the ETGE domain of DPP3 and KEAP1 (Hast et al., 2013). In addition, as seen for PALB2, SQSTM1, and WXT, DPP3 competes with NRF2 for KEAP1 binding sites so that its increased expression correlates with an increase in NRF2 activity (Hast et al., 2013).

This accumulating body of evidence therefore highlights the importance of KEAP1 binding partners in the activity of NRF2. Changes in expression of these proteins could directly influence transcription of NRF2 target genes. There are a number of additional pathways that impact on NRF2 transcription directly which will be discussed below.

1.6.1.1.3 Transcriptional regulation of NRF2

NRF2 can regulate its own transcription via the ARE sequence in the NRF2 promoter. (Kwak et al., 2002). In addition to the ARE sequence, three xenobiotic response element-like elements (XREL1, XREL2 and XREL3) are also present within its promoter region (Miao et al., 2005). These were shown to be functional, although XREL2 is the most potent (Miao et al., 2005). These are

activated by the aryl hydrocarbon receptor (AHR) inducer TCDD (2, 3, 7, 8-tetrachlorodibenzo-p-dioxin) (Miao et al., 2005). AHR, when activated by ligands such as TCDD, heterodimerizes with AHR nuclear translocator (ARNT). These heterodimers activate XRE, which induce expression of phase I drug metabolising enzymes such as cytochrome p450s (Rushmore and Kong, 2002) that promotes the generation of reactive intermediates which may activate the ARE in target genes (Bryan et al., 2013).

A number of studies have identified cross-talks between NRF2 and the transcription factor, NF- κ B. Indeed, nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) down-regulates the transcription of ARE-containing genes (Nair et al., 2008). The promoter region of NRF2 has been shown to contain an NF- κ B binding region (Nair et al., 2008), suggesting that NF- κ B has a role in NRF2 transcription. Furthermore, KEAP1 has been identified as a negative regulator of the NF- κ B pathway through its ability to bind to I κ B Kinase β (IKK β), resulting in its ubiquitination and degradation (Kim et al., 2010).

1.6.1.1.4 Post-translational modification of NRF2

NRF2 contains serine, threonine and tyrosine residues that could be phosphorylated by a number of different kinases. A number of pathways have been implicated in the phosphorylation of NRF2 including the mitogen-activated protein kinase cascades (MAPK), the phosphatidylinositol 3-kinase (PI3K/AKT), protein kinase C (PKC), GSK3 β pathway as well as ERK signalling pathways. The most predominant part of the NRF2 pathway affected by these phosphorylation's are the nuclear import/export of NRF2 itself or the interaction of KEAP1 with NRF2. These will be discussed below and are summarised in **Figure 1.13**.

NRF2 nuclear localisation

The tyrosine kinase Fyn has been shown to phosphorylate NRF2 at tyrosine-568, resulting in its nuclear export and degradation (Jain and Jaiswal, 2006). This was confirmed by mutational studies which revealed that mutations at this residue resulted in the nuclear accumulation of NRF2 due to loss of interaction with exportin chromosomal maintenance 1 (Crm1), also known as Exportin 1 (Jain and Jaiswal, 2006). Exportin Crm1 is the most prominent export protein for the transport of macromolecules across the nuclear membrane (Nguyen et al., 2012). In addition, 13 different locations within the Neh4 and Neh5 transcriptional activation domains of NRF2, have been identified as protein kinase casein kinase II phosphorylation sites *in vitro* (Apopa et al., 2008). These phosphorylations have been shown to enhance the translocation of

NRF2 into the nucleus to transcribe ARE responsive genes (Apopa et al., 2008). Lastly, phosphorylation of NRF2 by p38 mitogen-activated protein kinases prevents NRF2 nuclear localisation by promoting its association with KEAP1 (Keum et al., 2006).

Interaction of NRF2 with KEAP1

Studies have also shown the involvement of JNK and ERK in NRF2 activation through phosphorylation. Indeed, ERK is able to phosphorylate NRF2, resulting in the dissociation of NRF2 from KEAP1. NRF2 is subsequently imported into the nucleus (Cullinan et al., 2003). PKC, specifically PKC delta, phosphorylates serine 40 on NRF2, which lies within the Neh2 domain (Huang et al., 2002, Numazawa et al., 2003, Niture et al., 2009). Phosphorylation at this site disrupts its interaction with KEAP1, leading to stabilisation of NRF2 (Huang et al., 2002, Numazawa et al., 2003, Niture et al., 2009), thereby increasing expression of ARE-containing genes (Huang et al., 2002). However, other reports also suggest that this phosphorylation only induces stability of NRF2 and its nuclear localisation requires dissociation from KEAP1 via antioxidant modifications of the 151 cysteine residues of KEAP1 (Niture et al., 2009, Bloom and Jaiswal, 2003).

In addition to cytoplasmic protein kinases, EGFR has also been shown to have a role in NRF2 activation. A study by Yamadori and colleagues suggested that NRF2 is regulated in an EGFR ligand-dependent manner (Yamadori et al., 2012). They demonstrate that a constitutively active EGFR leads to constitutive activation of NRF2 while the inhibition of EGFR and downstream mediators MEK1/2 and PI3K results in a decrease in NRF2 expression. Furthermore, they propose NRF2 as a critical molecular target to improve sensitivity to EGFR-TKIs in NSCLC. An additional study by Huo and colleagues showed that EGFR associates with KEAP1 in the nucleus (Huo et al., 2010). Upon ligand binding, EGFR internalises and translocates to the nucleus to fulfil its role in gene transcription, DNA repair and DNA replication (Wang et al., 2010c, Han and Lo, 2012, Wang and Hung, 2012). Nuclear EGFR interacts with and phosphorylates Y206, Y263, and Y334 residues on nuclear KEAP1. Phosphorylation at these sites results in a reduction in nuclear KEAP1, thereby enhancing NRF2 transcriptional activity (Huo et al., 2014).

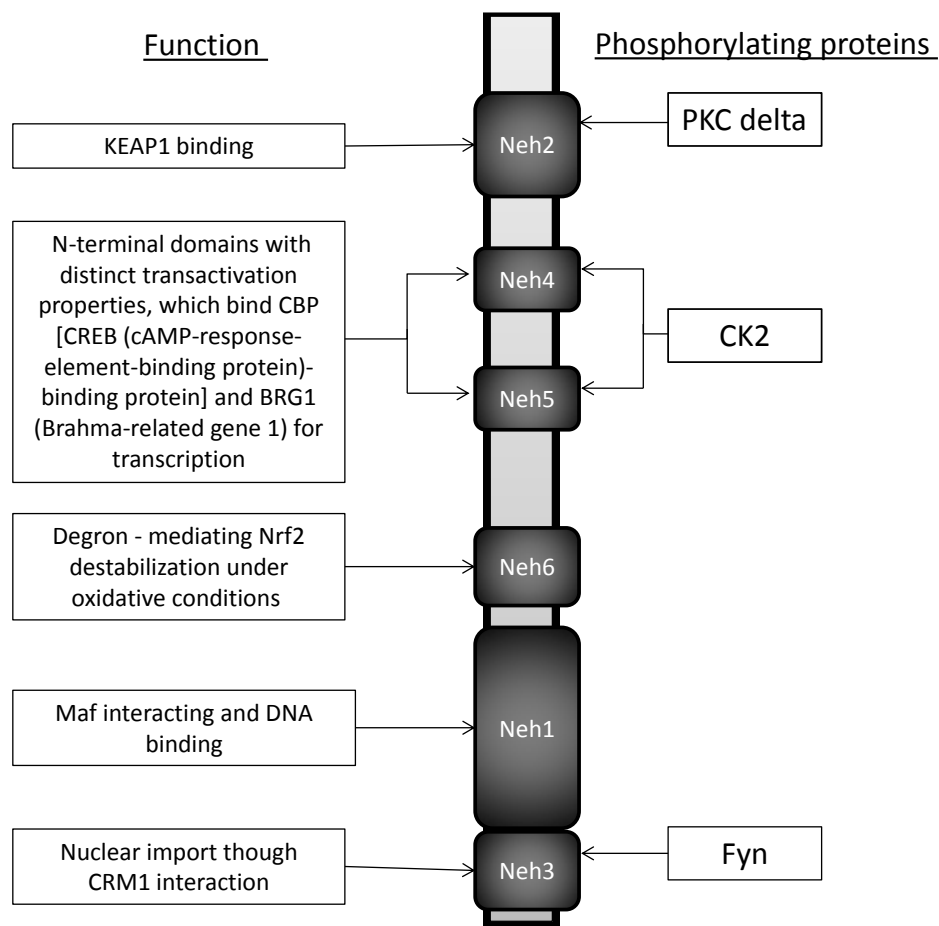


Figure 1.13 Structure of NRF2, domains and phosphorylation sites. NRF2 is comprised of 6 Neh domains 1-6. The Neh2 domain allows NRF2 to bind to KEAP1 facilitating its degradation. Whereas Neh1 enables NRF2 to interact with Maf proteins required for DNA binding. The Neh3 region is required for interaction with the nuclear import protein CRM1, which allows NRF2 to cross the nuclear membrane. A number of proteins including Fyn, CK2, and PKC delta are known to phosphorylate NRF2, their function has been associated with interfering with NRF2/KEAP1 or import of NRF2 into the nucleus.

1.6.2 The role of phosphatidylcholine metabolism in drug resistance

Phosphatidylcholine and phosphatidylethanolamine are major components of biological membranes (Gibellini and Smith, 2010). Increased levels of these molecules have been implicated in oncogenic development and tumour progression (Baenke et al., 2013). This is thought to help satisfy the demand for lipids in highly proliferative tissue. However, in addition to this a number of enzymes within both the synthesis and degradation pathways have been implicated in drug resistance. An example of this is choline kinase, the first enzyme of the CDP-choline/Kennedy pathway (Gibellini and Smith, 2010). It has been shown to be overexpressed in a number of cancers including lung, prostate and breast (Shah et al., 2010, Ramirez de Molina et al., 2007, Challapalli et al., 2015). Overexpression of choline kinase correlates with poor prognosis, disease progression (Shah et al., 2010, Glunde and Bhujwalla, 2007) and resistance to the thymidylate synthase inhibitor 5-fluorouracil. The latter was associated with increased rhodamine-123 efflux (Shah et al., 2010), suggesting that choline kinase can modulate multidrug resistance protein 1 (MDR1) or other ABC transporters, although the mechanism remains to be elucidated (Shah et al., 2010). These and other findings lead to the development of small molecular inhibitors of choline kinase alpha and a first-generation example of these compounds is the competitive inhibitor MN58b that shows anti-tumoural activity (Hernandez-Alcoceba et al., 1997, Hernandez-Alcoceba et al., 1999, Sanchez-Martin et al., 2005). Moreover, a second-generation inhibitor, RSM-932A (TCD-717), has now completed phase I clinical testing (Lacal and Campos, 2015, ClinicalTrials.gov, 2014).

The phosphatidylcholine degradation pathway has also been implicated in cancer progression through the generation of second messenger molecules. Phosphatidylcholine is hydrolysed by phospholipase enzymes, which results in the production of a number of second messengers such as phosphatidic acid (PA) and diacylglycerol (DAG) (Besterman et al., 1986). Indeed phospholipase D (PLD), which catalyses the cleavage of phosphodiester bonds in phosphatidylcholine and phosphatidylethanolamine resulting in the release of choline and phosphatidic acid, has been shown to induce DNA synthesis and cell proliferation (Knauss et al., 1990). Increase in phospholipase activity has been found in multidrug-resistant breast cancer cells (Welsh et al., 1994). PLD was also shown to increase resistance to rapamycin treatment (Chen et al., 2003), although the underlying mechanism for this is unclear. Nevertheless, here again, it is thought to involve an increase in DNA synthesis and cell proliferation via a phosphatidic acid-driven mechanism. The activity of PLD has been shown to increase following

EGF stimulation and, therefore, this enzyme may have a role in EGFR-driven cancers (Fisher et al., 1991).

In addition to PLD, PLC, of which there are six isoforms (β , γ , δ , ϵ , ζ , η), hydrolyses phosphatidylcholine and phosphatidylethanolamine. This results in the release of phosphocholine and the second messenger diacylglycerol (DAG). Increased DAG has been shown to activate conventional (α , β_I , β_{II} , and γ) and novel (δ , ϵ , η) PKCs, (Sharkey et al., 1984). The activation of PKC induces the expression of the MDR1 gene through the RAF-MAPK pathway, affecting the cellular response to drug treatments (Yang et al., 2001).

The degradation of phosphatidylcholine and phosphatidylethanolamine by phospholipase A results in the formation of free fatty acids (FAs). FAs have been implicated in a number of signalling pathways including the activation of AMPK, ERK, GPCR, Toll-like receptor 4/NF- κ B, Src/JNK, and PKC as well as sphingomyelinase/ceramide signalling (Huang and Freter, 2015). Therefore, activation of these pathways may lead to an increase in a number of cellular survival responses to promote drug resistance.

Taken together, these data highlight the importance of metabolic pathways in oncogenic development and their potential role in resistance to targeted compounds such as erlotinib. However, until now, nothing is known about a direct role of metabolic changes in mediating resistance to erlotinib in EGFR-T790M mutant-expressing lung cancer cells. In this thesis, we used metabolomics analysis to address this issue.

1.7 Metabolomics in cancer

Hanahan and Weinberg have proposed changes in metabolism or deregulated cellular energetics to be a hallmark of cancer (Hanahan and Weinberg, 2011). Indeed, they suggested that a well-documented feature of neoplastic transformation is alterations in metabolic pathways that facilitate survival (Hsu and Sabatini, 2008, Hanahan and Weinberg, 2011). The highly proliferative nature of cancer cells leads to a continuous need for carbohydrates, amino acids and fatty acids to synthesise DNA, RNA, protein and lipid required for increasing the biomass. Cancer cells have an increased rate of nutrient consumption but also need to maintain a pool of substrates which are required for *de novo* synthesis.

The most well document change in metabolism is “the Warburg effect,” in which, cells rely primarily on mitochondrial oxidative phosphorylation for the generation of energy (Warburg, 1956b). Metabolism in cancer cells is adapted to facilitate the uptake and incorporation of nutrients to increase cellular biomass required for cellular division (Vander Heiden et al., 2009). Oncogene status

and tumour microenvironment select for alterations in metabolism which are beneficial for the cell survival and growth (Vander Heiden et al., 2009). Several signalling pathways implicated in cellular proliferation also have role in the regulation of metabolic pathways.

One metabolic protein which has a key role within glycolysis has been shown to be regulated by several tyrosine phosphorylated proteins, is the M2 isoform of the glycolytic enzyme pyruvate kinase (PK-M2). This enzyme catalyses the last step of glycolysis, and therefore has a pivotal role in tumour metabolism and growth (Christofk et al., 2008b). PKM2 is regulated through binding to and fructose-1,6-biphosphate, an intermediate of glycolysis (Christofk et al., 2008c). However, fructose-1,6-biphosphate can be displaced by tyrosine-phosphorylated proteins such as FGFR, resulting in an increase in lactate production and decrease in oxygen consumption (Christofk et al., 2008c, Hitosugi et al., 2009). In addition to regulation through phosphorylation, other proteins have been identified to have a role in transcription of PK-M2. mTOR has been shown to induce expression of PKM2 via binding with hypoxia-inducible factor 1 at the promotor region of PKM, (Sun et al., 2011). A number of additional proteins have also been suggested to have a role in PKM2 regulation, including EGFR, c-MYC (Yang et al., 2012b, David et al., 2010).

Other signalling molecules have been highlighted to have a role in metabolism including P53 which has a role at multiple stages of glycolysis and oxidative phosphorylation (Puzio-Kuter, 2011). Mutations which occur within this protein have been shown to result in changes in metabolism which are advantageous to cancer cells. An example of which is loss of P53, which prevents the expression of cytochrome c oxidase, thereby interfering with mitochondrial respiratory chain (Matoba et al., 2006). P53 also affects TP53-induced glycolysis and apoptosis regulator through inhibition of glycolysis by decreasing fructose-2,6-bisphosphate levels, a stimulator of glycolysis as well as an inhibitor of gluconeogenesis (Kawauchi et al., 2008).

In addition to these downstream proteins, RTKs have also shown to have a role within cell metabolism. EGFR signalling has been shown to increase aerobic glycolysis as well as activate de novo pyrimidine synthesis in EGFR-mutated lung adenocarcinoma cells (Makinoshima et al., 2014). Makinoshima and colleagues went on to show that EGFR inhibitors such as gefitinib and erlotinib were able to repress aerobic glycolysis in these EGFR mutated cell lines (Makinoshima et al., 2014). Thereby highlighting that EGFR not only has a role within metabolism, but also that this can be modulated by targeting the RTK with small molecular inhibitors.

Due to the importance of metabolism in the pathology of cancer and other diseases, a number of techniques such as mass-spectrometry and nuclear magnetic resonance (NMR) have been used to

compare normal with diseased state and monitor disease progression and the development of drug resistance.

1.7.1 Mass-spectrometry

Mass-spectrometry (MS) can be coupled with liquid chromatography (LC), gas chromatography (GC) or high-performance liquid chromatography (HPLC) to generate metabolic data, but GC-MS has proven to have the highest resolving power (O'Hagan et al., 2007). However, a number of compounds cannot be analysed directly due to their polarity (Lenz and Wilson, 2007). Also, while it has a lower resolving power than GC-MS, LC-MS is often the method of choice due to the small amount of sample required for analysis and the minimal number of steps involved in sample preparation.

All MS machines proceed through 5 basic features to enable sample analysis: 1) The vaporised sample is injected into the machine; 2) The sample is ionised resulting in the formation of positive ions; 3) The positive ions are accelerated by an electric field; 4) The newly formed positive ions are deflected by a magnetic field. 4) Ions are then detected based on their mass/charge ratio. A basic overview of the MS machine is shown in **Figure 1.14**.

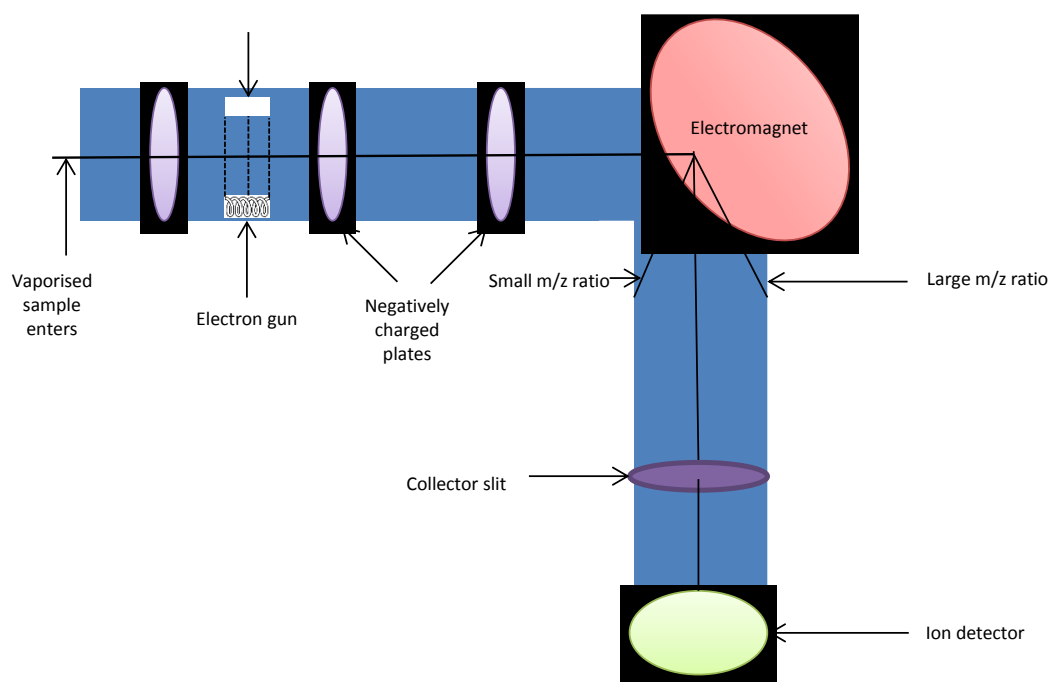


Figure 1.14 Schematic representation of a Mass spectrometer.

1.7.2 Nuclear magnetic resonance

NMR is a technique which detects molecules based on their magnetic properties (**Briefly summarised in figure 1.15**). A number of nuclei can be used for this purpose, including ^1H , ^{31}P , and ^{19}F as well as stable isotope labelled ^{13}C and ^{15}N . The most predominantly used is ^1H -NMR, which produces high resolution spectra and high sensitivity. The instrumentation used has vastly improved in recent years with the advent of high-field, ultra-shielded magnets and cryoprobes that have increased their sensitivity.

The technique exploits the proton spin and changes to its behaviour when a magnetic field is applied. Simplistically, when a compound is pulse-radiated, the low energy nuclei absorb the waves and flip to a higher energy level. The release of this energy leads a change in the magnetic momentum that is translated into a peak in the NMR spectrum. The amount of energy which is required to raise a proton to a higher energy level and the strength of the magnetic field experienced by the nucleus is dependent on the chemical environment of the nucleus itself. Therefore, the combination of peaks produced on a spectrum are characteristic of the proton's chemical environment and this can be used to identify the detected molecule.

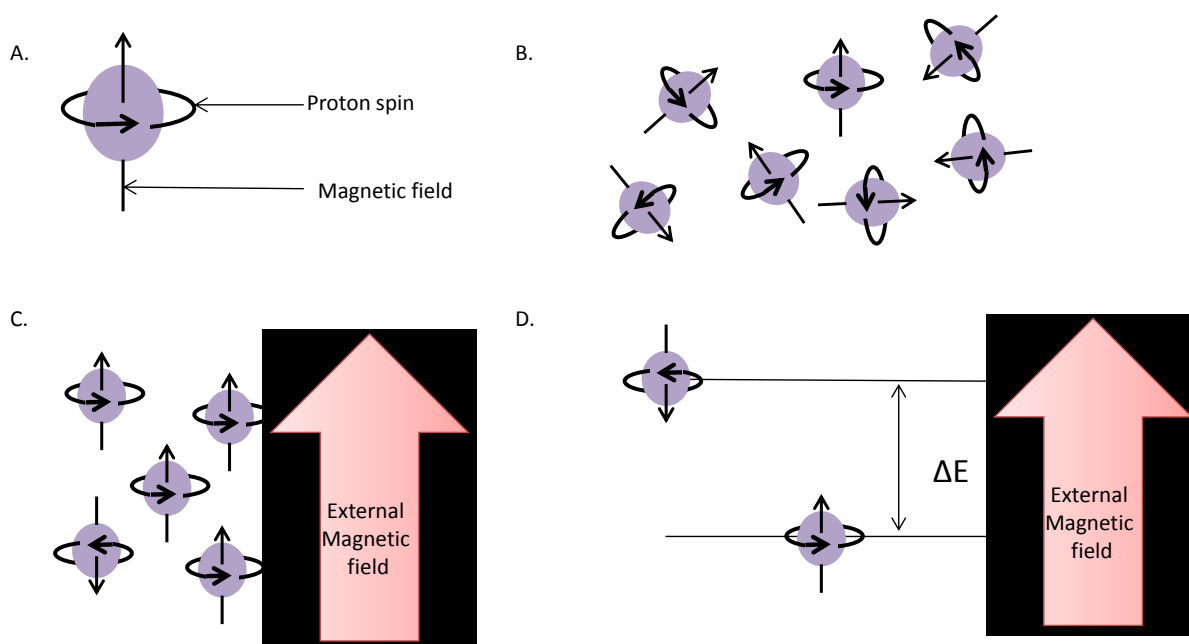


Figure 1.15 Hydrogen nuclei behaviour when external magnet is applied. (A) Hydrogen nuclei (protons) spin and thereby generate a small magnetic field. (B) Magnetic fields of spinning protons are arranged randomly in the absence of an external magnetic fields. (C) When a magnetic field is applied the majority of protons align with its direction but some oppose it. (D) Those which oppose the magnetic field have a higher energy than that which align with it.

1.7.3 Data analysis

Data analysis methods which are used in the comparison of disease vs normal or resistant to drug vs sensitive, use methods which are based on metabolomic fingerprinting. The most commonly used methods for this type of analysis include unsupervised, principal component analysis (PCA) and supervised methods such as partial least squares with discriminate analysis (PLS-DA), orthogonal PLS-DA and orthogonal partial least squares (Mao et al., 2009, Maher et al., 2007, Kinross et al., 2008, Cloarec et al., 2005, Nicholson et al., 1999). The use of these analysis methods enables the identification of features which contribute most to variations or separation between samples, prior to further analysis.

Unsupervised methods such as PCA, are commonly used in metabolic studies such as disease models and drug toxicity studies, where metabolites of interest are not known. PCA is an algorithm which reduces a high-dimensional data set to a smaller number of dimensions, to identify the dimensions responsible for the majority of the variation within the data set. Each of the principle components is a linear combination of the original variables. The principle components (PCs) are orthogonal (uncorrelated), with the first few containing the largest amount of variation, with each following PC containing and decreasing amounts of variation (Nicholson et al., 1999). Once the PCs have been identified they can be plotted on a PCA map. The data points generally appear as clusters on a PCA map, with the clusters representing a metabolic fingerprints. These fingerprints can then be used to identify potential biomarkers, which show the strongest variation. Despite the efficiency with which PCA can identify clusters and outliers, it is often only used to indicate future directions of data processing as its accuracy can be improved by further analysis with supervised methods.

Supervised analysis is commonly used to maximise separation between classes in order to identify robust biomarkers (Mao et al., 2009). Supervised methods include partial least squares with discriminate analysis (PLS-DA), orthogonal PLS-DA, and orthogonal partial least squares. PLS-DA utilises the produced spectra as well as descriptor (e.g. class) in n-dimensional space (Nicholson and Lindon, 2008). The presence of noise is an issue for this analysis method, as it can make it difficult to interpret data. In order to reduce the noise, which is common within biological samples, additional methods are needed such as orthogonal signal correction (OSC) to improve the integrity of the dataset as well as increase accuracy (Gavaghan et al., 2002, Griffin, 2006, Mao et al., 2009). Methods such as orthogonal PLS-DA is an extension of PLS-DA that integrates an OSC filter and models the variation to X and Y separately from uncorrelated variation (Bylesjö et al., 2006). This method produces a model which is easier to interpret.

There are a number of other methods which can be utilised dependent on the requirements of the chosen dataset. For example, geometric trajectory analysis can be used when tracking sequential time points on a PCA map in the case of disease progression tracking (Keun et al., 2004).

1.7 The Thesis

In this thesis, we hypothesize that acquisition of the second resistance mutation, T790M, in EGFR will induce specific metabolic changes that may constitute a signature for, and/or offer actionable mechanistic understanding of erlotinib resistance.

Here, we will:

- Firstly, determine if there are changes in metabolic pathways between erlotinib-resistant EGFR-T790M expressing and erlotinib-sensitive single activated mutant EGFR expressing NSCLC cell line pairs using ¹H-NMR and MS.
- Validate these changes *in vitro* and identify if modulation of the identified pathways alters response to erlotinib treatment.
- Identify if *in vitro* findings extend to the *in vivo* setting in animal models and/or in human tissue material.
- Use reverse phase protein array (RPPA) to identify signalling pathways changed in EGFR-T790M containing cells and attempt to link these to the previously highlighted metabolic changes.
- Validate the RPPA findings and identify if targeting of these pathways alters response to erlotinib treatment.

Chapter 2.0 Materials and methods

2.1 Material

Table 2.1 Equipment and software

Item	Company
7900 HT Fast-Real-Time PCR system	Applied Biosystems, UK
Adobe Illustrator CS6	Adobe Systems Incorporated, San Jose, CA, USA
Aire Laboratory CO2 Incubators	NuAire, USA
Aushon 1740 Arrayer Platform	Aushon
Avid nitrocellulose slide	Grace Biolabs
FACSCanto A flow cytometer	BD Biosciences, UK
FIJI ImageJ	NIH, USA
FlowJo version 7.6.5	Tree Star Inc., USA
Fusion SOLO Chemiluminescence Imager	PEQLAB, UK
FUSION-CAPT software	PEQLAB, Erlangen, Germany
GraphPad software	La Jolla, CA, USA
hybridisation cassette	ArrayIt Corporation
iBlot® Dry Blotting System	Invitrogen, UK
Image Studio Lite software	LI-COR Biosciences, Lincoln, NE, USA
Inopsys 710 slide scanner	Inopsys
Inverted research microscope IMT-2	Olympus, Tokyo, Japan
KONICA SRX-101A film processor	Konica Minolta
Microsoft Office	Microsoft, USA
Milli-Q® Integral Water Purification Systems	Merck Millipore Billerica, MA, USA
Mini-PROTEAN® 3 cell and Mini Trans-Blot module	Bio-Rad Laboratories LTD, UK
MP225 General Purpose GLP pH /mV / T Meter	Mettler-Toledo, Greifensee, Switzerland
Nalgene, Mr. Frosty	Sigma-Aldrich, St. Louis, MO, USA
NanoDrop 1000 Spectrophotometer	Thermo Fisher Scientific, UK
qbasePLUS 2.1 software	Biogazelle, Belgium
SDS v2.3	Applied Biosystems, UK
Sorvall® Legend RT centrifuge	Thermo Fisher Scientific, UK
Sunrise™ microplate absorbance reader	Tecan Trading AG, Switzerland
Veriti® Thermal Cycler	Applied Biosystems, USA
VORTEX GENIE 2	Scientific Industries, Bohemia, NY, USA

Table 2.2 Reagents

Item	Company
1 Kb Plus DNA Ladder	Thermo Fisher Scientific, UK
Acetic acid (glacial)	Thermo Fisher Scientific, UK
AllStars Negative Control siRNA	QIAGEN, UK
Ammonium persulfate (APS)	Sigma-Aldrich, Germany
Ampicillin	Sigma-Aldrich, Germany
Attractene Transfection Reagent	QIAGEN, UK
Avid Nitrocellulose slides	Grace Biolabs, US
Bio-Rad protein assay reagent	Bio-Rad Laboratories Ltd, UK
Bovine serum albumin (BSA)	Sigma-Aldrich, Germany
Bromophenol blue sodium salt	Sigma-Aldrich, Germany
Buthionine sulfoximine (BSO)	Sigma-Aldrich, Germany
Crystal violet	Sigma-Aldrich, Germany
DAF-FM probe	Thermo Fisher Scientific, UK
Dasatinib	Selleckchem
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Germany
Dithiothreitol (DTT)	Fluka Chemicals, Switzerland
Dorsomorphin	Selleckchem
Dulbecco's Modified Eagle's Medium (DMEM)	Sigma-Aldrich, Germany
EDTA-free protease inhibitor	Roche
Enhanced chemiluminescence system (ECL) reagent	Thermo Fisher Scientific, UK
Epidermal Growth Factor human (EGF)	Sigma-Aldrich, Germany
Erlotinib	Selleckchem
Ethacrynic acid (EA)	Sigma-Aldrich, Germany
Ethanol	Sigma-Aldrich, Germany
Ethylenediamine tetra acetic acid (EDTA)	Thermo Fisher Scientific, UK
Etomoxir	Tocris Bioscience
etomoxir	Tocris Bioscience
Fast SYBR® Green PCR Master Mix	Applied Biosystems, USA
Foetal Calf Serum (FCS)	First Link Ltd., UK
FUJIFILM High contrast X-ray film (blue sensitive)	FUJIFILM, Photon Imaging Systems Ltd, UK
GelDoc-It®2 Imager	UVP, USA
Gibco™ Opti-MEM™	Thermo Fisher Scientific, UK
Glycerol	Thermo Fisher Scientific, UK
Glycine (electrophoresis grade)	Sigma-Aldrich, Germany
High Capacity cDNA Reverse Transcription Kit	Applied Biosystems, USA
Hydrochloric acid 37%	Thermo Fisher Scientific, UK
Isopropyl alcohol	Thermo Fisher Scientific, UK
L-Glutamine GIBCO	Invitrogen, UK
Lipofectamine™ RNAiMAX	Invitrogen, UK
LY2603618	Eli Lilly
Methanol	Thermo Fisher Scientific, UK
Nitrocellulose iBlot® membranes	Invitrogen, UK

Item	Company
Nocodazole	Sigma-Aldrich, Germany
One Shot® TOP10 Chemically Competent E.coli	Invitrogen, UK
PageRuler™ Prestained Protein Ladder	Fermentas, UK
Phosphate buffered saline (PBS)	OXOID, UK
Poly-L-lysine	Sigma-Aldrich, Germany
Polyvinylidene difluoride (PVDF) iBlot® membranes	Invitrogen, UK
Protease inhibitor mix	Hoffmann-La Roche, Switzerland
ProtoGel® Acrylamide 30%	National Diagnostics, UK
PureLink® HiPure Plasmid Filter Maxiprep Kit	Invitrogen, UK
RNeasy® kit	QIAGEN, UK
RPMI 1640	Sigma-Aldrich, Germany
Skim milk powder	AppliChem GmbH, Germany
Sodium azide	Sigma-Aldrich, Germany
Sodium chloride	Thermo Fisher Scientific, UK
Sodium Dodecyl Sulfate (SDS)	Thermo Fisher Scientific, UK
Sodium fluoride	Sigma-Aldrich, Germany
Sodium orthovanadate	Sigma-Aldrich, Germany
Superblock T20	Thermo Fisher Scientific, UK
SYTOX® Green	Invitrogen, UK
TEMED (N,N,N',N'-tetramethylethylene diamine)	Sigma-Aldrich, Germany
Tris-(hydroxymethyl) aminomethane	VWR International, Belgium
Trypsin	Sigma-Aldrich, Germany
Tween 20	Sigma-Aldrich, Germany
VX970	Vertex Chemical

Table 2.3 Antibodies used in western blotting

Antibody	Company
ACC	Cell Signaling
AKT	Cell Signaling
AMPK	Cell Signaling
ATR	Santa Cruz
CHK1	Cell Signaling
ERK	Cell Signaling
HSP90	NeoMarkers
KEAP1	Proteintech Europe
Lamin	Santa Cruz
NRF2	Santa Cruz
P53	Santa Cruz
pACC	Cell Signaling
pAKT	Cell Signaling
PALB2	Novus
pATR	Cell Signaling
pERK	Cell Signaling
p-P53 (Ser15)	Cell Signaling
SQSTM1	Abcam
Tubulin	Sigma
WEE1	Cell Signaling
YES	Cell Signaling

2.2 Methods

2.2.1 Cell lines and cultures

PC9, PC9ER, H3255 and H1975 were kindly gifted from Julian Downward (Cancer research UK/London Research Institute, London, UK). The PC9, H3255 and H1975 gifted from Julian Downward were from the CRUK cell bank where they were authenticated and mycoplasma tested. The PC9ER cell line was made within Julian Downwards lab the method by which this was achieved is outlined in de Bruin *et al* 2014. PC9ER cells were generated by continued culturing of PC9 cells with 1µM erlotinib. The surviving clones were then picked and cultured in the presence of 1µM erlotinib for 3 months. Cells were authenticated and presence of the T790M mutation identified through DNA sequencing. HEK293 cells were purchased from ATCC. All cell lines used are listed below.

Table 2.4: Cell lines utilised in this study including details of the cell lines including their origin and culturing conditions.

Cell lines	Type	Culture	Medium
PC9	NSCLC, Human adenocarcinoma	Adherent	RPMI
PC9ER	NSCLC, Human adenocarcinoma	Adherent	RPMI
H3255	NSCLC, Human adenocarcinoma	Adherent	RPMI
H1975	NSCLC, Human adenocarcinoma	Adherent	RPMI
HEK293	Human Embryonic Kidney	Adherent	RPMI
HEK293 – EV	Human Embryonic Kidney	Adherent	RPMI
HEK293 – L858R	Human Embryonic Kidney	Adherent	RPMI
HEK293 – L858R + T790M	Human Embryonic Kidney	Adherent	RPMI

PC9, PC9ER, H3255 and H1975 cell lines were maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 50U/ml penicillin and 50 µg/ml streptomycin. PC9ER and H1975 were maintained with the addition of erlotinib (2µm) (Selleckchem) for 2 weeks, 1 month prior to experimental use. All 4 cell lines were kept at 5% CO₂ at 37°C in a humidified atmosphere. HEK293 cells stably transfected with empty vector, EGFR L858R, EGFR L858R + T790M were incubated at 5% CO₂ and 37°C. Dulbecco's Modified Eagle's medium (DMEM) was

used, supplemented with 20 % FCS, 3mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin and cell selection via puromycin treatment.

All cell lines were maintained at a sub-confluent density, typically passaged twice a week. This was achieved by initially washing the cells with phosphate-buffered saline (PBS) before incubation in 2.5 mg/ml trypsin in 0.02% EDTA, 0.09% NaCl at 37°C. Once cells have detached from the flask trypsin was inhibited by addition of culture media. Trypsin was removed by centrifugation and cells were passaged into new culture flasks at a suitable ratio. All cells were mycoplasma tested on a monthly basis to ensure the absence of contamination.

2.2.2 RNA interference

Cells were reverse transfected and therefore, plated at the time of transfection. The density of cells seeded is dependent on the format used for 6 well plates 110 thousand cells were used 1.5 million cells were used for 10 cm dishes. Cells transfected for use in survival assays were reseeded 24 hours (hrs) post transfection. Cells were then transfected with 20 nM siRNA (Dharmacon) using Lipofectamine™ RNAiMAX transfection reagent (Invitrogen), which was diluted in OptiMEM reduced serum medium (Invitrogen). In the case of a 6 well plate 0.17 µl siRNA and 2.5 µl Lipofectamine™RNAiMAX of was added to 250 µl OptiMEM. This solution was then incubated for 10 minutes (min) at room temperature to allow for complex formation. This was then added drop-wise to the well, giving a final volume of 2.5 ml. As a control for these experiments AllStars Negative Control siRNA (QIAGEN, Netherlands) was used. The sequences for siRNA used in this thesis are detailed in **table 2.5**.

Table 2.5 siRNA sequences

Supplier	Target gene	Dharmacon siGENOME siRNA ID	Sequence
ThermoFisher scientific	BHMT	D-008422-01	UGAAGGAGAUGCUUUGGUA
		D-008422-03	GAGUGAAACUGAAGUCAAA
		D-008422-05	GUUGAAAACCUUGAUAGCAU
		D-008422-19	GAGAAGAGGGGCUACGUAA
ThermoFisher scientific	CHDH	D-008123-01	GCAAGCGGCUCUCUGGAA
		D-008123-02	GGAAACAGGUGGGUUAUC
		D-008123-03	GAGUGGCUCUGGAAAUUCA
		D-008123-04	GGACAUCCAGUCCAUUUC
ThermoFisher scientific	CHKA	D-006704-01	GAAUUAAGUUUGCCAGUAU
		D-006704-03	GAGCAAACAUCGGAAGAU
		D-006704-04	GAAUACAGCAGUUACAAUU

Supplier	Target gene	Dharmacon siGENOME siRNA ID	Sequence
ThermoFisher scientific	CHKB	D-006704-17	UGAAAUGUCCUGCGGGAUA
		D-006705-01	GGAAUGGCCUUUCUACAAA
		D-006705-02	GUGAGUGGGUUUAUGAUUA
		D-006705-03	CCACGAAGAUGGCGCAAUU
		D-006705-04	CAUAGAAUUUGGUUACUUG
ThermoFisher scientific	CHPT1	D-009775-01	GAACUUAUCCUGACUGGUU
		D-009775-06	UUCAGGCAUGUUGAGAUU
		D-009775-19	GCGCUCAUUGGCAGACUUA
		D-009775-20	CUUCAUUUGAUUAGGUGAU
ThermoFisher scientific	EGFR-T790M	TMOSLR-002091	CCACCGUGCAGCUCAUCAUGCUU
		TMOSLR-002093	CCGUGCAGCUCAUCAUGCAGCUU
		TMOSLR-002095	UGCAGCUCAUCAUGCAGCUCAUU
		TMOSLR-002097	GCUCAUCAUGCAGCUCAUGCCUU
Qiagen	GCLC	Hs_GCLC_5	TAGGATCAGTAAATCCCATA
		Hs_GCLC_4	AAGGCTTTCTTTGGTAGGTAA
		Hs_GCLC_6	CCGGATCATATTTACATGGAT
		Hs_GCLC_7	CATCGACTTGACGATAGATAA
ThermoFisher scientific	GCLM	D-011670-19	CCGACAUUAUCUACUUAAA
		D-011670-20	GGUAUUCGGUCAUUGUGAA
		D-011670-21	GAAUGAAUGGAGUUCCTAA
		D-011670-22	GGCAGAUUUUCUUCGUCA
ThermoFisher scientific	GDPD1	D-018992-02	GAAGCGGUAAUAAUCGAGAA
		D-018992-03	CAACUGGGGUCAAUGUAAA
		D-018992-04	CCUAACUGCUCGAGGCAUU
		D-018992-17	CCUUUCAGCAUGCGGUUAA
ThermoFisher scientific	GPX1	D-008982-01	GCAAGGUACUACUUAUCGA
		D-008982-02	UGAAUUCCCUCAAGUACGU
		D-008982-03	GGAGAACGCCAAGAACGAA
		D-008982-04	GCAACCAGUUUGGGCAUCA
ThermoFisher scientific	GPX2	D-011675-01	GAAGCAGCAUCCUGUCUUC
		D-011675-02	GAAGGUAGAUUUCAAUACG
		D-011675-03	CAGGAGAACUGUCAGAAUG
		D-011675-04	GCAGGGCCGUGCUGAUUGA
ThermoFisher scientific	GPX3	D-006485-01	GUACGGAGCCCUACCAUU
		D-006485-02	GGAUGUCAAUGGAGAGAAA
		D-006485-03	AGGAAGAGCUUGCACCAUU
		D-006485-04	GAGGCUUUGUCCCUAAUUU
ThermoFisher scientific	GSR	D-009647-01	GAACACAGCUGUCCACUCU
		D-009647-02	GAUUAUGGCUUCCAAGUU
		D-009647-03	GAUACGGCAUGAUAAAGGUA
		D-009647-04	UCAAUUGGCGUGUUAUUAA
ThermoFisher scientific	GSS	D-009586-01	CAAAGAAGCUGGCAAGAUC
		D-009586-02	CGACGAACAUUUGAAGAU

Supplier	Target gene	Dharmacon siGENOME siRNA ID	Sequence
ThermoFisher scientific	GSTM1	D-009586-03	CUACAUGCCUCGUCAGUAC
		D-009586-04	GCAUCUACUUCGAACCAAA
		D-011118-01	CAGCUACAAUCCAGAAUUU
		D-011118-03	CAAUCCAGAAUUUGAGAAA
		D-011118-04	GGGCAUGAUCUGCUACAAU
ThermoFisher scientific	GSTpi	D-011118-14	GGCCAUGGUUUGCAGGAAA
		D-011179-01	GCAGGAGGGCUCACUCAA
		D-011179-02	CCUACACCGUGGUCUAUUU
ThermoFisher scientific	GSTpi	D-011179-03	ACGGAGACCUCACCCUGUA
		D-011179-04	GCUGAUCCAUGAGGUCCUA
ThermoFisher scientific	GSTZ1	D-011290-01	GGCAAUUGCUGAAAGAUUC
		D-011290-02	CCACUGAGCUGAGGGCCUA
		D-011290-03	GCACAGCGGGCAUUAUCUG
		D-011290-04	GCUUUAACGCCUGGAGCA
ThermoFisher scientific	KEAP 1	D-012453-01	GGACAAACCGCCUUAUUUC
		D-012453-02	CAGCAGAACUGUACCUGUU
		D-012453-03	GGGCGUGGCUGUCCUCAAU
		D-012453-04	CGAAUGAUCACAGCAAUGA
ThermoFisher scientific	LDHA	D-008201-02	GGCAAAGACUAUAAUGUAA
		D-008201-03	GAAGAGGCCCGUUUGAAGA
		D-008201-04	CUAGCUGGCUUGGAAGAU
		D-008201-17	UAAGGGUCUUUACGGAAUA
ThermoFisher scientific	NFE2L2	D-003755-01	GAGAAAGAAUUGCCUGUAA
		D-003755-02	CCAAAGAGCAGUUCAUGA
		D-003755-04	UAAAGUGGCUGCUCAGAAU
		D-003755-05	UGACAGAAGUUGACAAUUA
ThermoFisher scientific	PALB2	D-012928-01	GAAGCAACCUCUCCUCUUU
		D-012928-02	GAUAAUGACUUGUCUAGGA
		D-012928-03	GCAGUGAACUUACUACUCA
		D-012928-04	GAAGUCACCUCACACAAU
ThermoFisher scientific	PCYT1A	D-008642-01	GGAGAGGGUUGACAAAGUA
		D-008642-02	UGAAACAUAUGCUGAAAGA
		D-008642-04	GGAGAAGUCCCGAGAAUUC
		D-008642-17	UGUGAGAGUUUAUGCCGAU
ThermoFisher scientific	PCYT1B	D-009611-02	GGACAUCAUUAACCAGAAU
		D-009611-03	AAAGAGCCAUGAUCUAAU
		D-009611-17	GGAAAUAACUAAUGCCU
		D-009611-18	CUUAGUCACCUGUGCGAAA
ThermoFisher scientific	SiT790M 1	Custom Oligo	CCACCGUGCAGCUCAUCAUGCUU
ThermoFisher scientific	SiT790M 2	Custom Oligo	CCGUGCAGCUCAUCAUGCAGCUU
Dharmacon	WEE1	LU-005050-00-002-05	AAUAGAACAUCUCGACUUA

Supplier	Target gene	Dharmacon siGENOME siRNA ID	Sequence
Thermofisher scientific	YES	LU-005050-00-002-06	AAUAUGAAGUCCCGUAUA
		LU-005050-00-002-07	GAUCAUAUGCUUAUACAGA
		LU-005050-00-002-08	CGACAGACUCCUCAAGUGA
		D-003184-05	GAAGGACCCUGAUGAAAGA
		D-003184-07	UCAAGAAGCUCAGAUAAUG
		D-003184-08	CAGAAUCCCUCCAUGAAUU
		D-003184-09	GCGACUAGAGGUUAAACUA

2.2.3 Bacterial transformation and Plasmid preparation

The amplification of plasmids containing the chosen gene of interest was done by heat shock into chemically competent bacteria. One Shot® TOP10 Chemically Competent E.coli cells (Invitrogen) were used 2.5 µl of plasmid was mixed with 25 µl competent bacteria which were subsequently incubated once for 30 min. The bacteria were then placed into 42°C for 30 seconds before being placed back on ice. The transformed bacteria were then mixed with 250µl of Super Optimal broth Catabolite (SOC) repression media for 1 hour (h) at 37°C to allow cells to recover. The bacteria cell suspension was then added to 250 ml of Luria-Bertani (LB) broth containing 100 µg/ml Ampicillin which was then grown overnight at 37°C while shaking. Plasmid was amplified and extracted from bacteria cultures using the PureLink® HiPure Plasmid Filter Maxiprep Kit (Invitrogen) carried out by following the manufacturer's instructions.

2.2.4 Plasmid transfections

HEK-293 cells were used for plasmid transfections using the DNA transfection reagent Attractene (QIAGEN) following manufacturers guidelines. The amount of DNA varied dependent on the size of the dish used for transfection. In the case of 15 cm dishes 15 µg of plasmid was used. Plasmid transfection reagent for 15 cm dishes contained 37 µl of Attractene reagent with 1 ml of OptiMEM (Thermo Fisher) and 10 µg of plasmid and was incubated for 10 mins at room temperature. Once the complexes had formed they were added drop-wise to the plate. The overexpression was then measured at 24 hrs post transfection. The Negative control for the plasmids used in this thesis was pBABE as this was the backbone for EGFR L858R and EGFR L858R + T790M plasmids.

2.2.5 Cell proliferation and viability assay

Cells were seeded 24 hours post transfection into clear 96 well plates. If required cells were treated with increasing concentration of drug e.g. Erlotinib (Selleckchem). Forty-eight hours following treatment cells were washed twice with Phosphate buffered saline (PBS) prior to staining and fixation with 25% methanol solution containing 0.08% crystal violet solution for 30 minutes. The plates were then washed in water to remove the excess crystal violet and then air dried. Quantitative data was obtained by solubilisation of crystal violet precipitates in 10% (v/v) acetic acid while gently shaking at room temperature for 30 min. The intensity of the staining was then measured with the use of the sunrise (TECAN) absorbance microplate reader at the absorbance 595 nm. The mean and standard error of the mean (SEM) of each independent experiment was then calculated. Each treatment response to drug was then normalised to the vehicle control with each condition present with at least three biological repeats.

2.2.6 Cell extracts preparation

Cells were washed using cold PBS and then placed on ice. The cells were then lysed with the use of radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCL pH 7.4, 0.5% (v/v) NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM Na₃VO₄ and cOmplete and Mini, EDTA-free protease inhibitor cocktail (Roche)). Samples were then incubated on ice for 10 minutes. Samples were then centrifuged at 16.2 RCF for 20 min at 4 °C to remove DNA and the protein lysates were then placed into fresh tubes. The protein concentration was then quantified using Bradford assay following the manufacturer's instructions. The samples were then normalised to a typically protein concentration of 1 µg/ul and the addition of Laemmli buffer [0.25 M Tris HCl, pH 6.8, 8% (w/v) SDS, 40% glycerol, 0.042% (w/v) bromophenol blue, 10% β-mercaptoethanol] followed by boiled at 95 °C for 5 min prior to use or short term storage at -20°C.

2.2.7 SDS PAGE and western blotting

The mini-PROTEAN II gel electrophoresis system (Bio-Rad) was used. The stacking gels were prepared with 5% stacking gel ((v/v) acrylamide, 0.125 M Tris, pH 6.8, 0.1% (w/v) SDS, 0.075% (v/v) ammonium persulphate (APS) and 0.083% (v/v) Tetramethylethylenediamine (TEMED)) and a 10% acrylamide resolving gel (10% (v/v) acrylamide, 0.375 M Tris pH 8.8, 0.1% (w/v) SDS, 0.06% (v/v) APS, 0.07% (v/v) TEMED). When lower molecular weight proteins are required 12 % acrylamide resolving gel was used (12% (v/v) acrylamide, 0.375 M Tris pH 8.8, 0.1% (w/v) SDS, 0.06% (v/v) APS, 0.07% (v/v) TEMED). Electrophoresis was performed using 1x SDS-PAGE running buffer (2.5 mM Tris, 0.2 M Glycine and 0.1% (w/v) SDS) at 100 mV until the dye front

has reached the bottom of the gel. In order to identify protein size PageRuler™ Plus Prestained Protein Ladder (Fermentas) was resolved alongside the protein samples.

The gels were electrophoretically transferred onto nitrocellulose membranes (Invitrogen) using the Invitrogen iBlot® Dry Blotting System (Invitrogen). Once transferred the membranes were blocked for 1-2 h at room temperature in Tris-buffered saline/Tween (TBST) (0.01 M Tris, pH7.4, 75 mM NaCl, 1.25 mM EDTA, and pH 8.0, 0.1% (v/v) Tween 20) containing either 5% (w/v) fat-free milk or 5% bovine serum albumin (BSA). The membranes are then incubated overnight in primary antibody diluted in 5% (w/v) BSA/TBST, containing 0.04% sodium azide, or if specified by the manufacturer, 5% (w/v) fat-free milk/TBST. The membranes were then washed four times in TBST to remove unbound antibody. HRP-conjugated secondary antibody was then diluted in 5% (w/v) fat-free milk/TBST and the membrane subsequently incubated for 1 h at room temperature. The membranes are then washed 4 times with PBST at room temperature over the course of 1 h. The secondary antibody is then detected using enhanced chemiluminescence (ECL) solution (Thermo-fisher Scientific), according to the manufacturer's instructions. The luminescence was then detected via imaging the chemiluminescence using Fusion Solo Chemiluminescence Imager (PEQLAB) or by exposure of the membranes to X-Ray film (FUJIFILM) then developing the film using a Konica SRX-101A film processor.

2.2.8 Quantitative Real-time PCR (qPCR)

2.2.8.1 RNA extraction

RNA was extracted from cells using the RNeasy® Mini kit (QIAGEN) following the manufacturer's protocol. The cells were lysed in 350 µl RLT buffer and scraped from the plate and then mixed with equal volume of 70% (v/v) ethanol before being loaded into the RNeasy column and centrifuged at 13,000 g for 15 seconds. The bound RNA then washed initially by 700 µl of RW1 buffer and then twice with 500µl of RPE buffer. The column was then spun at high speed for 1 min to remove excess buffer remaining in column. The RNA was then eluted in RNAase-free water.

The concentration of RNA extracted was then quantified using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). The RNA was then transcribed by High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer protocol as follows. A 20 µl reaction containing 1 x RT Buffer, 1 x Deoxynucleotide (dNTP) mixture, 1 x RT Random primers, 1 µl MultiScribe™ Reverse Transcriptase (50 U/µl) and 1 µg of RNA were

combined. Veriti® Thermal Cycler (Applied Biosystems) was used for reverse transcription. The program selected was as follows: Initially 10 min at 25°C followed by 120 min at 37°C. This allowed for the reverse transcription of the RNA into cDNA. The final step results in the deactivation of the reverse transcriptase by heating to 85°C for 5 min. The samples were then stored at -20°C for long term storage or 4°C for short term.

2.2.8.2 Quantitative Real-time PCR (qPCR)

The levels of gene expression were quantified using cDNA obtained from RNA conversion, which was subsequently analysed using quantitative real-time (qPCR) on the Applied Biosystems 7900HT Real-time PCR System. An estimated total of 1 µg of cDNA (1:1 ratio of conversion assumed) was obtained from the conversion, was then diluted to 2 ng/ul with the use of nuclease-free water. A 20 µl reaction mixture was then made of 5 µl of diluted cDNA, 10 µl 2x Fast SYBR® Green PCR Master Mix, 0.6 µl of both forward and reverse qPCR primers (5 µM stock) with the addition of 3.8 µl nuclease-free water. qPCR was then carried out on an Applied Biosystems 7900HT Real-time PCR System with the thermal cycler conditions were as follows: Initial activation of 20 sec at 95 °C, 40 cycles; denaturation for 1 min at 95 °C; annealing / extension for 20 sec at 60 °C; final melting curve was carried out for 15 sec at 95 °C and then 15 sec at 60 °C. Every reaction was present in triplicate. The quantification of gene expression was performed using qbase+ software. The housekeeping genes used were hypoxanthine phosphoribosyl-transferase (HPRT), TATA Box Binding Protein (TBP) and Vinculin. Analysis of relative gene expression was done in qbasePLUS 2.1 software (Biogazelle) with the use of the 2(-Delta Delta C (T)) method (Livak & Schmittgen 2001). The sequences of all primers used in this thesis are in **table 2.6**.

Table 2.6 Primer sequences

Gene	Protein	Forward primer	Reverse primer
GSS	glutathione synthetase	Qiagen primers (sequences unavailable)	
GSR	glutathione reductase		
GCLc	glutamate-cysteine ligase, catalytic subunit		
GPX1	Glutathione peroxidase 1		
GSTPi	Glutathione S-transferase pi 1		
GCLm	Glutamate-cysteine ligase modifier subunit		
GPX3	glutathione peroxidase 3		

GPX2	glutathione peroxidase 2		
GGT	Gamma-Glutamyltransferase 1		
GSTzi	Glutathione S-transferase zeta		
GSTm1	Glutathione S-transferase m1		
EGFR	Epidermal growth factor receptor		
ACTB	Beta actin	TTCTACAATGACTGCGTG	GGGGTGTGAAGGTCTCAA
CHKA	Choline kinase alpha	GAGGACGAGTTCCACATCATG	ATACAGCCGCAGGAGCACTTTC
CHKB	Choline kinase beta	GGTCCAGTTTACGGCGATAC	CCTCTCATGGTGAACAGCAA
CHPT1	Cholinephosphotransferase 1	CTTCTGGCTTGTTTCCCATC	CATCTCCTACTGTCCCACGG
HPRT	Hypoxanthine-guanine phosphoribosyltransferase	Qiagen primers (sequences unavailable)	
KEAP1	Kelch-like ECH-associated protein 1	CAGATTGGCTGTGTGGAGTT	GCTGTTCGCAGTCGTAATTG
NRF2	Nuclear Factor, Erythroid 2-Like 2	GAGAGCCCACTCTTCATTGC	TGCTCAATGTCCTGTTGCAT
PCYT1A	phosphate cytidyltransferase 1, choline, alpha.	GTTCTTCCAAAGTGCAGCGCT	AGGAGTTCCTCTGCTGGCTTCT
PCYT1B	phosphate cytidyltransferase 1, choline, beta	ACCAACTGCCAGTGTCAAGCAC	TGCATGACCTGAGTGGAAGAGG
PEMT	Phosphatidylethanolamine N-methyltransferase	CTCTAAGCGTCACCATCCTGCT	GGTGTCCAGGCTCTCCATCCT
PLA2G15	Phospholipase A2 Group XV	ACCAACTGCCAGTGTCAAGCAC	TGCATGACCTGAGTGGAAGAGG
PLA2G6	Phospholipase A2 Group VI	CCTCATCGTGTTCCGAGCAGAA	CACGGTTCTCAGCAGAGTCAAG
PLD1	Phospholipase D1	TCCTGAAACGCCCACTGGTTGA	ATGCCAAGAGCGAGTTCCACTCT
PLD2	Phospholipase D2	TGCCACAGCAGCAAAGTAAC	CCCAGGCAGAGACTTCCTAC
SQSTM1	Sequestosome 1	CTGGGACTGAGAAGGCTCAC	GCAGCTGATGGTTTGGAAT
TBP	TATA box binding protein	TGCACAGGAGCCAAGAGTGAA	CACATCACAGCTCCCCACCA
VCL	Vinculin	CTTTGCTGCTACAGGGGAAG	GGATAGGGACGGGAAGTT

2.2.9 Glutathione (GSH) colourmetric assay

Cells were seeded into 6 well plates 24 hours prior to collection at a density of 160,000 cells per well. As additional positive and negative controls, cells were treated for 24 hours with either 50

μ M ethacrynic acid (EA) or 40 μ M buthionine sulfoximine (BSO). Cell lysates were collected and then sonicated in 50 mM PBS and 1 mM EDTA. The concentration was determined using Bradford assay according to the manufacture's protocol and protein was subsequently normalised to 1 μ g/ μ l of protein. The samples GSH levels were then quantified using an adapted version of manufacturer's protocol (BioAssay systems). In summery the basis for this assay is the use of reagent A which is a modified 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) method which combines deproteination and detection into one reagent. The DTNB solution is able to react with reduced glutathione to form a yellow product. Samples were prepared by the addition of 120 μ l of cell lysates with 120 μ l of buffer A. Then 200 μ l of this solution (lysates + buffer A) was vortexed until homogenous. The addition of 100 μ l of buffer B was added and then incubated for 25 minutes. Samples were then placed into a clear bottom 96 well plate. The blank was prepared by adding 100 μ l of 50 mM PBS and 1 mM EDTA with 100 μ l of reagent A and 100 μ l of reagent B. The calibrator which acts as a positive control is prepared by the addition of 200 μ l of distilled water to 100 μ l of calibrator. The plate was then read at an OD412nm using the Sunrise™ microplate absorbance reader (Tecan).

2.2.10 Reverse Phase Protein Array

2.2.10.1 Sample preparation

The cells were lysed on ice for 20 min using cold lysis buffer (1% Triton X-100, 50mM HEPES, pH 7.4, 150mM NaCl, 1.5mM MgCl₂, 1mM EGTA, 100mM NaF, 10mM Na pyrophosphate, 1mM Na₃VO₄, 10% glycerol, containing freshly added protease and phosphatase inhibitors (Roche)). The cell lysates were then collected post centrifuged at 12,000 g for 10 minutes at 4°C.

2.2.10.2 Microarray layout

The protein lysates were spotted at four dilutions 2.0mg/ml (4/4), 1.0mg/ml (3/4), 0.5mg/ml (2/4), 0.25mg/ml (1/4) in PBS with 10% Glycerol in arrays consisting of 20 x 20 spots at a 250 μ m spot-to-spot distance. The array was carried out using Aushon 1740 Arrayer Platform using 8 x 85uM pins, two rounds of spotting were carried out. The sample dilution series were carried out in triplicate on each array, on each 1 pad Avid nitrocellulose slide (Grace Biolabs). Sample dilution series were spotted in triplicate on each array, with 1 array per pad, on 1 pad Avid Nitrocellulose slides (Grace Biolabs).

2.2.10.3 Assay procedure

After slides had been spotted the chips were blocked with superbloc T20 (Thermo Fisher) buffer. Each individual array was separated using a hybridisation cassette (ArrayIt Corporation). The arrays were incubated with primary analyte specific antibody in superbloc buffer overnight at 4°C. A list of antibodies which were used in this study is detailed in **appendix table 8.1**. The slides were then washed with TBS-Tween three times for 10 minutes. The slides were then incubated with fluorescence Dylight-800-labeled anti-species antibodies in superbloc for 1 hour at room temperature in the dark. The slides were then washed three times with TBS-T. The slides were then washed with distilled water and spun to remove excess moisture. The arrays were then imaged using Inopsys 710 slide scanner. In order to identify non-specific signals one array omitted primary antibody from the first incubation step for one array.

2.2.10.4 Data analysis

Microarray images were analysed using Mapix software (Inopsys). The spot diameter was set to 120 µm. Fluorescence intensity for each spot on the array was measured. The linear fit was found using the fluorescent signal from each dilution series of all samples on the array. The flag system was then used to analyse samples where $R^2 > 0.9$ was deemed good, >0.8 was deemed acceptable and <0.8 was considered poor and therefore excluded from further data analysis. The median values from the 4 point dilution series were calculated using the measure of fluorescence intensity. The sample data was then grouped using a GAL file and the mean and standard deviation values were calculated for all samples across the triplicates. The mean data for each of the array is then normalised to the total amount of protein which is identified from fastgreen stain. The nonspecific signal values are then subtracted from each of the samples.

2.2.11 FLOW cytometry analysis of nitric oxide species

PC9 and PC9ER cells were seeded into a 6 well plate, 200,000 cells per well and allowed to adhere overnight. The cells were then washed with cold PBS and then trypsinized at 37°C in the presence of 5% CO₂. The trypsin was neutralised with RPMI and cell suspension was then washed with cold PBS three times to remove any RPMI present. The cells were then incubated with 10 µM DAF-FM for 1 hour at 4°C. The DAF-FM probe is a cell permeable probe which was for quantify the levels of nitric oxide (NO) present within each sample. The probe is non-fluorescent until it reacts with NO to form a fluorescent benzotriazole. As a negative control unstained cells for each cell line were used. The excess DAF-FM was removed by washing 5

times with cold PBS. The samples were then analysed on FACSCanto A flow cytometer and FlowJo version 7.6.5 (Tree Star Inc.).

2.2.12 DNA isolation

Cells were seeded into 6 well plates 200,000 cells per well and allowed to adhere overnight at 37°C in the presence of 5% CO₂. The cells were then washed with cold PBS and then trypsinized until all cells were detached. The trypsin was neutralised with RPMI and cell suspension was then washed and then resuspended in cold PBS three times to remove any RPMI present. In order to extract DNA from the cells QIAamp® DNA Mini and Blood Mini kit (Qiagen) was used the protocol is as followed. The cell suspension was then added to RNase and DNase free microcentrifuge tubes containing 20 µl of proteinase K. The cells were then lysed using 200 µl of AL buffer and pulse vortexed for 15 seconds. The cells were then Incubated at 56°C for 10 min. Ethanol was then added to the lysates the samples were then again mixed by pulse-vortexing. The solution was then added to QIAamp Mini spin column and centrifuged at 6,000 g for 1 min. The buffer AW1 was added to the QIAamp Mini spin column and it was then centrifuged for 1 min at 6000g. The buffer AW2 was then added to the QIAamp Mini spin column and then it was centrifuged at 20,000 g. The spin column was then centrifuged at full speed for 1 minute to remove excess buffer from the spin column. The DNA was then eluted in 200 µl of AE buffer.

2.2.13 PCR

PCR was performed on genomic DNA isolated using QIAamp® DNA Mini and Blood Mini kit as described as above or plasmid DNA. The reaction mixture was as follows: 1 x Phusion HF, 200 µM dNTPs, 0.5 µM forward Primer, 0.5 µM reverse Primer, 200 ng of Template DNA or 10 ng plasmid DNA, Phusion DNA Polymerase 0.5 µl per 50 µl reaction and distilled RNase and DNase free water. The PCR reaction mixture was then run on Veriti Thermal Cycler the program run is as follows: Initial denaturation step of 98°C for 30 minutes, followed by 35 cycles of a denaturing steps at 98°C for 10 sec, annealing temperature is primer T_m dependent within the range of 45-72°C, 72°C extension step for 30 seconds per kb. Finally a last denaturation step at 72°C for 10 minutes the product can then be stored at 4°C.

2.2.14 DNA gel electrophoresis

Samples were run on a 1 % Agarose gel in Tris-acetate-EDTA (TAE) buffer (0.04 M Tris - Acetate 0.001 M EDTA) in replacement of the commonly used ethidium bromide, SYBR® Safe DNA Gel Stain (Thermo Fisher) was used at a 1:10,000 dilution. Gel Loading Dye (NEB) was added to each

of the sample prior to loading and Quick-Load® 1 kb DNA Ladder used (NEB). The gel was run at 90 volts (V) until the dye front was 80 % of the way down the gel. The gel was then imaged on the GelDoc-It®2 Imager (UVP, USA). Samples were then purified and sequenced using Beckman Coulter Genomics (Takeley, UK).

2.2.15.1 Mass spectrometry (Metabolon)

PC9, PC9ER, H3255 and H1975 cells were seeded into 15 cm 37°C in the presence of 5% CO₂. The cells were then washed with cold PBS and then trypsinized until all cells were detached. The trypsin was neutralised with RPMI and cell suspension was then washed and then re-suspended in cold PBS three times to remove any RPMI present. The samples were then sent to Metabolon (Durham, US) for MS analysis. The methods for which have been explained briefly in a number of publications (DeHaven et al., 2010, Evans et al., 2009, Evans et al., 2012). Metabolon platform utilises both (Ultra Performance Liquid Chromatography) UPLC/MS and GC/MS. The spectra which are then produced by these techniques are compared to an in-house library. These libraries include data such as retention time, reference spectra from mass scan as well as fragmentation of molecules. The initial chromatography is used to separate metabolites followed by electro spray ionization the newly charged molecules then pass through the spectrometer. Metabolon uses of both GC-MS and UPLC-MS, which use chromatography for separation prior to MS in order to identify a large number of metabolites (Lawton et al., 2008). GC-MS combines gas chromatography with mass spectrometry using electro ESI-MS/MS. Molecular mass can then be detected by electron impact ionization and chemical ionisation. The use of chemical ionization has shown to be more successful in providing information to identify molecules of unknown mass. UPLC-MS is an improvement on the capabilities of liquid chromatography mass spectrometry. It is extremely powerful as it has high sensitivity and selectivity and as such it has been used in many applications (Ardrey, 2003). When compared to GC, UPLC is not as sensitive as a separation technique, however, it requires limited sample preparation (Wilson et al., 2005). Both separation techniques are useful for the separation of different substrates. In this study Metabolon identified 395 metabolites of several classes of molecules including amino acid, peptides, carbohydrate, energy, lipid, nucleotide, cofactors and vitamins, and xenobiotics.

The cell lysates were treated with methanol, shaken for 2 minutes, followed by centrifugation. The extract was divided into three parts UPLC-MS/MS where analysis was based on positive ions, UPLC-MS/MS where analysis was based on negative ions and one for GC-MS analysis. Experimental controls consisted of water samples for blanks and a standard spiked into every

analysis to allow instrumental monitoring. The samples as well as controls were randomized through the run. The UPLC/MS utilised ESI and the instrument was set up to identify positive ions from acidic extracts or negative ions from basic extracts. The extracts are then loaded into columns with the addition 95% methanol containing 0.1% formic acid or 6.5mM ammonium bicarbonate.

GC-MS was dried under a vacuum pump for a minimum of 18 hours prior to derivatized in dried nitrogen. The derivatized samples were then separated on a 5% phenyldimethyl silicone column with helium used as a gas carrier. The temperature ramp was 60° c to 340° c across a 17 minute period. Samples were then analysed with a resolving power using electron impact ionization and a 50-750 atomic mass unit scan range.

The metabolites were subsequently analysed by an automatic comparison with experimental samples to the reference library based on retention time, molecular weight, and in-source fragments (Suhre et al., 2011). The identification of metabolites was based on comparison to the mass spectrometry library of more than 2,400 purified standards. The concentrations of the analysed metabolites are reported as relative concentrations.

2.2.16 ¹H NMR

2.2.16.1 Extraction of the intracellular metabolites

The cells were 1×10^7 cells per condition were trypsinized and washed three times in ice-cold PBS prior to the extraction of metabolites. The cells were then re-suspended in 0.6 ml cold H₂O with methanol solution (1:2). The cells were then put under three freeze-thaw cycles and then sonicated in an ice bath for 15 minutes in 1 min pulses followed by 1 min rests. The samples were then centrifuged at 3200 g at 4°C for 10 min. The samples were then freeze-dried followed vacuum-driven methanol evaporation. The lyophilized samples were then stored at -80°C.

2.2.16 ¹H NMR profiling

The freeze-dried intracellular metabolites were extracted by dissolving in 600 µl of phosphate buffer 0.1 M (pH 7.4, 99.9% D₂O) containing 0.001% sodium 3-trimethylsilyl-1-[2,2,3,3-²H₄] propionate (TSP). The samples were then centrifuged at 12,000 g at 4°C for 10 min, the samples were then vortexed and the supernatant was then transferred to 5 mm NMR tubes for NMR detection. The 1D ¹H-NMR spectra were acquired using Bruker AVIII 600 MHz NMR spectrometer which is equipped with a cryogenic probe (BrukerBiospin, Germany) at 298 K. The first increment of Nuclear Overhauser Effect Spectroscopy (NOESY) pulse sequence with continuous wave irradiation on the water peak during recycle delay and mixing time for water

suppression was employed. The recycle delay of 2 sec and mixing time of 100 ms were set. The 90° pulse was adjusted to 10 µs approximately and 64 scans were then collected into 32,000 data points with the spectral width of 20 ppm. In order to assign metabolites a number of 2D-NMR spectra were used including ^1H - ^1H COSY, ^1H - ^1H TOCSY, ^1H J-resolved, ^1H - ^{13}C HSQC and ^1H - ^{13}C HMBC in a process previously described by Dai and colleagues (Dai et al., 2010) .

2.2.16.1 NMR data analysis

Using the software AMIX package (v3.8, BrukerBiospin) the spectral region from δ 0.5 to 9.5 was then integrated into bins with the width of 0.002 ppm. Each of the bin areas were normalised to the total area of the respective spectrum. The multivariate data analysis was performed using software SIMCA-P+ (v 12.0, Umetrics, Sweden). This was done using orthogonal projection to latent structure-discriminant analysis (OPLS-DA) 34 with Pareto variance (Par) scaling and 7-fold cross validation. The parameter R2X was the variation of X which is explained by the model. The predictability of the model is represented by Q2. In order to assess the validity of the model was made confirmed by CV-ANOVA ($p < 0.05$). Using an in-house script within MATLAB 7.1 to assist the biological interpretation generated from the models. The loadings were back-transformed and then plotted with colour- coded OPLS-DA coefficients. The colour code corresponded to the absolute value of the OPLS-DA coefficients ($|r|$), which indicated the contribution of each variable to explain the intergroup differentiation. The value of $|r|$, greater than 0.602, was considered to be significant ($n = 10$, $p < 0.05$).

2.2.17 Animal experiment

A total of 5×10^6 PC9ER cells were injected subcutaneously into the flank of BALB/c nude mice. The tumours were grown until they reached 100 mm³. The mice were then randomised into 3 groups of 10. The mice were then intraperitoneal injected with 25 mg/kg/day Erlotinib/0.5% w/v methylcellulose and/or 6 mg/kg/day ethacrynic acid/1% Tween 80 in distilled water. The treatments were administered daily from day 7 to day 26. The tumours were measured using caliper and the tumour volumes were then calculated using the following equation $V = \frac{1}{2} \times L \times W^2$ (Length, W; width of tumour).

2.2.18 Tissue mRNA extraction and qPCR

The origin of tissues and techniques used are as previously reported (de Bruin et al., 2014). In short, samples were obtained from EGFR-mutant lung adenocarcinoma patients with acquired erlotinib resistance under Human Investigations Protocol #111000928 (Yale Cancer Center, New

Haven, CT). Those were reviewed by a pathologist to ensure adequate tumour content. Tumour areas were circled and micro dissection performed to enrich for tumour content.

2.2.19 Tissue mRNA extraction and RNA-Seq

The Illumina TruSeq RNA Sample Preparation Kit was used for RNA tissue extraction and analysis done as previously described (Huang et al., 2012). A brief outline will follow: Total mRNA was converted into a library of template molecules. Sequence reads were then generated using Illumina HiSeq 2000 with TruSeq v3 reagent kits and software. The sequences were then mapped to the human reference genome using TopHat (Trapnell et al., 2009), followed by generation of a list of total number of uniquely mapped reads for each gene present using open-source tool HTSeq-count (v. 0.5.3p3) available from the European Molecular Biology Laboratory (EMBL). The determination of differentially expressed genes was determined using R package DESeq (Wang et al., 2010a). The method used to identify genes which were differentially expressed was MA-plot-based method with technical Replicates (MATR).

2.2.20 Subcellular fractionation

Cells were seeded onto 15cm dishes and left until cells were 80-90% confluent. The cells were then washed with cold PBS and lysed in a fractionation lysis buffer (10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH7.6, 3mM MgCl₂, 40mM KCl, 5% glycerol, 1% Triton X-100, protease and phosphatase inhibitors) on ice for 10 min. The cell lysate were centrifuged at 2,500 rpm at 4°C for 5 min to collect the cytoplasmic fraction (supernatant). The pellet was washed in a fractionation wash buffer (10mM HEPES pH7.6, 1.5mM MgCl₂, 10mM KCl, protease and phosphatase inhibitors) and centrifuged at 2,500 rpm at 4°C for 5 min. The pellet was re-suspended in a fractionation extraction buffer (20mM HEPES pH7.6, 1.5mM MgCl₂, 420mM NaCl, 25% glycerol, 0.2mM EDTA, protease and phosphatase inhibitors) and probe sonicated for 15s on ice. The lysate was then centrifuged at 13,300 rpm at 4°C for 10 in to collect the nuclear fraction (supernatant). Then the samples were processed the same way as in SDS-PAGE and western blotting section mentioned above.

2.2.21 Statistical Analysis

All data was normalised to the relevant control values. The error which is displayed unless otherwise stated represents +/- the standard error of the mean (SEM). GraphPad was used to carry out all statistical analysis other than MS and NMR. In the case of only two data sets Students T-Test was performed (two sample equal variance; two tailed distribution) and

statistical significance was determined by a p value which was lower than 0.05. When required and differences are calculated for more than two data sets the significance was determined using ANOVA, Bonferroni's multiple comparison test. The degrees of significance is noted as follows; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, ****, $P < 0.0001$.

**Chapter 3.0 Metabolomic studies identified
increased phosphatidylcholine degradation in
erlotinib resistant cell lines**

3.1 Introduction

Hanahan and Weinberg have described changes in metabolism or deregulated cellular energetics to be a hallmark of cancer (Hanahan and Weinberg, 2011). A well-documented feature of neoplastic transformation is alterations in metabolic pathways that facilitate survival (Hsu and Sabatini, 2008, Hanahan and Weinberg, 2011). The highly proliferative nature of cancer cells results in the continuous need for carbohydrates, amino acids and fatty acids to synthesise DNA, RNA, protein and lipid to increase biomass. Cancer cells have an increased rate of nutrient consumption but also need to maintain a pool of substrates which are required for de novo synthesis. In addition to the well-known 'Warburg effect', re-programming in lipid metabolism is an important underlying feature of malignant growth (Menendez and Lupu, 2007). Cancer cells require fatty acid for the synthesis of membranes but also for the generation of lipid signalling molecules which enhance cell proliferation.

A number of lipid metabolites have been detected to have increased levels in cancer cells including phosphocholine (Eliyahu et al., 2007, Nakagami et al., 1999, Glunde et al., 2011). Phosphocholine is a precursor of membrane lipid synthesis and is modified in the Kennedy pathway to result in the formation of Phosphatidylcholine, a major component in biological membranes. It has been suggested to be utilised in cancer to satisfy the demand for lipids in highly proliferative tissue (Trousil, 2014). In addition to this, increase in phosphocholine and other lipid membrane precursors may have additional implications within oncogenic development, as well as, disease progression (Baenke et al., 2013). A number of enzymes involved in the phosphatidylcholine synthesis pathway (Kennedy Pathway) have been associated with drug resistance, including choline kinase (Janardhan et al., 2006). Choline kinase is the first enzyme within the phosphatidylcholine synthesis pathway and converts choline into phosphocholine. It has been known to be over-expressed in a number of cancers including lung, prostate and breast (Shah et al., 2010, Ramirez de Molina et al., 2007, Challapalli et al., 2015). In both lung and breast cancer choline kinase alpha over-expression has been demonstrated to correlate with poor prognosis and disease progression (Shah et al., 2010, Glunde and Bhujwalla, 2007). However, the mechanism(s) by which choline kinase displays this phenotype are relatively unknown.

The degradation pathway for phosphatidylcholine has also been implicated in cancer biology. Phosphatidylcholine is hydrolysed by phospholipase enzymes, which results in the production of a number of second messenger molecules such as phosphatidic acid (PA) and diacylglycerol

(DAG). Moreover, degradation of phosphatidylcholine by phospholipase D (PLD) that results in the formation of the second messenger PA, which has been shown to induce DNA synthesis and cell proliferation (Knauss et al., 1990). Therefore, it is not surprising that PLD has been proposed as a novel therapeutic target in cancer treatment (Bruntz et al., 2014). Interestingly, EGF stimulation activates phospholipase D (PLD) and therefore, may have a prominent role within EGFR driven cancers (Fisher et al., 1991). The production of the second messenger DAG in phosphatidylcholine hydrolysis is predominantly associated with phospholipase C (PLC). Increased levels of DAG result in the intracellular re-localisation and activation of protein kinase C (PKC) (Sharkey et al., 1984). It has long been established that PKCs have a role within carcinogenesis with activation of PKC-alpha and beta isoenzymes have frequently being associated with the malignant phenotype (Koivunen et al., 2006). However, it is important to note that some PKC have anti-cancer properties such as PKC-delta (Koivunen et al., 2006). The degradation of phosphatidylcholine does not only produce second messenger molecules but also results in the formation of free fatty acids (FAs). Huang and Freter suggest that FAs have a role in a number of signalling pathways including the activation of adenosine monophosphate (AMP)-activated protein kinase, extracellular signal-regulated kinase (ERK), GPCR signalling, Toll-like receptor 4/NF- κ B, Src-JNK, and PKC signalling, as well as, sphingomyelinase-ceramide signalling (Huang and Freter, 2015).

A number of studies have utilised metabolomics to identify possible erlotinib resistance mechanisms. Indeed, Fan and colleagues utilised Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS)-based metabolomic analysis to study lung lesions (Fan et al., 2009). In this work they identified changes in phospholipid metabolism and suggested metabolic analysis could be used to differentiate early stage erlotinib sensitive and insensitive NSCLC (Fan et al., 2009).

In this thesis our studies relate to the development of erlotinib resistance driven by the T790M additional mutation, occurring in 50% of NSCLC that become resistant to first-line EGFR TKIs such as erlotinib. In view of the importance of changes in metabolism associated with oncogenic development, progression and the development of drug resistance, we have utilised ^1H -NMR to study the metabolic profiles of erlotinib-resistant and sensitive cell line pairs. This identified a number of altered metabolic pathways in erlotinib-resistance cells. One of the most prominent pathways identified was the phosphatidylcholine pathway. There was a significant increase in both choline and phosphocholine levels in erlotinib-resistant cell lines. Therefore, we used RNA

interference to target key enzymes within this pathway and assess how this impacted on erlotinib resistance. However, targeting these enzymes did not affect the survival of our cell lines to erlotinib treatment. As mass spectrometry is known to have higher sensitivity compared to NMR, it was subsequently used to identify additional changes which may occur within this pathway. A number of key metabolites in phosphatidylcholine degradation pathway were identified to be increased within resistant cell lines. However, modulation of key enzymes in this pathway by RNA interference also failed to alter survival to erlotinib treatment.

3.2 ¹H-NMR-based metabolic profiling identified changes in a number of metabolites in erlotinib resistant cell lines.

Two erlotinib resistant/sensitive cell line pairs were utilised for metabolic analysis. The first pair is made of the isogenically matched PC9 (erlotinib sensitive) and PC9ER (erlotinib resistant) cells that harbours the Δ E746-A750 deletion mutation in EGFR with the additional EGFR/T790M mutation in the PC9ER cell line. The second cell line pair is made of the H3255 (erlotinib sensitive) and the genetically unrelated H1975 (erlotinib resistant) cell lines, sharing the L858R activating mutation, but with an additional T790M mutation in the H1975 cells. The response of the two cell line pairs to erlotinib was assessed by performing cell survival assays (**Figure 3.1A and B**). As expected, PC9 and H3255 showed significantly greater sensitivity to erlotinib compared to their resistant counterparts. It was previously suggested that T790M-mediated EGFR resistance was due to an increased affinity for ATP. This would thereby infer that treatment with irreversible inhibitors such as 324674 should be able to restore killing in resistant cells. However, both PC9ER and H1975, continued to display significant erlotinib resistance in the presence of 324674 compared to their respective sensitive counterparts (**Appendix figure 8.1**). This suggests that additional resistance mechanisms, distinct from altered ATP competition, might be involved in the resistance of T790M-containing cell lines to erlotinib.

Changes in metabolism have frequently been implicated in tumour progression and drug resistance in many cancer types. Therefore, we used metabolic profiling to identify these additional resistance mechanisms. One-dimensional ¹H-NMR analysis of aqueous cell extracts from all 4 cell lines was performed. Thirty six metabolites were identified (**Figure 3.1C**) and each were assigned a corresponding number as shown in **appendix Table 8.2**. Orthogonal projections to latent structure discriminant analysis (OPLS-DA) showed clear separation between the erlotinib resistant and erlotinib sensitive cell lines (**Figure 3.2**). This highlighted statistically significant changes in the levels of 14 metabolites, in both cell line pairs, between erlotinib-sensitive and resistant cell lines, including glutathione, amino acids, nucleotides as well as choline metabolites (**Table 3.1**).

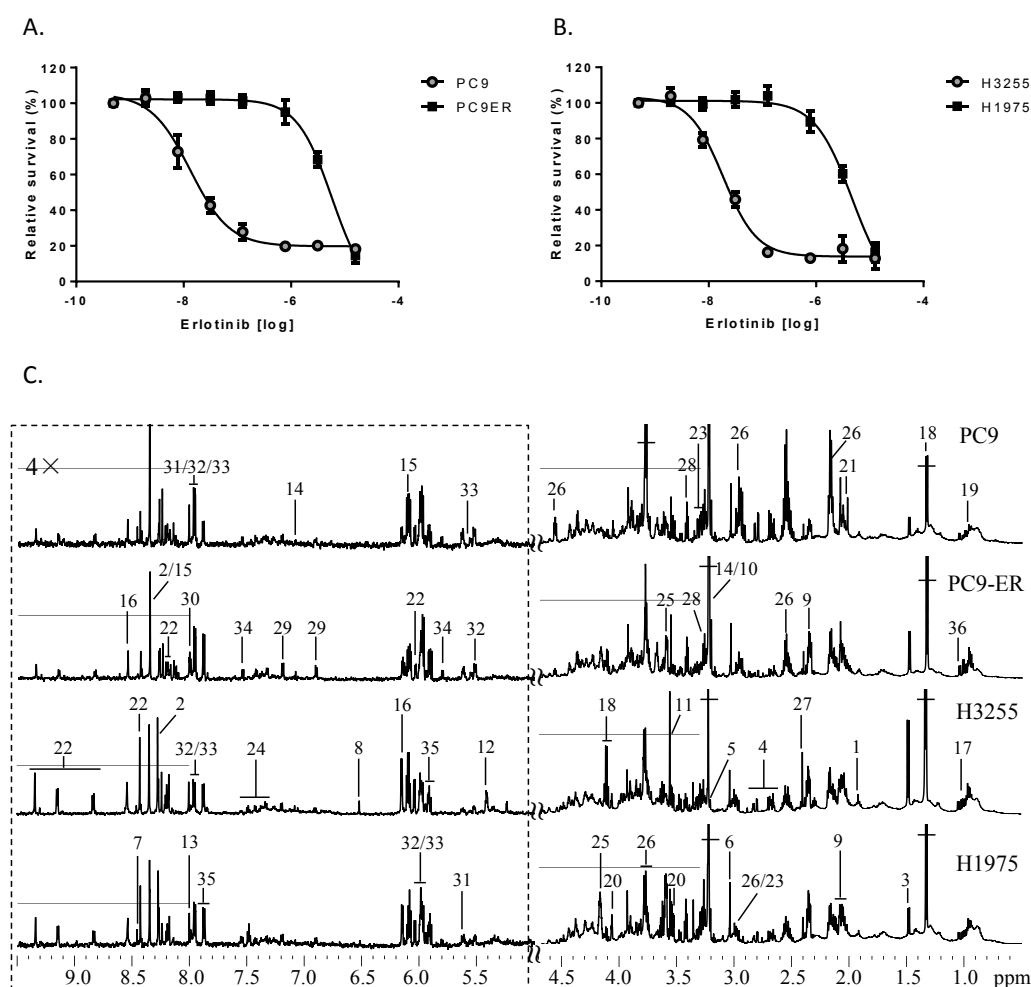


Figure 3.1: Erlotinib resistance confers an altered metabolite profile. Cell survival was measured in response to increasing concentrations of erlotinib treatment (48 hours) and quantified by crystal violet staining **(A)** PC9 and PC9ER **(B)** H3255 and H1975. Figures represent the mean of 3 independent biological experiments $\pm$ SEM. **(C)** Typical 600 MHz ^1H -NMR spectra of aqueous extracts from PC9, PC9ER, H3255 and H1975 cells. The region enclosed in the dashed box (δ 5.0-9.5) is vertically expanded four times ($\times 4$). Data is representative of 10 independent experiments. The identification of peak numbers is shown in **Appendix table 8.2**. ^1H -NMR analysis was carried out by Hongde Li at the Chinese Academy of Sciences, China.

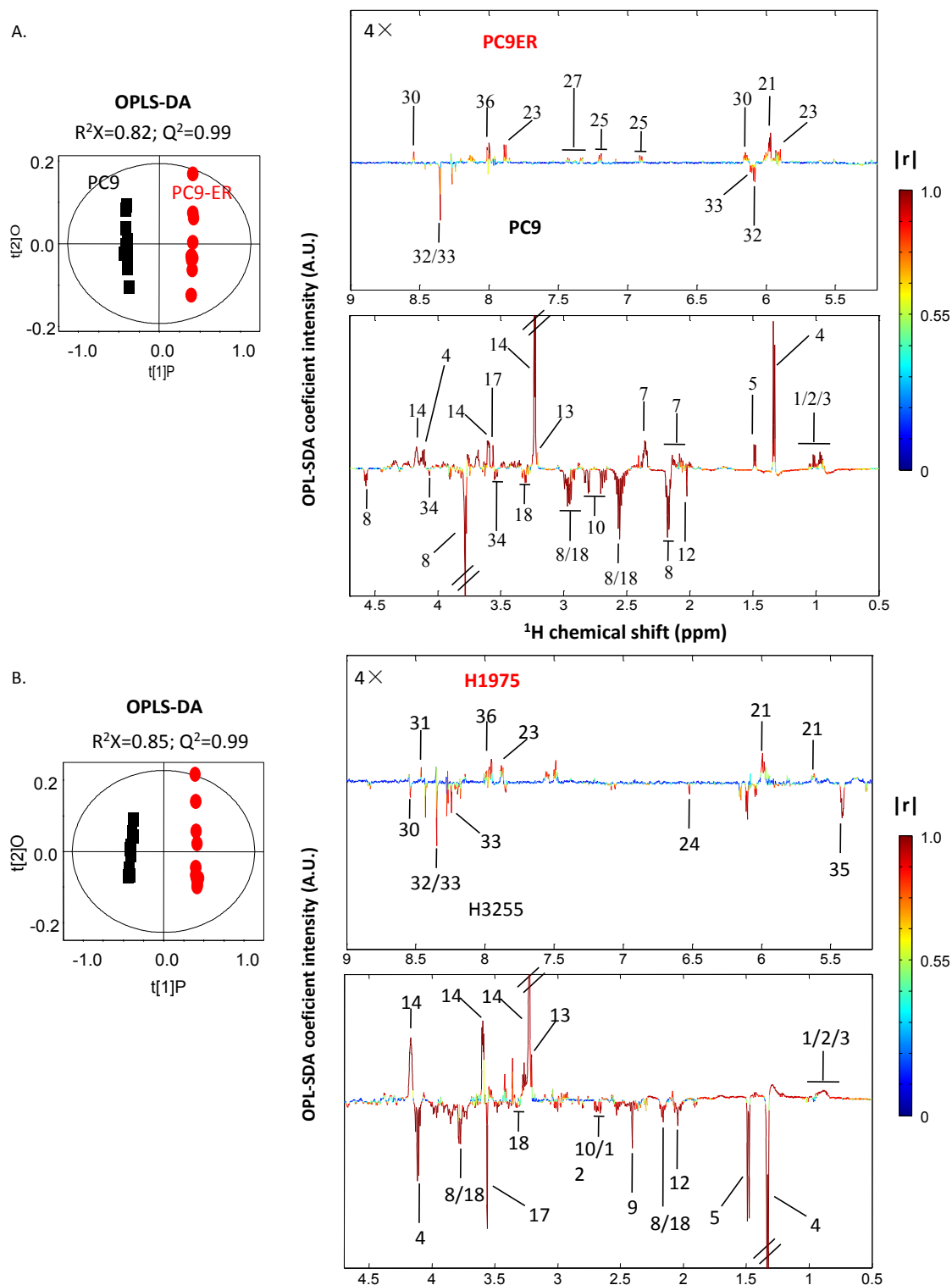


Figure 3.2: Orthogonal projections to latent structures discriminant analysis (OPLS-DA) demonstrated clear distinction between erlotinib resistant and erlotinib sensitive cell lines. OPLS-DA score plots showing (left) and coefficient plots (right) for ^1H -NMR spectra of aqueous cellular extracts from (A) PC9 and PC9ER (B) H3255 and H1975. Models validated by CV-ANOVA, (A) $p=2.36 \times 10^{-17}$ and (B) $p=3.04 \times 10^{-19}$. The Q^2 is 0.99 for both models. The colour scale for coefficient plots reflects the differences in the contribution of metabolite variations between groups. $|r|$ cut-off value is 0.602 ($n=10$, $p<0.05$). The identification of peak numbers is shown in **Appendix table 8.2**. ^1H -NMR analysis was carried out by Hongde Li at the Chinese Academy of Sciences, China.

Table 3.1: ¹H-NMR analysis of Metabolites that had consistently altered abundance between erlotinib resistant and erlotinib sensitive cell lines. Metabolites identified from ¹H-NMR which were similarly regulated in both cell line pairs (erlotinib-sensitive vs resistant cells). Key refers to the peaks labelled on **Figure 3.1C** and **Figure 3.2**. ¹H-NMR analysis was carried out by Hongde Li at the Chinese Academy of Sciences, China.

Key	Metabolite	Variation tendency	Chemical shift	Coefficient PC9 vs ER	Coefficient 1975 vs 3255
2	Adenosine	Decreased	6.087	0.81	0.72
4	Aspartate	Decrease	2.805	0.99	0.96
5	Choline	Increase	3.211	0.71	0.95
15	Inosine	Decrease	6.111	0.86	0.93
17	Isoleucine	Increase	1.019	0.99	0.87
19	Leucine	Increase	0.961	0.99	0.80
21	N-acetyl aspartate	Decrease	2.023	0.99	0.99
23	GSSG	Decrease	3.327	0.90	0.79
25	Phosphocholine	Increase	3.227	0.98	0.99
26	GSH	Decrease	4.571	0.96	0.69
30	UDP	Increase	7.995	0.96	0.93
31	UDPG	Increase	5.607	0.72	0.87
35	Uridine	Increase	5.909	0.87	0.68
36	Valine	Increase	1.053	0.97	0.87

3.3 Targeting of phosphatidylcholine synthesis pathway does not affect erlotinib resistance.

NMR identified the levels of both choline and phosphocholine as being significantly increased in erlotinib resistant cell lines. **Figure 3.3** shows a schematic representation of the phosphatidylcholine pathway, highlighting metabolites that were increased with erlotinib resistance (red text) with key synthesizing (white boxes) and catabolising (grey boxes) enzymes. The basal levels of a number of key enzymes were assessed by qPCR in both erlotinib resistant and sensitive cell line pairs to gain an insight as to which enzymes might be key to the increased levels of choline and phosphocholine in the erlotinib resistant cells. In addition, the enzymes were silenced using siRNAs to access if their modulation affected sensitivity to erlotinib treatment and qPCR was performed to ensure adequate knockdown. A number of these enzymes will be discussed in the following section.

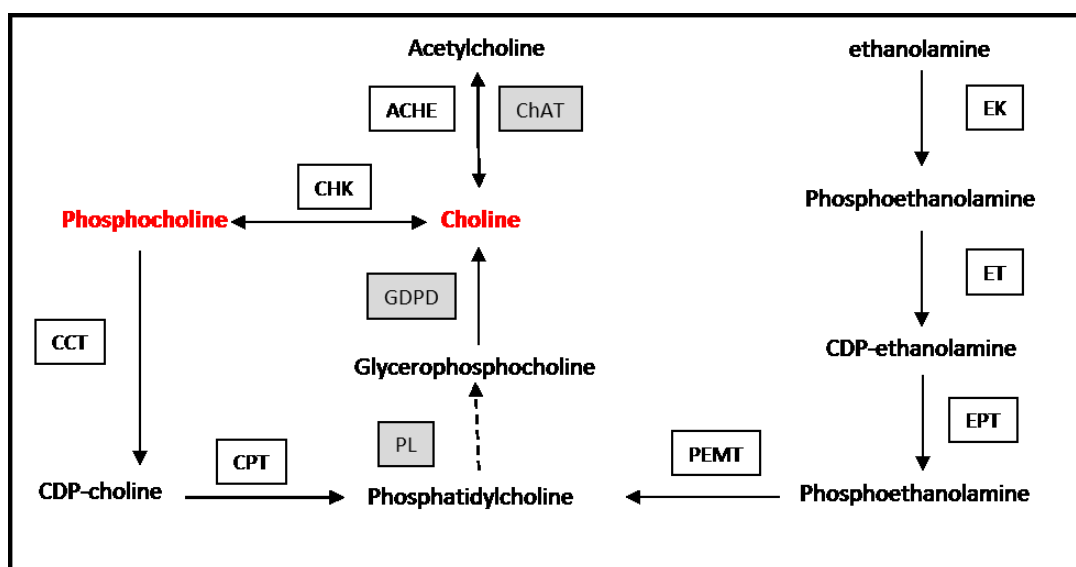


Figure 3.3: Simplified schematic representation of the Phosphatidylcholine metabolic pathway. Choline, phosphocholine are highlighted in red, as they showed a significant increase in the resistant cell line based on the ^1H NMR analysis. Enzymes are denoted in white and grey boxes for synthesising enzymes and degradation enzymes respectively. Acetylcholine is converted to choline by acetylcholinesterase (ACHE). This reaction can be reversed by choline acetyltransferase (ChAT). Choline is phosphorylated by choline kinase (CHK) to form phosphocholine. Phosphocholine is converted to CDP-choline by the enzyme CTP:phosphocholine cytidyltransferase (CCT) the rate limiting enzyme within this pathway. Choline phosphotransferase (CPT) the last enzyme in the Kennedy pathway leads to the formation of Phosphatidylcholine. Phosphatidylcholine can also be formed by the alternative PEMT pathway. Ethanolamine is phosphorylated in an ATP-dependent manner by ethanolamine kinase (EK) to form Phosphoethanolamine. Phosphoethanolamine is then converted to CDP-ethanolamine by Phosphorylethanolamine Transferase (ET). Ethanolamine:1,2-diacylglycerol ethanolamine-phosphotransferase (EPT) then catalyses the production of phosphoethanolamine from CDP-ethanolamine. Finally, Phosphoethanolamine is then converted to phosphatidylcholine by the transferase enzyme phosphatidylethanolamine N-methyltransferase (PEMT). Phosphatidylcholine can be converted into glycerophosphocholine by a number of phospholipase (PL) enzymes. Glycerophosphocholine is then hydrolysed to produce choline by the enzyme Glycerophosphodiester phosphodiesterase (GDPD).

3.3.1 Choline kinase altered mRNA expression in erlotinib-resistant H1975 cell line

Choline kinase (CHK) is the enzyme which catalyses the first reaction in the phosphatidylcholine biosynthesis pathway, leading to the formation of phosphocholine. There are three known isoforms of CHK which include CHK α -1, CHK α -2 and CHK β (Aoyama et al., 2004). These are encoded by two different genes choline kinase alpha (CHK α) and choline kinase beta (CHK β). The different isoforms are thought to have different functions.

CHK α is primarily associated with phosphorylation of both choline and ethanolamine. CHK α levels were shown to have a decreased mRNA expression in PC9ER compared to PC9 (**Figure 3.4A**). However, H1975 had an increased mRNA expression compared to H3255 (**Figure 3.4B**). Despite inconsistent changes at the mRNA level between cell line pairs, changes in the levels/activity of the enzymes may still occur at a translational level. Hence, we used siRNAs to target CHK α in our cell lines. H3255 could not be tested owing to its very low transfection efficiency. PC9, PC9ER and H1975 cells were transfected with siRNAs against CHK α and survival to erlotinib treatment was assessed by crystal violet assay (**Figure 3.4 C, D and E**). The knockdown of CHK α was confirmed by qPCR (**Figure 3.4F, G and H**). Despite obtaining an efficient knockdown for CHK α in all cell lines, downregulation of this enzyme did not modulate the sensitivity to erlotinib treatment.

CHK β is the enzyme which catalyses the phosphorylation of ethanolamine but not choline. The mRNA expression of CHK β showed no significant difference between PC9 and PC9ER cells (**Figure 3.5A**). However, there was a significant increase of around 6 fold in the mRNA levels of CHK β in H1975 compared to H3255 cells (**Figure 3.5B**). However, despite the increase at the mRNA level, siRNA-mediated knockdown of this enzyme did not change cellular survival to erlotinib treatment (**Figure 3.5C, D and E**). The knockdown was confirmed by qPCR (**Figure 3.5F, G and H**). CHK α -1, CHK α -2 and CHK β are reported to be active when they are in hetero/homo and oligomeric form (Aoyama et al., 2004). Therefore a double knockdown was performed for CHK α and CHK β (**Appendix figure 8.2**). However, this also failed to modulate the response to erlotinib treatment.

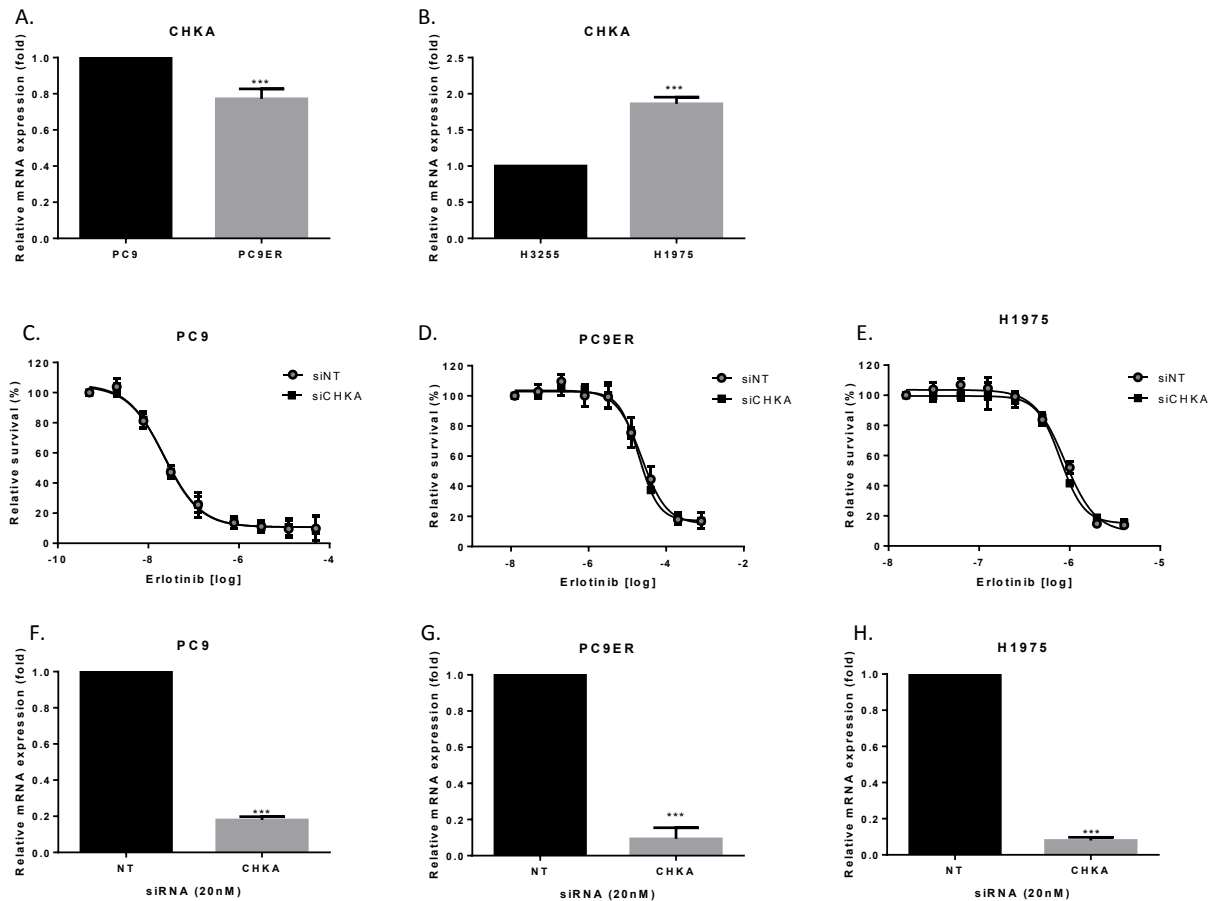


Figure 3.4: Effect of silencing CHKA on cell survival to erlotinib treatments. (A,B) Basal mRNA levels of CHKA were quantified by qPCR in both cell line pairs. (C) PC9, (D) PC9ER and (E) H1975 were transfected with siRNA against CHKA or the non-targeting (NT) control. Twenty-four hours post transfection cells were treated with increasing concentrations of erlotinib (48h) prior to fixation and crystal violet staining. The obtained values were normalised against the DMSO control. siRNA efficiency was confirmed by qPCR in (F) PC9, (G) PC9ER and (H) H1975. Figures represent the mean of three biological repeats with each replicate done in technical triplicate ($\pm$ SEM). Statistical analysis was done using Student's *t*-test. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

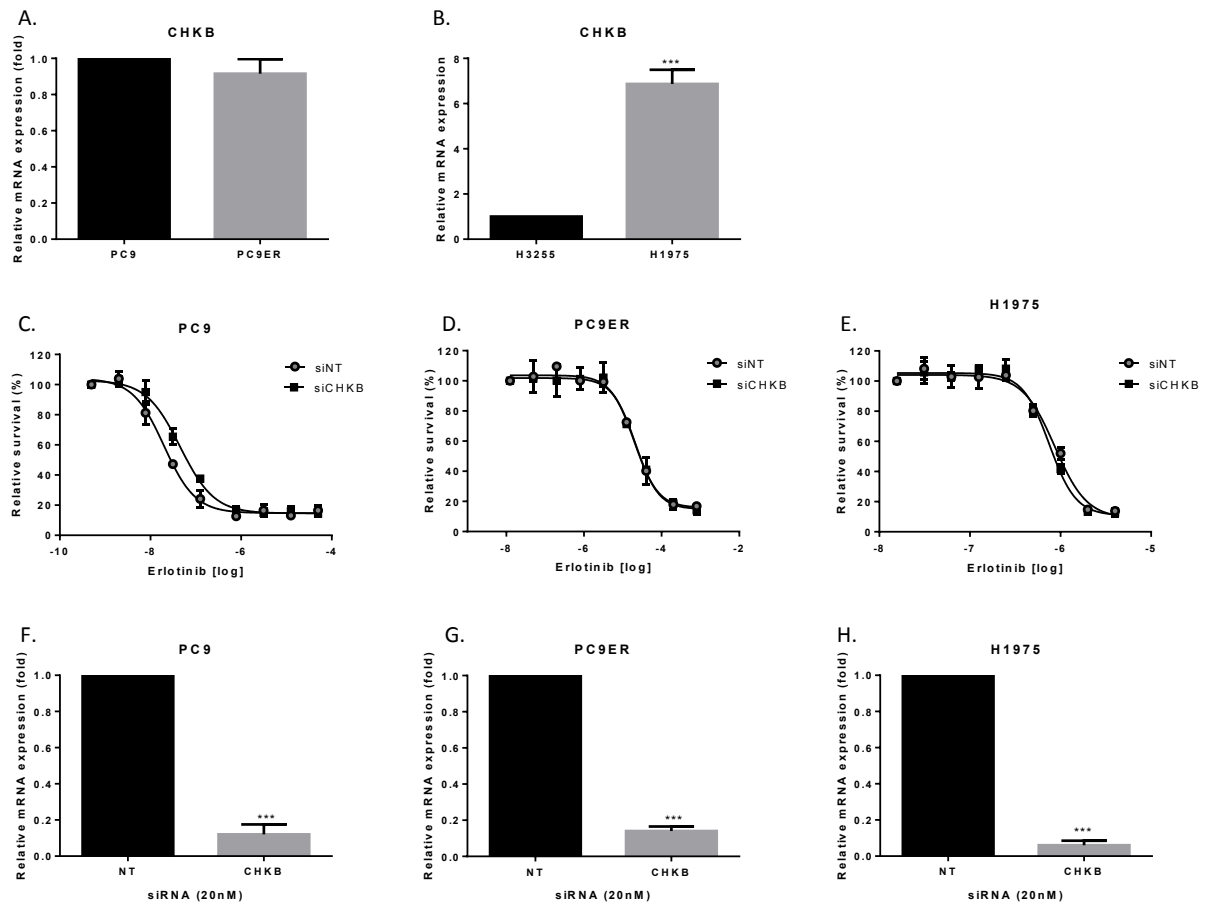


Figure 3.5: Effect of silencing CHKB effect on cell survival to erlotinib treatment. (A, B) Basal mRNA levels of CHKB were quantified by qPCR in both cell line pairs. (C) PC9, (D) PC9ER and (E) H1975 were transfected with siRNA against CHKB or the NT control. Twenty-four hours post transfection cells were treated with increasing concentrations of erlotinib (48h) prior to fixation and crystal violet staining. The obtained values were normalised against the DMSO control. siRNA efficiency was confirmed by qPCR in (F) PC9, (G) PC9ER and (H) H1975. Figures represent the mean of three biological repeats with each replicate done in technical triplicate ($\pm$ SEM). Statistical analysis was done using Student's *t*-test. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$

3.3.2. Phosphate cytidyltransferase 1 (PCYT1) does not affect survival to erlotinib treatment.

PCYT1 catalyses the formation of CDP-choline and diphosphate from Cytidine triphosphate (CTP) and choline phosphate. There are 2 known isoforms of this gene, PCYT1A and PCYT1B. The basal mRNA levels of both PCYT1A (**Figure 3.6A**) and PCYT1B (**Figure 3.7A**) showed no significant difference between PC9 and PC9ER cells. However, there was a significant increase in transcript levels of both PCYT1A (**Figure 3.6B**) and PCYT1B (**Figure 3.7B**) in the erlotinib-resistant H1975, when compared to the sensitive H3255, cells. We therefore, tested if this transcriptional upregulation was involved in the response to erlotinib using siRNAs to target either PCYT1A (**Figure 3.6C, D and E**) or PCYT1B (**Figure 3.7C, D and E**) in PC9, PC9ER and H1975 cells. Unfortunately, despite efficient knockdown of the genes (**Figure 3.6F, G, H and Figure 3.7F, G and H**), no significant change in sensitivity to erlotinib treatment was observed in any of the cell lines.

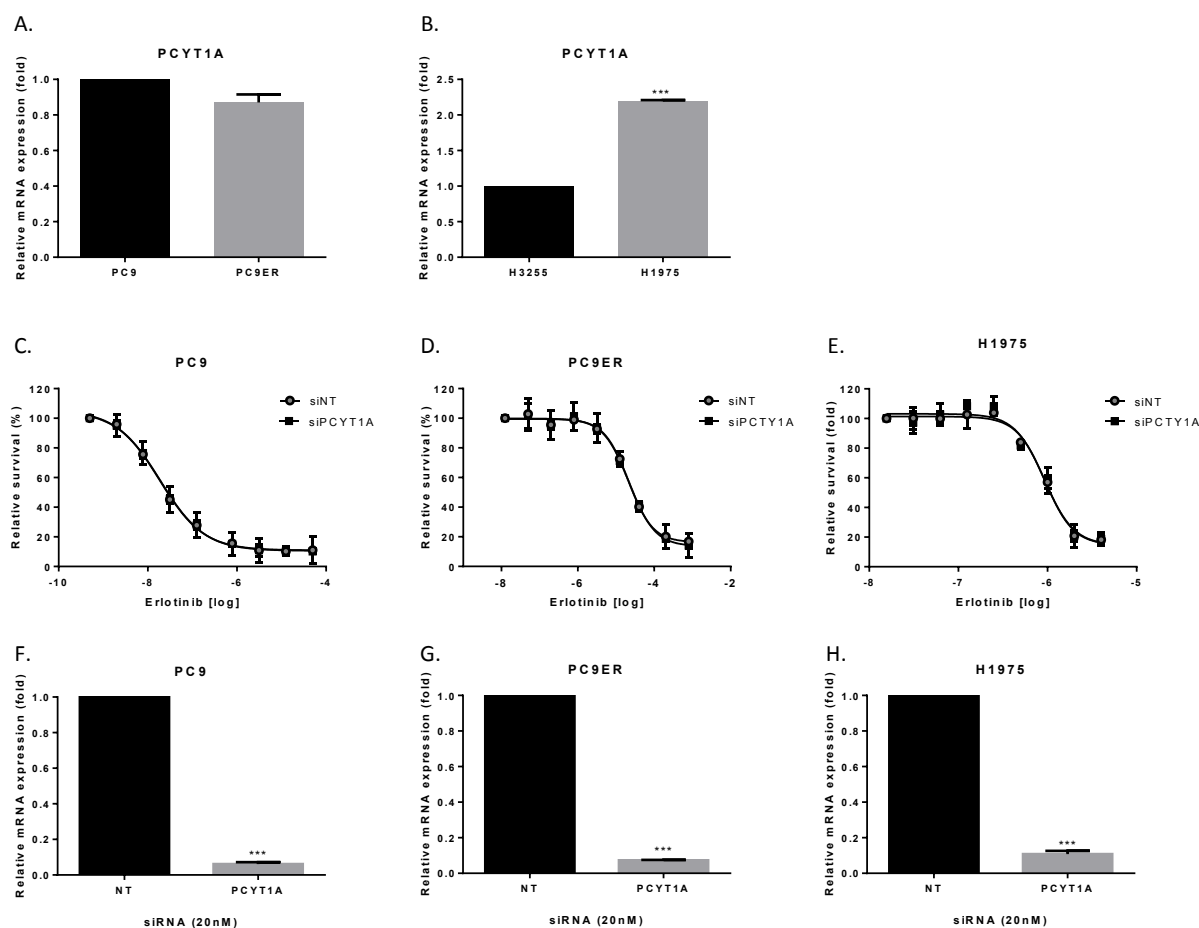


Figure 3.6: Effect of silencing PCYT1A on cell survival to erlotinib treatments. (A, B) Basal mRNA levels of PCYT1A were quantified by qPCR in both cell line pairs. **(C) PC9, (D) PC9ER and (E) H1975** were transfected with siRNA against PCYT1A or the NT control. Twenty-four hours post transfection cells were treated with increasing concentrations of erlotinib (48h) prior to fixation and crystal violet staining. The obtained values were normalised against the DMSO control. siRNA efficiency was confirmed by qPCR in **(F) PC9, (G) PC9ER and (H) H1975**. Figures represent the mean of three biological repeats with each replicate done in technical triplicate ($\pm$ SEM). Statistical analysis was done using Student's *t*-test. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$

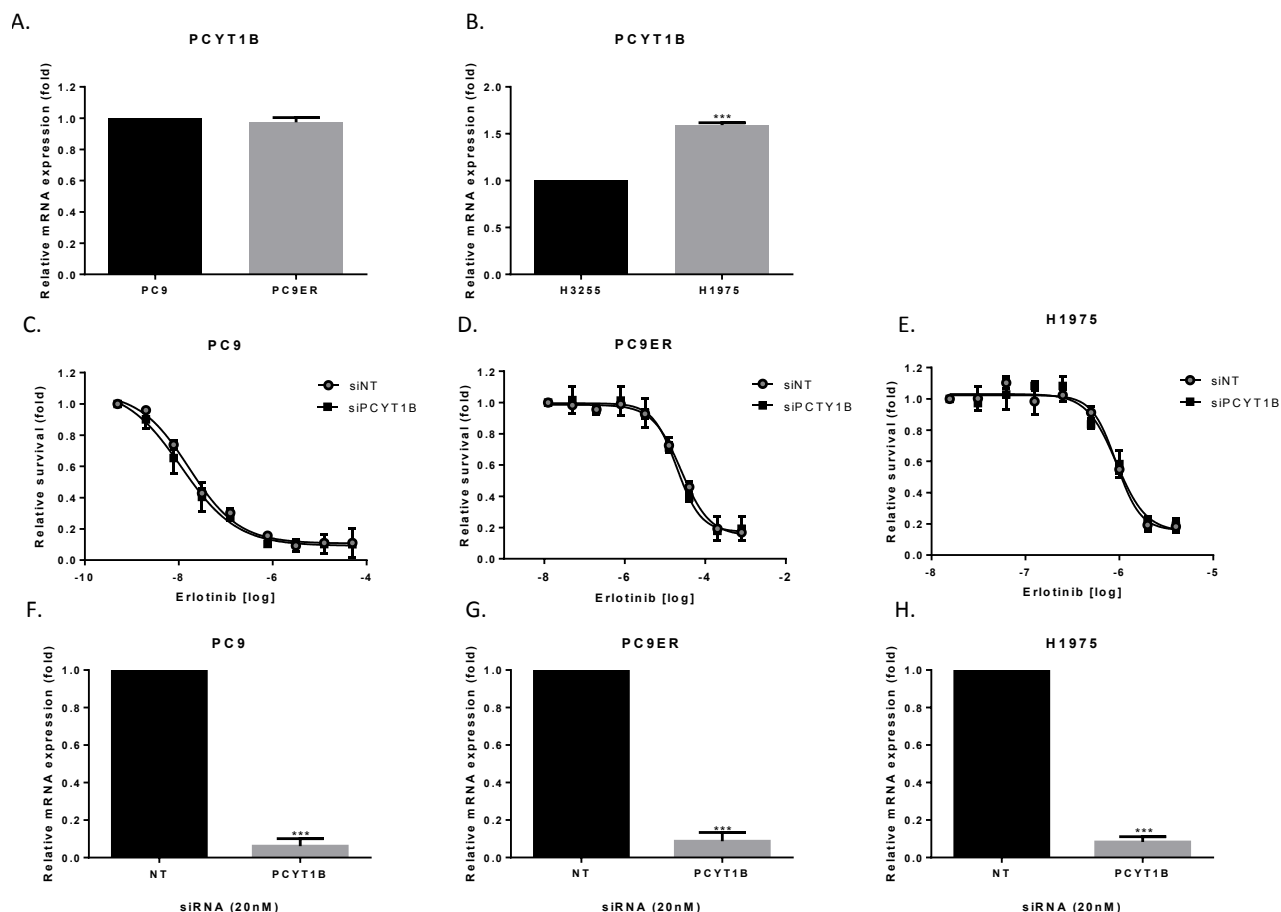


Figure 3.7: Effect of silencing PCTY1B on cell survival to erlotinib treatments. (A, B) Basal mRNA levels of PCYT1B were quantified by qPCR in both cell line pairs. **(C) PC9, (D) PC9ER and (E) H1975** were transfected with siRNA against PCYT1B or the non-targeting (NT) control. Twenty-four hours post transfection cells were treated with increasing concentrations of erlotinib (48h) prior to fixation and crystal violet staining. The obtained values were normalised against the DMSO control. siRNA efficiency was confirmed by qPCR in **(F) PC9, (G) PC9ER and (H) H1975**. Figures represent the mean of three biological repeats with each replicate done in technical triplicate ($\pm$ SEM). Statistical analysis was done using Student's *t*-test. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$

3.3.3 Choline Phosphotransferase 1 (CHPT1) does not affect survival to erlotinib treatment.

Choline Phosphotransferase 1 is encoded by the gene CHPT1. CHPT1 catalyses the last step within this pathway, whereby it combines phosphocholine to the hydroxyl group on the 3rd carbon of diacylglycerol (DAG), resulting in the formation of phosphatidylcholine (Cansev et al., 2008). The mRNA levels of CHPT1 did not show any change between PC9 and PC9ER cells (**Figure 3.8A**). However, H1975 cells showed a significant increase in transcript levels of CHPT1 compared to H3255 cells (**Figure 3.8B**). siRNA-mediated knockdown of CHPT1 had no effect on survival to erlotinib treatment (**Figure 3.8C, D and E**) despite efficient knock down (**Figure 3.8F, G and H**).

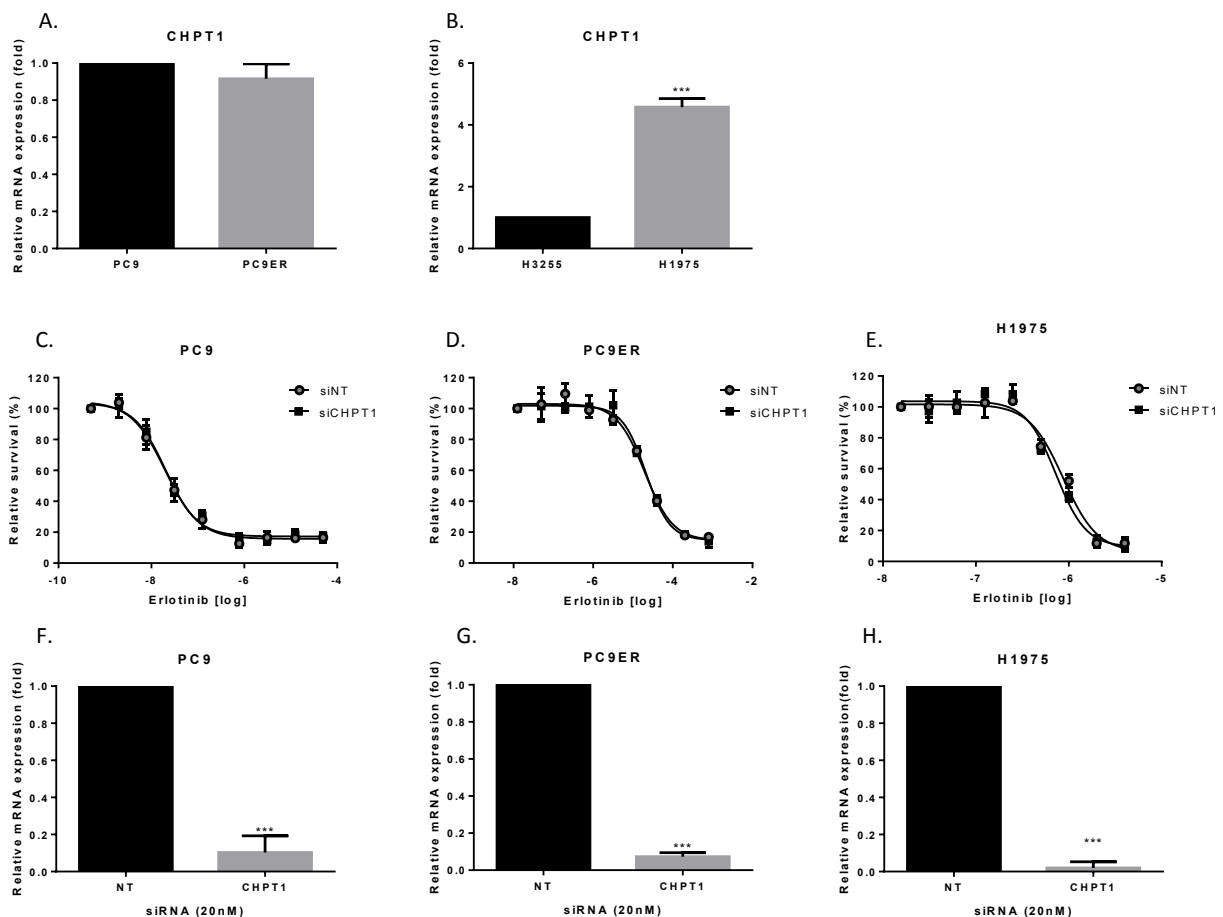


Figure 3.8: Effect of silencing CHPT1 on cell survival to erlotinib treatments. (A, B) Basal mRNA levels of CHPT1 were quantified by qPCR in both cell line pairs. **(C)** PC9, **(D)** PC9ER and **(E)** H1975 were transfected with siRNA against CHPT1 or the NT control. Twenty-four hours post transfection cells were treated with increasing concentrations of erlotinib for (48h) prior to fixation and crystal violet staining. The obtained values were normalised against the DMSO control. siRNA efficiency was confirmed by qPCR in **(F)** PC9, **(G)** PC9ER and **(H)** H1975. Figures represent the mean of three biological repeats with each replicate done in technical triplicate ($\pm$ SEM). Statistical analysis was done using Student's *t*-test. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$

3.3.4 Phosphatidylethanolamine N-Methyltransferase (PEMT) does not affect survival to erlotinib treatment.

PEMT encodes the gene phosphatidylethanolamine N-Methyltransferase which converts phosphatidylethanolamine to phosphatidylcholine. The basal quantification of PEMT levels mRNA showed a decrease in PC9ER compared to PC9 cells (**Figure 3.9A**), while the converse relationship was observed for the H3255/H1975 cell line pair with increased levels found in the resistant H1975 cell line (**Figure 3.9B**). The siRNA-mediated downregulation of PEMT did not affect survival to erlotinib in any of the cell lines tested (**Figure 3.9 C, D and E**). This was despite efficient knock down as confirmed by qPCR (**Figure 3.9 F, G and H**).

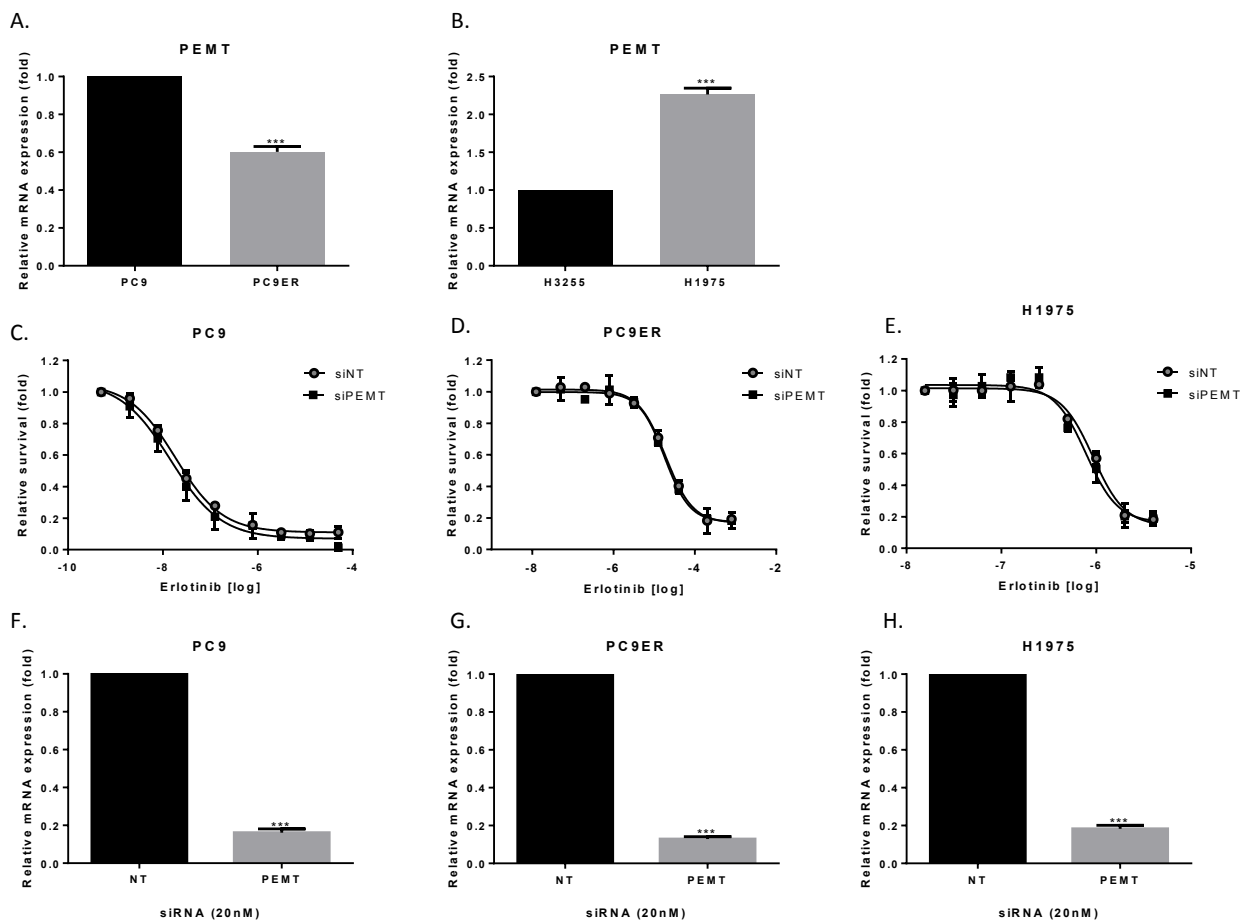


Figure 3.9: Effect of silencing PEMT on cell survival to erlotinib treatments. (A,B) Basal mRNA levels of PEMT were quantified by qPCR in both cell line pairs. **(C)** PC9, **(D)** PC9ER and **(E)** H1975 were transfected with siRNA against PEMT or the non-targeting (NT) control. Twenty-four hours post transfection cells were treated with increasing concentrations of erlotinib (48h) prior to fixation and crystal violet staining. The obtained values were normalised against the DMSO control. siRNA efficiency was confirmed by qPCR in **(F)** PC9, **(G)** PC9ER and **(H)** H1975. Figures represent the mean of three biological repeats with each replicate done in technical triplicate ($\pm$ SEM). Statistical analysis was done using Student's *t*-test. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

Overall, our data indicate a possible increase in the phosphatidylcholine synthesis enzymes in H1975 compared to H3255. This was not mirrored in the PC9 and PC9ER cell line pairs. In addition, decreasing the levels of these enzymes using RNA interference did not affect survival to erlotinib treatment. This was despite a consistent increase in choline and phosphocholine in both resistant cell lines compared to their sensitive counterpart. Therefore, further metabolomic studies were needed to increase our understanding of the changes in choline metabolism.

3.4 Mass spectrometry confirmed an increase in metabolites of the phosphatidylcholine bio-synthetic pathway in H1975 cells compared to its sensitive counterpart.

Mass spectrometry was used to quantify the metabolic changes which occur within our cell line pairs. Although, this confirmed changes in the phosphatidylcholine pathway in resistance cell lines, the majority of the changes identified within the phosphatidylcholine synthesis pathway (**Figure 3.10A**) were not consistent between both cell line pairs (**Figure B-G**). Only choline showed a significant increase in both the resistant cell lines (**Figure 3.10B**). Ethanolamine did however show a general trend of increase in resistant cell lines compared to their sensitive counterparts, but this was not significant between H3255 and H1975 cells (**Figure 3.10C**). Interestingly, contrary to the results obtained by NMR, phosphocholine was found to be decreased in PC9ER cells compared to PC9 cells (**Figure 3.10D**). In addition, phosphoethanolamine also showed lower levels in PC9ER compared to PC9 cells (**Figure 3.10E**). Although PC9 and PC9ER showed no difference in their levels of cytidine diphosphate-choline (CDP)-choline (**Figure 3.10 F**), CDP-ethanolamine was significantly decreased in PC9ER compared to PC9 cells (**Figure 3.10G**). The levels of keys synthesising metabolites were decreased in PC9ER compared to PC9 cells, while H1975 showed increased levels compared to H3255 cells in all metabolites tested (**Figure 3.10 B, C, D, E, F and G**) although this was not significant for ethanolamine (**Figure 3.10C**). This may suggest that there is differential regulation within this pathway between our two cell line pairs.

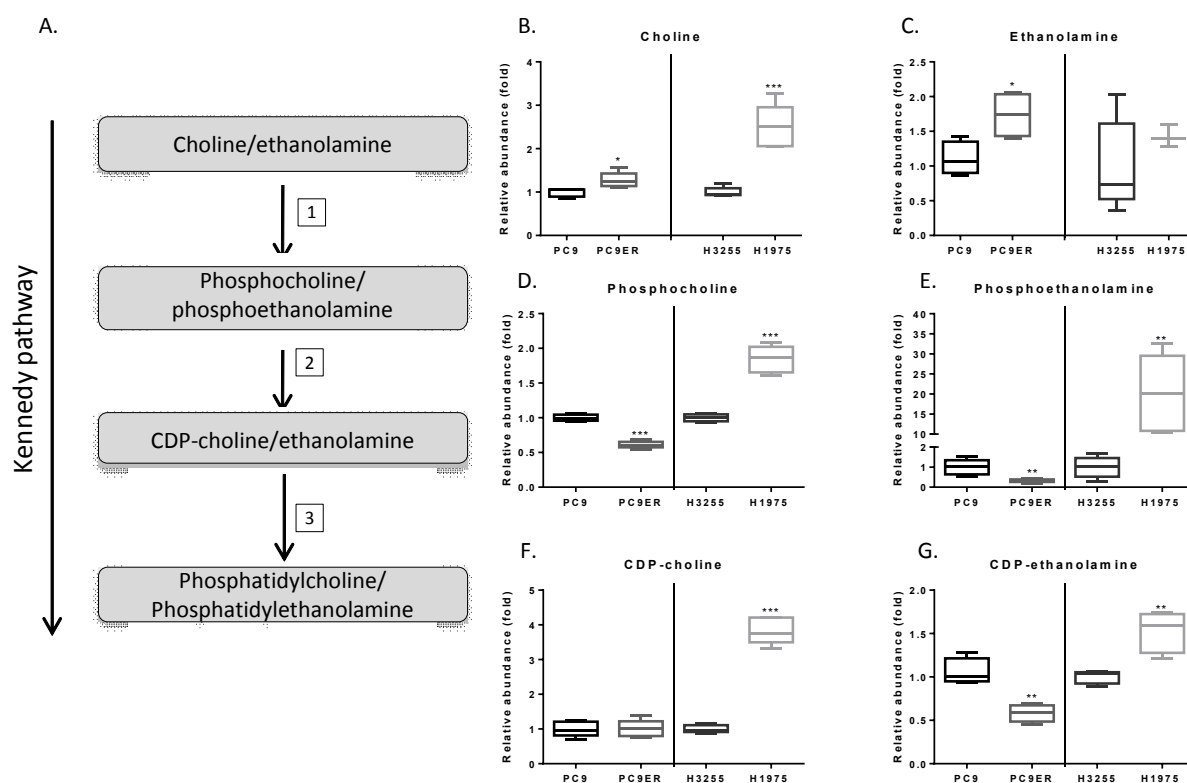


Figure 3.10: Erlotinib resistance affects abundance of metabolites involved in phosphatidylcholine synthesis pathway. (A) Schematic diagram of the Kennedy pathway with enzymes denoted as follows (1) Choline kinase (CK), (2) CTP:phosphocholine cytidyltransferase (CCT), (3) Choline phosphotransferase (CPT). Mass spectrometry analysis of (B) choline, (C) ethanolamine, (D) phosphocholine, (E) phosphoethanolamine, (F) cytidine diphosphate-choline (CDP)-choline, (G) CDP-ethanolamine. PC9ER and H1975 each normalised to their sensitive counterpart PC9 and H3255 respectively. All MS data are the average of five biological repeats. Statistical analysis was done using Student's t-test: * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$. Mass spectrometry analysis was carried out by Metabolon (Durham, US).

3.5 Mass spectrometry indicated an increase in phosphatidylcholine degradation pathway

In addition to the role of choline and ethanolamine in the phosphatidylcholine synthesis pathway they are also products of phosphatidylcholine degradation as can be seen in **Figure 3.11**. The increased abundance of choline and ethanolamine in erlotinib resistant cells may be due to enhanced degradation of phosphatidylcholine. Indeed, there are three key pathways involved in phosphatidylcholine degradation two of which have products including choline and ethanolamine (**Figure 3.11**). Therefore our studies concentrated on these two pathways. The mRNA levels of key enzymes within the pathway before and after silencing with siRNA were quantified and the effect on erlotinib resistance measured by crystal violet based survival assay.

3.5.1 Phospholipase D knockdown did not affect resistance to erlotinib.

Phospholipase D (PLD) catalyses the hydrolysis of phosphatidylcholine to produces phosphatidic acid and choline. It is able to mediate downstream signalling through the production of phosphatidic acid. However, it also interacts with a number of intracellular molecules, which in turn interact with and regulate EGFR function and therefore could be potentially an interesting target. There are two known isoforms of phospholipase D, PLD1 and PLD2. The basal mRNA levels of PLD1 were lower in both resistant cell lines compared to their sensitive counterparts (**Figure 3.12A and B**). However, siRNA-mediated knockdown of PLD1, did not modulate the sensitivity to erlotinib treatment in any of the cell lines tested (**Figure 3.12 C, D and E**). The knockdown was confirmed by qPCR (**Figure 3.12F, G and H**). PLD2 basal mRNA levels showed no change between either cell line pairs (**Figure 3.13A and B**). In addition, PLD2 knockdown did not affect survival to erlotinib treatment in any of the cell lines tested (**Figure 3.13 C, D and E**), despite efficient knockdown, which was confirmed by qPCR (**Figure 3.13F, G and H**). As both PLD1 and PLD2 show no change or lower mRNA levels in resistant cell lines and their targeting did not affect survival to erlotinib treatment other degradation pathways were studied.

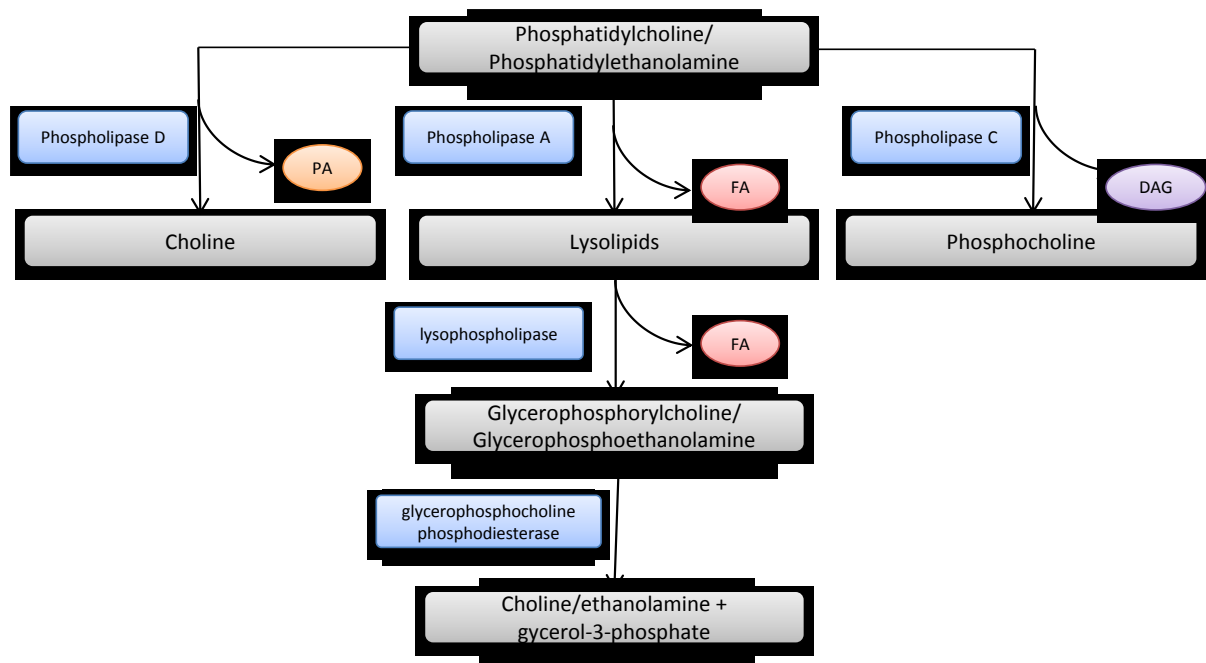


Figure 3.11: Schematic diagram of phosphatidylcholine degradation. Phosphatidylcholine is degraded by three principle degradation pathways. Phosphatidylcholine is hydrolysed by phospholipase D, which produces phosphatidic acid and soluble choline. Phospholipase A has two isoforms phospholipase A1 and Phospholipase A2. Phospholipase A1 (PLA1) catalyses the cleavage at the SN-1 position of phospholipids, producing fatty acid and a lysophospholipid. Phospholipase A2 specifically catalyses the hydrolysis of the SN-2 acyl bond of phospholipids, which results in the release of arachidonic acid and lysophospholipids. Lysophospholipase then hydrolyse the carboxylic ester bond, which results in the formation of glycerophosphorylcholine or glycerophosphoethanolamine. This is then further hydrolysed by glycerophosphocholine phosphodiesterase, producing choline or ethanolamine and glycerol-3-phosphate.

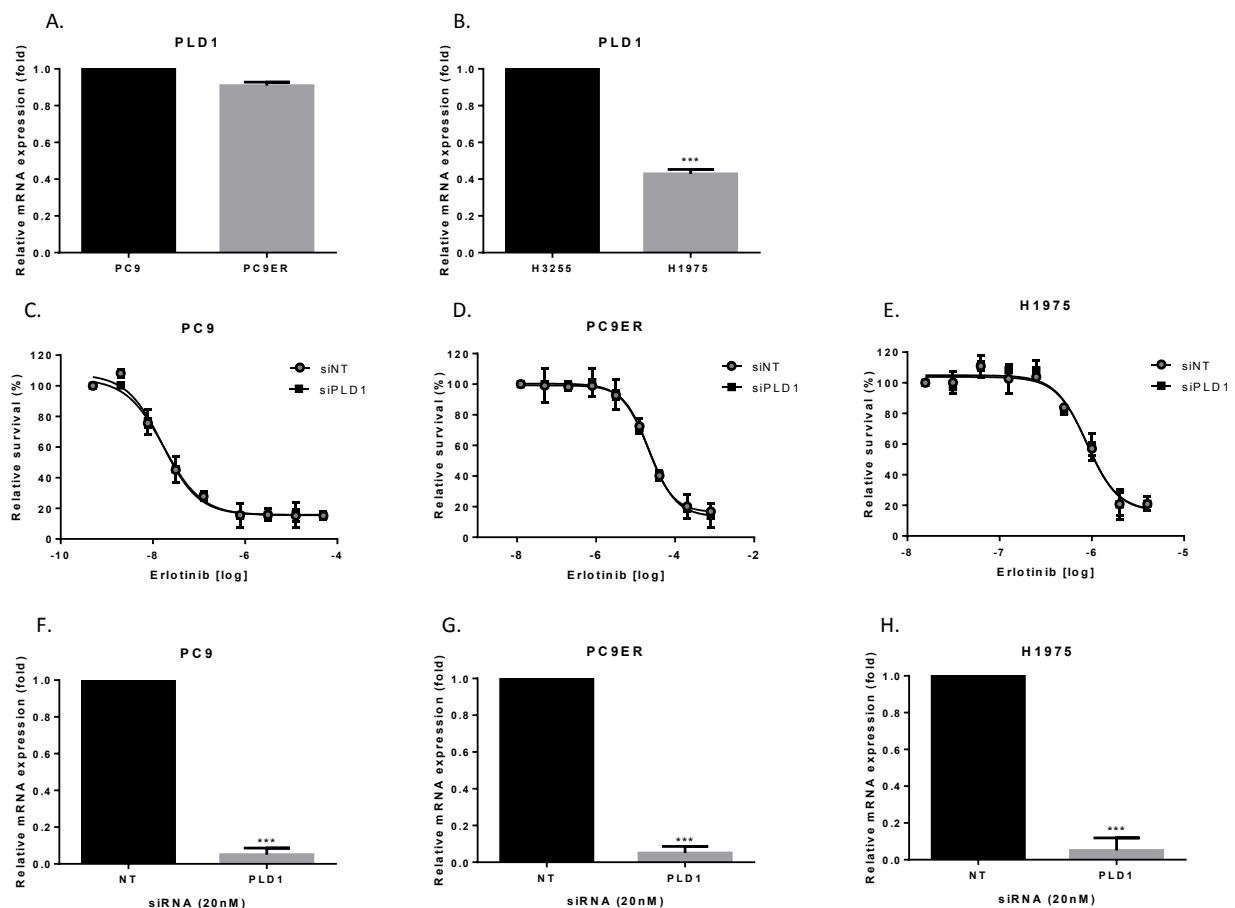


Figure 3.12: Effect of silencing PLD1 on cell survival to erlotinib treatments. (A, B) Basal mRNA levels of PLD1 were quantified by qPCR in both cell line pairs. (C) PC9, (D) PC9ER and (E) H1975 were transfected with siRNA against PLD1 or the NT control. Twenty-four hours post transfection cells were treated with increasing concentrations of erlotinib for (48h) prior to fixation and crystal violet staining. The obtained values were normalised against the DMSO control. siRNA efficiency was confirmed by qPCR in (F) PC9, (G) PC9ER and (H) H1975. Figures represent three biological repeats with error bars showing $\pm$ SEM. Statistical analysis were done using Student's *t*-test. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

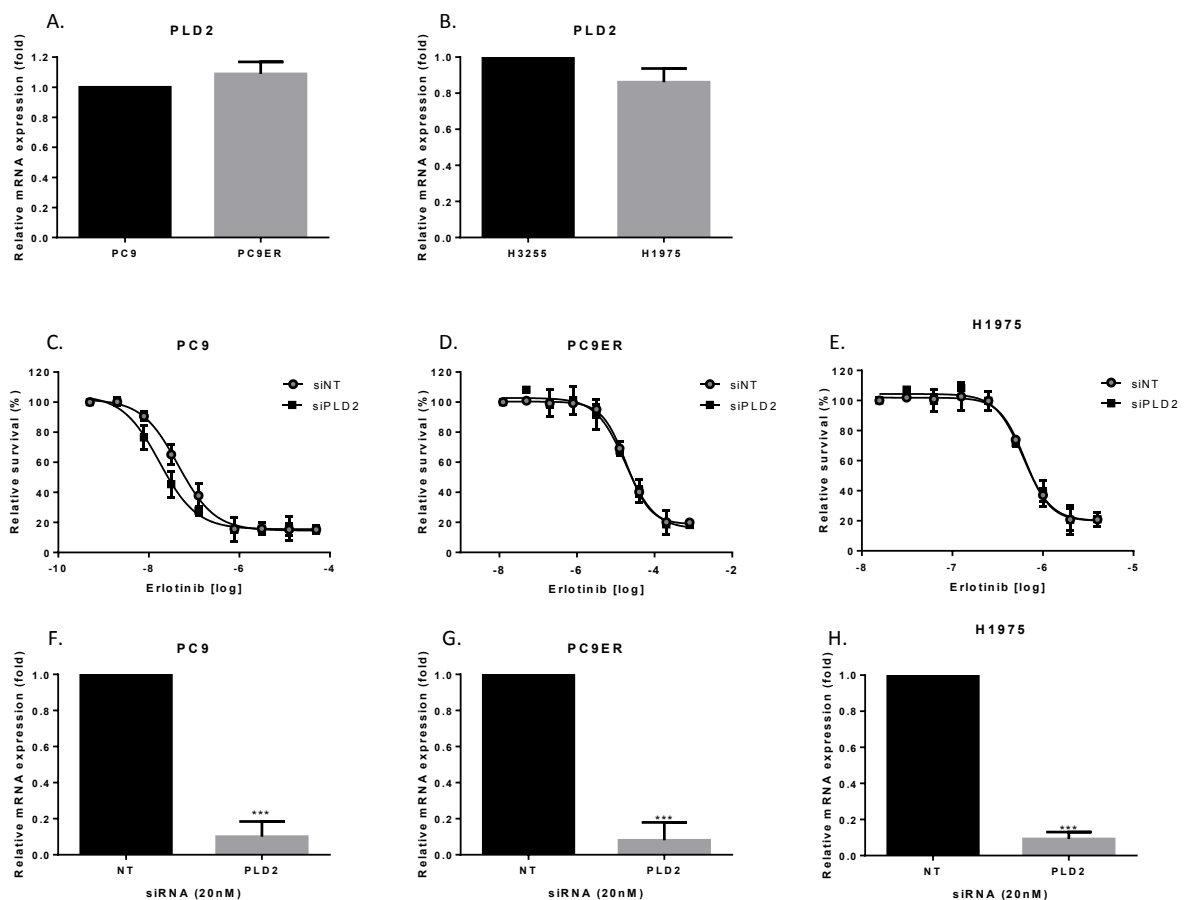


Figure 3.13: Effect of silencing PLD2 on cell survival to erlotinib treatments. (A, B) Basal mRNA levels of PLD2 were quantified by qPCR in both cell line pairs. (C) PC9, (D) PC9ER and (E) H1975 were transfected with siRNA against PLD2 or the NT control. Twenty-four hours post transfection cells were treated with increasing concentrations of erlotinib (48h) prior to fixation and crystal violet staining. The obtained values were normalised against the DMSO control. siRNA efficiency was confirmed by qPCR in (F) PC9, (G) PC9ER and (H) H1975. Figures represent the mean of three biological repeats with each replicate done in technical triplicate ($\pm$ SEM). Statistical analysis was done using Student's *t*-test. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$

3.5.2 Increased phosphatidylcholine degradation in erlotinib resistant cell lines

Analysis of mass spectrometry data revealed changes in some of the key metabolites in the phospholipase A driven degradation pathway (**Figure 3.14**). There is significantly higher abundance of both glycerophosphocholine (**Figure 3.14B**) and glycerophosphoethanolamine (**Figure 3.14C**) in both resistant cell lines. In addition, glycerol-3-phosphate (**Figure 3.14D**), choline (**Figure 3.14E**) and ethanolamine (**Figure 3.14 F**) were increased in resistant cell lines compared to their sensitive counterparts.

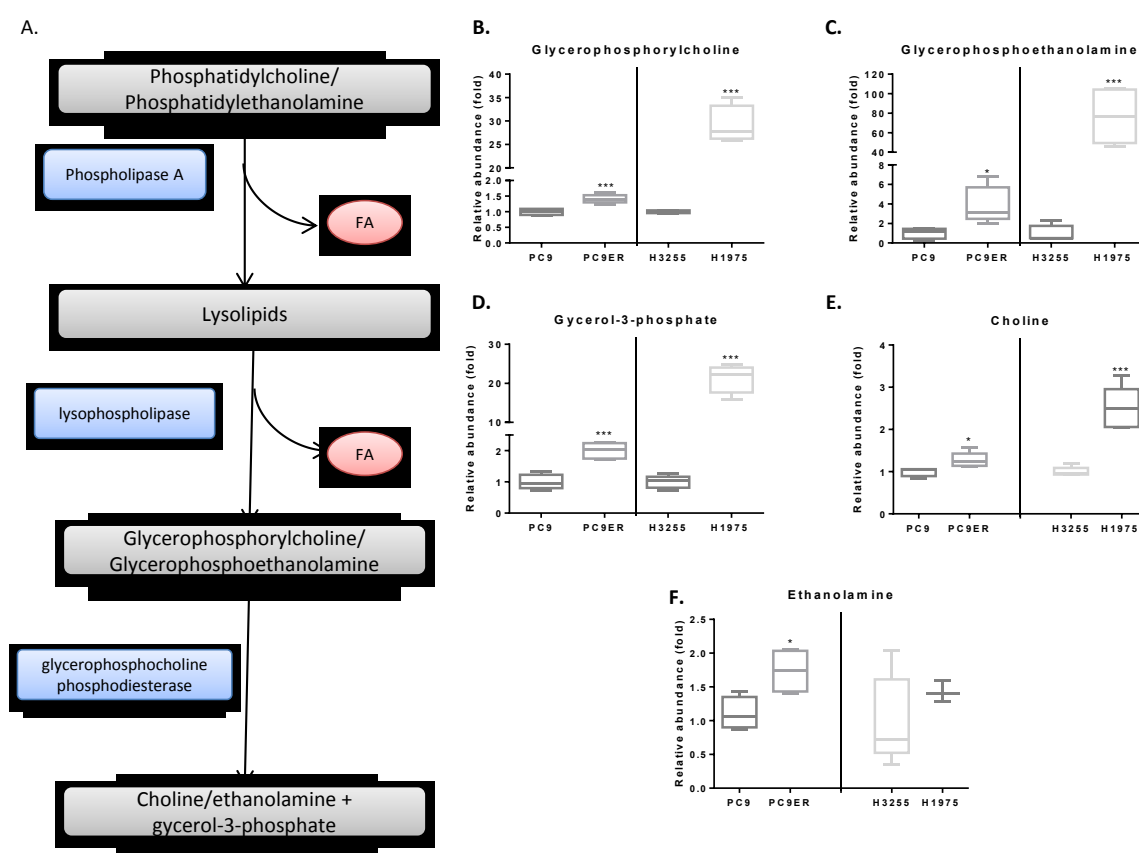


Figure 3.14: Mass spectrometry identified phosphatidylcholine metabolites were increased in erlotinib resistant cell lines. (A) Schematic diagram of phosphatidylcholine hydrolysis pathway mediated by phospholipase A. Metabolites shown in grey, enzymes in blue and fatty acid (FA) represented in pink. Quantitative data from MS showing the abundance of (B) Glycerophosphorylcholine, (C) Glycerophosphoethanolamine, (D) Glycerol 3-phosphate, (E) Choline, (F) Ethanolamine. PC9ER and H1975 were each normalised to their sensitive counterpart PC9 and H3255 respectively. All MS data were the average of five biological repeats. Statistical analysis was done using Student's t-test: * $P \leq 0.05$, ** $P \leq 0.01$. Mass spectrometry analysis was carried out by Metabolon (Durham, US).

3.5.3 siRNA targeting of Phospholipase A2, Group VI (PLA2G6) does not alter sensitivity to erlotinib treatment.

Phospholipase A (PLA) has two isoforms PLA1 and PLA2, both of which hydrolyse phospholipids to release fatty acids. In addition, PLA2 has a number of human isozymes which are grouped as follows Group I: PLA2G1B, Group II: PLA2G2A, PLA2G2C, PLA2G2D, PLA2G2E, PLA2G2F, Group III: PLA2G3, Group IV: PLA2G4A, PLA2G4B, PLA2G4C, PLA2G4D, PLA2G4E, PLA2G4F, Group V: PLA2G5, Group VI: PLA2G6, Group VII: PLA2G7, Group X: PLA2G10 and lastly Group XII: PLA2G12A, PLA2G12B. Due to the large number of isoforms which could be targeted and the cost involved, this study focused on PLA2G6. PLA2G6 is a cytosolic, calcium-independent phospholipase enzyme, which is expressed within a number of organs including the lung. It was chosen due to its functions as an A2 phospholipase by the hydrolysing the sn-1 acyl chain of phosphatidylcholine. In addition, it can function as a lysophospholipase by hydrolysing the sn-1 acyl chain of lysophosphatidylcholine (LPC) (Engel et al., 2010). It has also been shown to be significantly associated with risk of lung cancer (Hosgood et al., 2008). The basal mRNA levels of this enzyme quantified by qPCR were consistently higher in sensitive cell lines compared to their resistant counterpart (**Figure 3.15A and B**). However, siRNA-mediated targeting of PLA2G6 in PC9, PC9ER and H1975 did not alter the response to erlotinib (**Figure 3.15C, D and E**). Efficient knockdown of the gene was confirmed by qPCR (**Figure 3.15F, G and H**).

3.5.4 siRNA-mediated targeting of Lysophospholipase (PLA2G15) does not affect sensitivity to erlotinib treatment.

The lysophospholipase enzyme encoded by the phospholipase A2, group XV (PLA2G15) gene hydrolyses lysophosphatidylcholine to glycerophosphorylcholine and free fatty acid. There was no difference in mRNA levels for PLA2G15 between erlotinib resistant and sensitive cell lines as shown by qPCR analysis (**Figure 3.16A and B**). Nevertheless, this could not discount possible changes in protein levels or activity. Therefore, PC9, PC9ER and H1975 cells were transfected with siRNAs against PLA2G15 and survival to erlotinib treatment was assessed by crystal violet assays (**Figure 3.16C, D and E**). This showed that PLA2G15 down regulation did not lead to changes in survival following erlotinib treatment for any of the cell lines tested despite efficient knockdown confirmed by qPCR (**Figure 3.16F, G and H**).

Overall this data indicates that there is an increase in phosphatidylcholine degradation indicated by metabolomics analysis of erlotinib-resistant and sensitive cell line pairs. However, the mRNA quantification of key enzymes within this pathway, failed to show transcriptional up-regulation of any of these phosphatidylcholine degradation enzymes in resistant cell lines. Furthermore, targeting by siRNA-mediated knockdown of these enzymes did not affect survival to erlotinib treatment.

However, due to the large number of isoforms for each of the phospholipase enzymes there is a possibility of redundant functions between the isoforms and further studies would be required to dissect out this pathway.

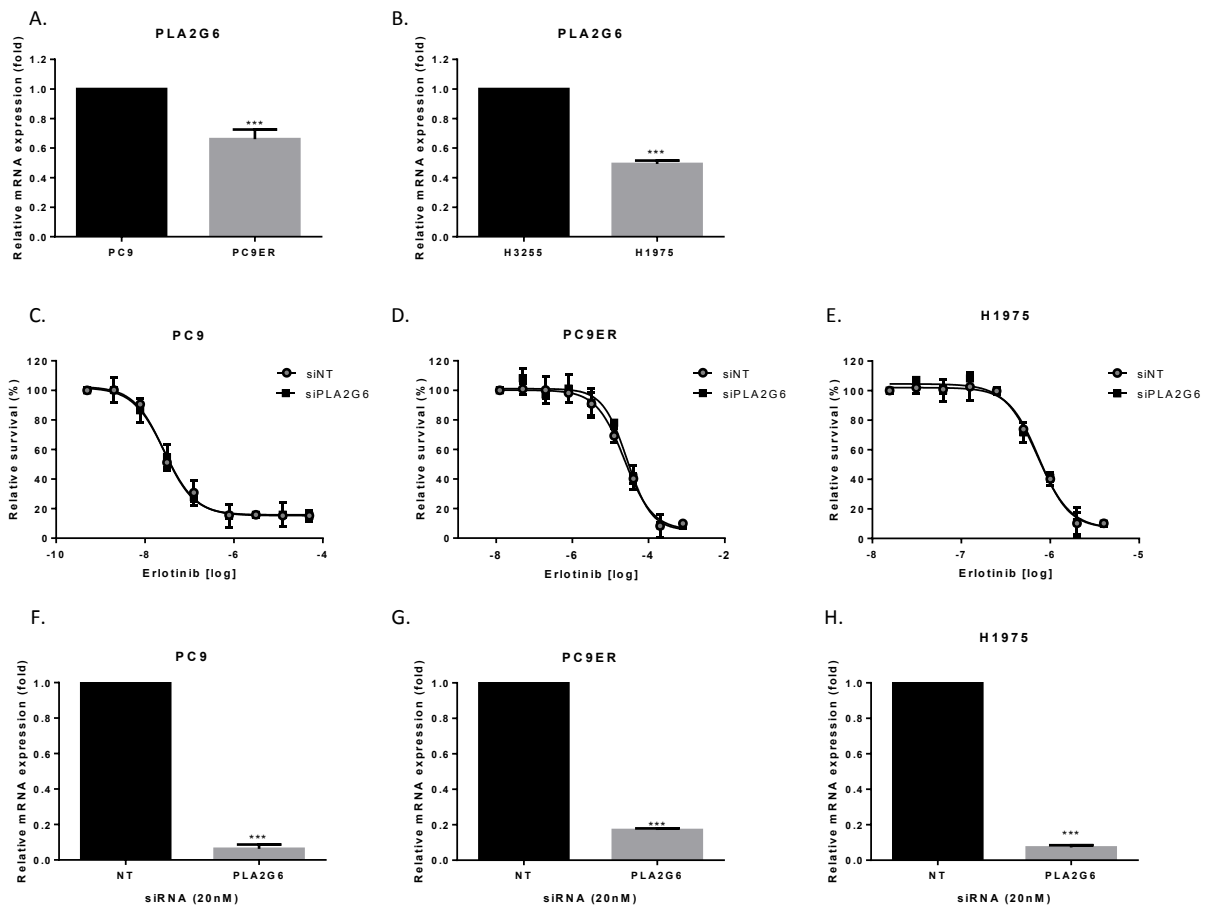


Figure 3.15: Effect of silencing PLA2G6 on cell survival to erlotinib treatments. (A, B) Basal mRNA levels of PLA2G6 were quantified by qPCR in both cell line pairs. (C) PC9, (D) PC9ER and (E) H1975 were transfected with siRNA against PLA2G6 or the NT control. Twenty-four hours post transfection cells were treated with increasing concentrations of erlotinib (48h) prior to fixation and crystal violet staining. The obtained values were normalised against the DMSO control. siRNA efficiency was confirmed by qPCR in (F) PC9, (G) PC9ER and (H) H1975. Figures represent the mean of three biological repeats with each replicate done in technical triplicate ($\pm$ SEM). Statistical analysis was done using Student's t-test. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

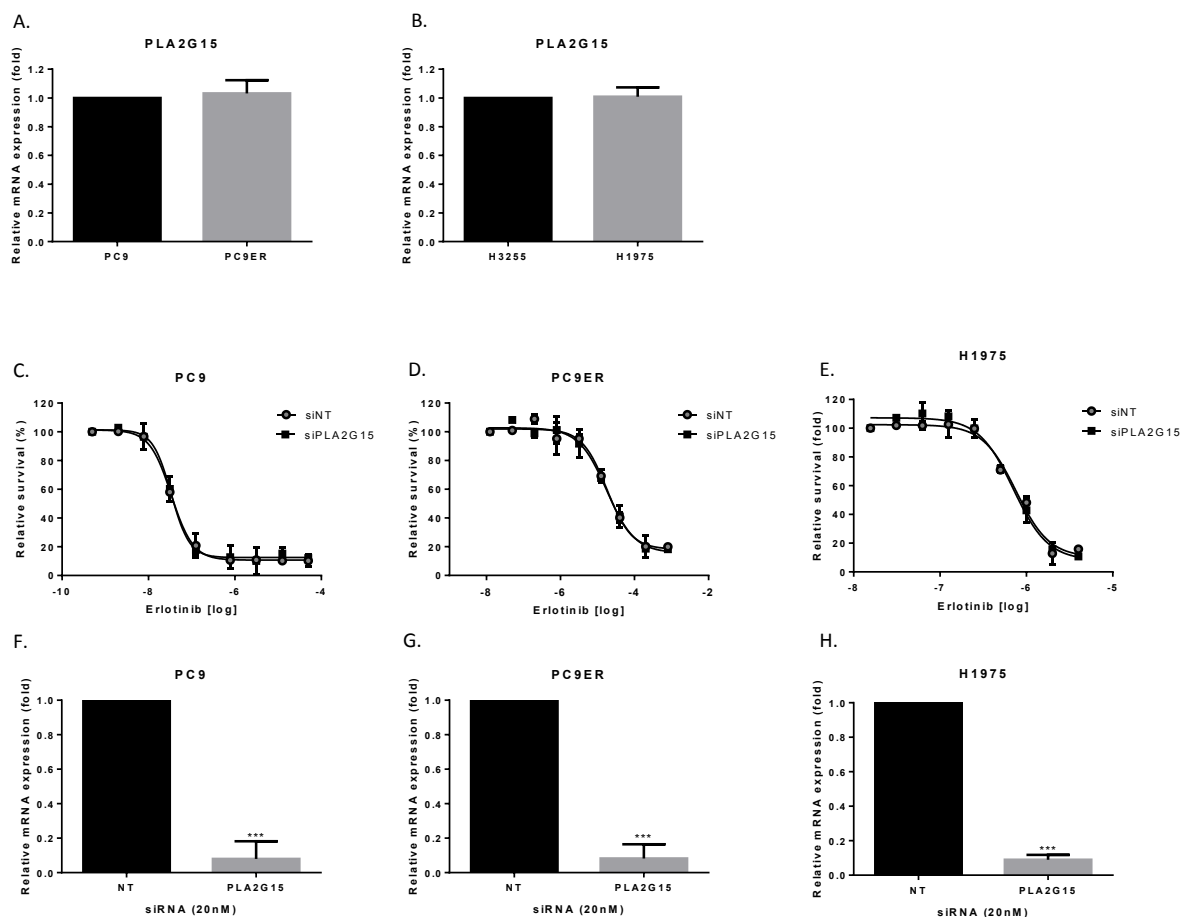


Figure 3.16: Effect of silencing PLA2G15 on cell survival to erlotinib treatments. (A, B) Basal mRNA levels of PLA2G15 were quantified by qPCR in both cell line pairs. (C) PC9, (D) PC9ER and (E) H1975 were transfected with siRNA against PLA2G15 or the NT control. Twenty-four hours post transfection cells were treated with increasing concentrations of erlotinib (48h) prior to fixation and crystal violet staining. The obtained values were normalised against the DMSO control. siRNA efficiency was confirmed by qPCR in (F) PC9, (G) PC9ER and (H) H1975. Figures represent the mean of three biological repeats with each replicate done in technical triplicate ($\pm$ SEM). Statistical analysis was done using Student's *t*-test. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$

3.6 Discussion

The aim of this thesis is to understand alternative resistance mechanisms to erlotinib which may occur as a result of the T790M mutation. This study used two cell line pairs with the resistant cell line harbouring the T790M mutation. These cell lines were chosen on the basis of the PC9/PC9ER and H3255/H1975 cell line pairs differing in their EGFR activating mutation while PC9ER and H1975 share the same secondary resistance T790M mutation. This minimised the chance of the metabolic differences observed between sensitive and resistant cells in both cell line pairs being driven by the EGFR activating event rather than the common resistance mutation. We initially used ^1H -NMR to metabolically profile these cell lines. Statistical analysis of our NMR data by OPLS-DA revealed significant differences in the metabolic signatures between the erlotinib-resistant cell lines and their respective sensitive counterparts (**Figure 3.2**). The metabolites that were found to be significantly altered between the cell line pairs are listed in **Table 3.1**. Two key metabolites, phosphocholine and choline were identified to be increased in our resistant cell lines. This confirms previous studies which correlated erlotinib resistance to increased phospholipid metabolites (Fan et al., 2009, Weaver et al., 2012).

Utilising qPCR, we were able to confirm an increase in the levels of several key synthesising enzymes in the phosphatidylcholine pathway in H1975 when compared to its sensitive counterpart (**Figure 3.4B, 3.5B, 3.6B, 3.7B and 3.9B**). This increase may or may not translate to changes in protein levels and/or activity of the enzymes. However, siRNA-mediated knockdown failed to alter the response to erlotinib treatment (**Figure 3.4C-E, 3.5C-E, 3.6C-E, 3.7C-E, 3.8 C-E and 3.9C-E**). Given the biological importance of this pathway, it is likely that downregulation of its key enzymes may result in the activation of alternative pathways and thereby negate the effect of our RNA interference on erlotinib resistance. Additionally, the effect of siRNA-mediated knockdown is short-lived and the length of time during which these enzymes must be reduced to observe a significant effect may be longer than what we achieve here. Hence, shRNA may be better adapted to address the potential role of these enzymes in T790M-mediated resistance. Taken together, we suggest that there is an increase in the phosphatidylcholine synthesis pathway in H1975 compared to H3255, although its involvement in erlotinib resistance is still currently unclear.

PC9 and PC9ER cells showed no difference in CHKB, PCYT1A, PCYT1B and CHPT1 basal mRNA expression levels (**Figure 3.4A, 3.5A, 3.6A, 3.7A and 3.9A**). CHKA other than PEMT was the only enzyme which showed a significant decrease in mRNA expression in PC9ER compared to PC9 cells (**Figure 3.4A**). In agreement with previously published results, transcripts levels of CHKA was found to be lower in PC9ER compared to the PC9 cells (Slade, 2013). The importance of CHKA in EGFR

signalling has also been demonstrated in breast cancer cell lines, where it was shown to complex with EGFR in a c-SRC-dependent manner (Miyake and Parsons, 2012). In addition, CHKA expression has been linked to decrease in glutathione levels, with silencing of CHKA resulting in decrease in a decrease of GSH in both its oxidised and reduced forms (Granata et al., 2015). Indeed, our ^1H NMR studies demonstrated PC9ER cells to have significantly lower levels of glutathione (**Table 3.1**). However, targeting of CHKA failed to alter response to erlotinib treatment (**Figure 3.4C and D**). As the knockdown was confirmed to be efficient at the mRNA level, this suggests that choline kinase may not have a role in erlotinib resistance. However, there may be additional explanations for this lack of impact on the response to erlotinib treatment such as alternative pathways, for example that of ethanolamine, being activated leading to compensation. Short-term siRNA-mediated knockdown may be insufficient to cause changes in metabolism. Due to the lack of consistency in the results obtained between our cell line pairs, we suggest that it is unlikely that phosphatidylcholine synthesis has a role in erlotinib resistance.

To gain further insights into the metabolic changes, we used mass spectrometry to get a detailed metabolic profile for these cell lines. This confirmed an increase in choline levels in both resistant cell lines compared to their sensitive counterpart (**Figure 3.10B**). Interestingly, this approach also confirmed that the levels of many of the metabolites in this synthesis pathway were lower in PC9ER compared to PC9 cells (**Figure 3. 10D, E and G**). In addition, there was a significant increase in ethanolamine in the PC9ER cell line suggesting that phosphatidylcholine synthesis is ethanolamine-driven rather than obtained through the classical Kennedy pathway. H1975 cells show an increase in many of the key metabolites involved within the synthesis pathway, suggesting an increase in phosphatidylcholine synthesis in H1975 compared to its sensitive counterpart. This further confirms that there is a lack of consistent changes in this pathway suggesting that it is not associated with resistance.

Choline and ethanolamine which had increased levels in both resistant cell lines are known products of phosphatidylcholine degradation (**Figure 3.14A**). Mass spectrometry data indicated that a number of metabolites involved in phosphatidylcholine degradation are increased in both the resistant cell lines (**Figure 3.14**). This pathway involves the use of a number of phospholipase enzymes. As mass spectrometry data showed the abundance of phosphocholine not to be consistent between our cell line pairs (**Figure 3.10E**), we hypothesised that that phospholipase C was unlikely to affect resistance and therefore excluded it from further studies. Phospholipase D (PLD) has previously been linked to drug resistance (Welsh et al., 1994, Chen et al., 2003, Fiucci et al., 2000). It is also known to be a key mediator of EGFR function in an EGF-dependent manner directly regulated by upstream binding

partners (Lee et al., 2009). Therefore, although it has not been directly linked to erlotinib resistance it became of interest in this study. The basal mRNA expression of PLD1 showed a decrease in H1975 compared to H3255 cells (**Figure 3.12B**). However, no difference was noted between PC9 and PC9ER cells (**Figure 3.12A**). PLD2 was not differentially expressed in either of the cell line pairs (**Figure 3.13 A, B**). While we cannot confirm what effect the differences in mRNA levels in our study have on protein expression, the knockdown of either PLD1 or PLD2 had no effect on survival to erlotinib treatment (**Figure 3.12 C-E, 3.13 C-E**).

The metabolites identified by mass spectrometry were predominantly involved in the degradation of phosphatidylcholine by phospholipase A. This degradation pathway involves three steps which lead to the formation of choline, ethanolamine and glycerol-3-phosphate. Our mass spectrometry data showed that the abundance of these was increased in both resistant cell lines (**figure 3.14**). Phospholipase A exists in both a cytosolic and secreted form. In a review by Park *et al* they highlighted that different isoforms of PLA2 may have differing function (Park et al., 2012). Indeed, they suggested that the cytosolic forms are predominantly involved in the generation of arachidonic acid to produce prostaglandin resulting in enhanced tumorigenesis (Park et al., 2012). This is in contrast to the secreted form which were suggested to have a tumour suppressive function (Park et al., 2012).

In this work we investigated the possible role of PLA2G6 in resistance to erlotinib. PLA2G6 is a IV cytosolic PLA2 protein and was chosen for this study due to its role as a phospholipase, hydrolysing the sn-1 acyl chain of phosphatidylcholine and also its function as a lysophospholipase able to hydrolyse the sn-1 acyl chain of LPC. A study by Li and colleagues showed that knockdown of PLA2G6 in ovarian cancer resulted in reduced tumorigenesis (Li et al., 2010a). However, despite PLA2G6 suggested role in tumorigenesis its function in resistance to RTK inhibitors such as erlotinib has not been established and therefore, provided an interesting target for this study.

The basal transcript level of PLA2G6, which encodes phospholipase A2, were significantly lower in resistant cell lines (**Figure 3.15 A and B**). This is in conflict with our mass spectrometry data that showed increased levels of a number of downstream metabolites in erlotinib-resistant cell lines (**Figure 3.14**). However, there are a number of phospholipase A isoforms and changes in an alternative form may be responsible for the increase in phosphatidylcholine degradation metabolites identified in the mass spectrometry data. As siRNA-mediated targeting of PLA2G6 did not affect survival to erlotinib treatment this further support this hypothesis (**Figure 3.15C, D and E**). In addition, the knockdown of a singular PLA may not be sufficient to lead to a response, as alternative PLAs may compensate for this.

Lastly this study looked at the Lysophospholipase enzyme, PLA2G15, which showed no significant difference in mRNA levels between resistant and sensitive cell lines (**Figure 3.15 A and B**). Although no change was noted at the mRNA level, this does not always correlate directly with protein expression. The knockdown of PLA2G15 did not affect the response to erlotinib treatment in any of the cell lines tested (**Figure 3.15 C, D and E**). However, again this may be as a result compensation from additional proteins with the ability to hydrolyze lysophosphatidylcholine to form glycerophosphorylcholine and a free fatty acid such as PLA2G6 (Engel et al., 2010).

Overall this data indicates there is an increase in the levels of phosphatidylcholine degradation in resistant cell lines. This was demonstrated by an increased abundance of a number of metabolites involved in its degradation. However, the targeting of enzymes within this pathway did not affect survival to erlotinib treatment. Therefore, a more comprehensive approach may be needed to correct the altered metabolic networks.

**Chapter 4.0 EGFR T790M-mediated erlotinib
resistance is functionally associated with decreased
intracellular levels of glutathione**

4.1 Introduction

Lung cancer is the commonest cancer killer with non-small cell lung cancer (NSCLC) comprising about 85% of cases. About 10% and 30% of NSCLCs in Western and Asian populations, respectively, express an activated mutant EGFR (EGFR_m) and 90% of these patients respond to ATP-competitive EGFR TKIs such as gefitinib or erlotinib (Mok et al., 2009, Lynch et al., 2004, Paez et al., 2004). Unfortunately, TKI-resistance develops within months, thereby reducing the survival benefits of these drugs. Several resistance mechanisms have been identified; including an additional T790M gatekeeper mutation within the mutant EGFR in 50% of patients (Kosaka et al., 2006, Balak et al., 2006, Kobayashi et al., 2005, Pao et al., 2005). The latter enhances kinase activity by increasing the affinity of EGFR for ATP, competing out TKI binding (Yoshikawa et al., 2013). This led to the development of compounds irreversibly interacting with EGFR_m/T790M, such as afatinib, 324674 and more recently AZD9291 and CO1686 (Walter et al., 2013b, Zhang et al., 2006a, Miller et al., 2012a). However, thus far clinical trials of afatinib failed to demonstrate improved response in EGFR_m/T790M patients, and although initial trials with the irreversible inhibitor AZD9291 showed great promises, additional resistance mechanisms to these inhibitors have already appeared (Miller et al., 2012a, Costa and Kobayashi, 2015). Consequently, there is great interest in further understanding the mechanisms of resistance that can occur in the presence of the T790M mutation.

Interestingly, changes in metabolite concentrations can specifically reflect the onset of therapy resistance, providing response/outcome biomarkers and novel therapeutic strategies to reverse resistance (Zhao et al., 2013, Sreekumar et al., 2009). Both ¹H-nuclear magnetic resonance (NMR) and mass spectrometry are efficient tools to investigate these metabolic changes (Boros et al., 2002, Wang et al., 2013, Tian et al., 2015, Serkova et al., 2007). Changes in metabolism have frequently been linked to tumour progression and drug resistance in various cancer types (Zhao et al., 2013). Therefore, in order to identify alternative resistance mechanisms, we previously carried out ¹H-NMR metabolic profiling of EGFR-driven NSCLC cells with (PC9ER and H1975) and without (PC9 and H3255) the T790M resistance mutation. This revealed that the levels of the antioxidant molecule glutathione (GSH), in both its reduced (GSH) and oxidised form (GSSG), was decreased in EGFR-T790M containing cells. This is in marked contrast to prior reports showing that increased levels of GSH are implicated in chemotherapy resistance, due to its function as an antioxidant as well as its ability to complex with chemotherapeutic drugs leading to their GST-mediated export (Spitz et al., 1993, Corti et al., 2010, Meister, 1988). Indeed, a number of chemotherapeutic compounds rely on the production of ROS to elicit their cytotoxic actions (Bracht et al., 2006, Backos et al., 2012). This process is counteracted by GSH and increased levels of GSH therefore may facilitate drug resistance.

However, in agreement with our preliminary data, in 2014 Serizawa *et al* also identified enhanced GSH degradation in erlotinib-resistant cell line PC9ER through mass spectrometry analysis. They identified this change to be a possible biomarker of resistance to erlotinib but did not determine whether it was functionally linked to resistance (Serizawa et al., 2014). In addition, strategies to reduce the intracellular levels of GSH have also been shown to be effective in the sensitisation of tumour cells to other anticancer drugs (Nagata et al., 2001, Wang et al., 2010b). However, the functional role and mechanism by which low GSH levels induce resistance to targeted therapies such as erlotinib remains to be clarified.

Here, we validate the decrease in GSH and GSSG in cells resistant to erlotinib previously observed using ¹H-NMR profiling. Further characterization revealed changes at the transcriptional level of a number of enzymes involved in GSH metabolism downstream of T790M mutation. The targeting of synthesizing or degrading enzymes within this metabolic pathway, through both siRNA and small-molecular compounds, was able to modulate not only GSH levels but also response to erlotinib treatment. These in vitro results were then validated in a mouse xenograft model, where dual treatment with GST inhibitor ethacrynic acid (EA) and erlotinib resulted in a significant reduction in tumour burden of erlotinib-resistant T790M EGFR expressing PC9ER xenografts.

4.2 Glutathione levels are reduced in erlotinib-resistant cell lines

Glutathione levels were identified to be decreased by ^1H -NMR in erlotinib-resistant cell lines. Glutathione exists in both an oxidised (GSSG) and reduced form (GSH) and a number of enzymes regulate the GSH pathway (**Figure 4.1A**, with enzymes involved in its formation shown in clear boxes and its degradation in grey). It is important to note that the stability of GSH/GSSG during sample processing and NMR can be variable because of its oxidoreductive activity largely due to the $-\text{SH}$ group. This may reduce the ability to distinguish between the oxidised and reduced form of GSH. However, this results held some validity as there was a decrease in both the oxidised and reduced forms of GSH, suggesting a decrease in total GSH. To validate the NMR data, the levels of GSH were quantified within the resistant (PC9ER and H1975) and sensitive (PC9 and H3255) cell line pair using a colorimetric assay and confirmed low GSH in the erlotinib-resistant cells (**Figure 4.1B and D**). In order to identify if these changes are a direct consequence of deregulation of enzymes involved in GSH metabolism, qPCR was performed to assess their basal mRNA levels (**Figure 4.1C and E**). The qPCR data indicated a significant decrease in the levels of glutathione cysteine ligase (GCL) in resistant cell lines. GCL is involved in the ATP-dependent condensation of cysteine and glutamate to form gamma-glutamylcysteine ($\gamma\text{-GC}$). GCL consists of two subunits, glutamate-cysteine ligase catalytic subunit (GCLc) and the regulatory subunit glutamate-cysteine ligase modifier subunit (GCLm). Both sub-units had decreased expression in resistant cells, although this was not significant for GCLm in the PC9/PC9ER cell line pair. The mRNA levels of the gene encoding glutathione-disulphide reductase (GSR), the dimeric disulfide oxidoreductase that utilises Flavin Adenine Dinucleotide (FAD) and NADPH to reduce GSSG to GSH, were significantly reduced in both resistant cell lines. Finally, the expression of glutathione synthetase (GSS), that catalyses the condensation of gamma-glutamylcysteine and glycine, to form glutathione, was also significantly decreased in both cell line pairs. The levels of enzymes involved in GSH degradation however, showed a variable pattern of expression (**Figure 4.1C and E**).

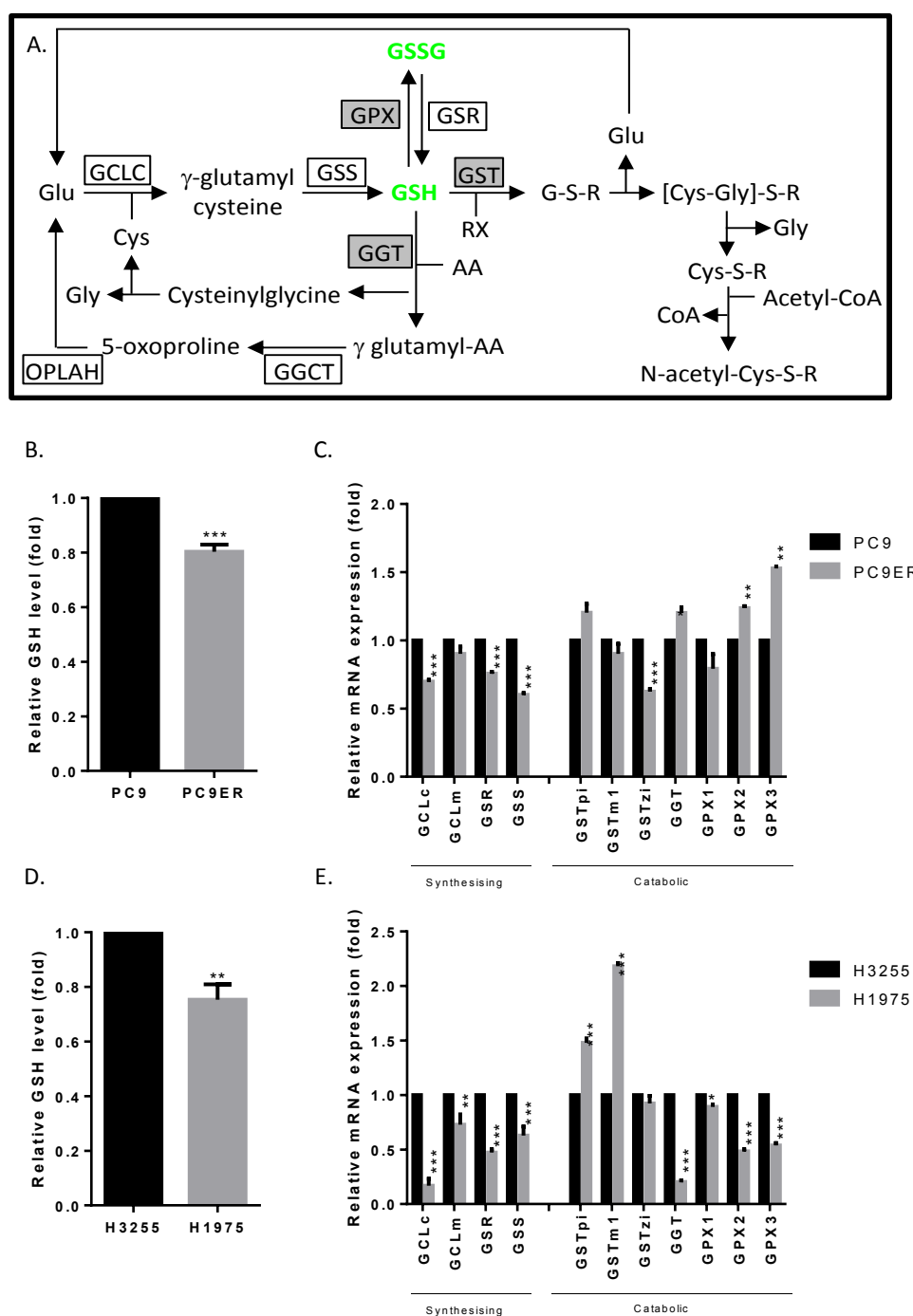


Figure 4.1: Decreased Glutathione in erlotinib-resistant cell lines. (A) Schematics of the GSH metabolic pathway. The white boxes represent the synthesising enzymes and grey boxes represent the catabolic enzymes. GSH levels were determined by colourimetric assay in (B) PC9 and PC9ER or (D) H3255 and H1975. Quantification of GSH metabolic enzymes by qPCR in (C) PC9 relative to PC9ER and (E) H3255 relative to H1975. Figures represent the mean of three biological repeats with each replicate done in technical triplicate ($\pm$ SEM). Statistical analysis was done using Student's *t*-test. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$. I am grateful to Mr William Stokes for carrying out the qPCR arrays in figure C and E.

In an effort to assess whether that these changes in mRNA levels were linked to the presence of the T790M mutation, siRNA against the T790M mutation were introduced in the erlotinib-resistant cell line PC9ER. The knockdown of the T790M mutation by T790M-targeting siRNAs was confirmed by qPCR quantification of EGFR expression (**Figure 4.2A**). This confirmed that the siRNA specifically targeted the EGFR expression in PC9ER cell line carrying the T790M mutation and not in the PC9 cell. The effect of the T790M downregulation on survival to erlotinib treatment was quantified by crystal violet assay (**Figure 4.2B**). This revealed that there was a significant decrease in survival to erlotinib treatment following T790M knockdown. However, it is important to note this did not sensitise PC9ER cells to levels similar these seen in PC9 cells. The targeting of T790M also significantly increased the levels of GSH in the PC9ER cell line (**Figure 4.2C**). This correlated with an increase in the transcription of the GSH synthesizing enzyme GSS and a decrease in the GSH catabolising enzymes GGT and GPX3 (**Figure 4.2D**).

To further study a direct correlation between GSH levels and the T790M mutation, HEK293 cells stably expressing EGFR-L858R, EGFR-L858R/T790M constructs or an empty vector control were used. The levels of EGFR were quantified by qPCR to confirm the mutated EGFR had been efficiently transfected (**Figure 4.3A**). Quantification of the relative GSH levels within these cells revealed a significant decrease with the presence of the T790M mutation (**Figure 4.3B**). This did not correlate with changes in transcription of GSH synthesizing or degrading enzymes (**Figure 4.3C**).

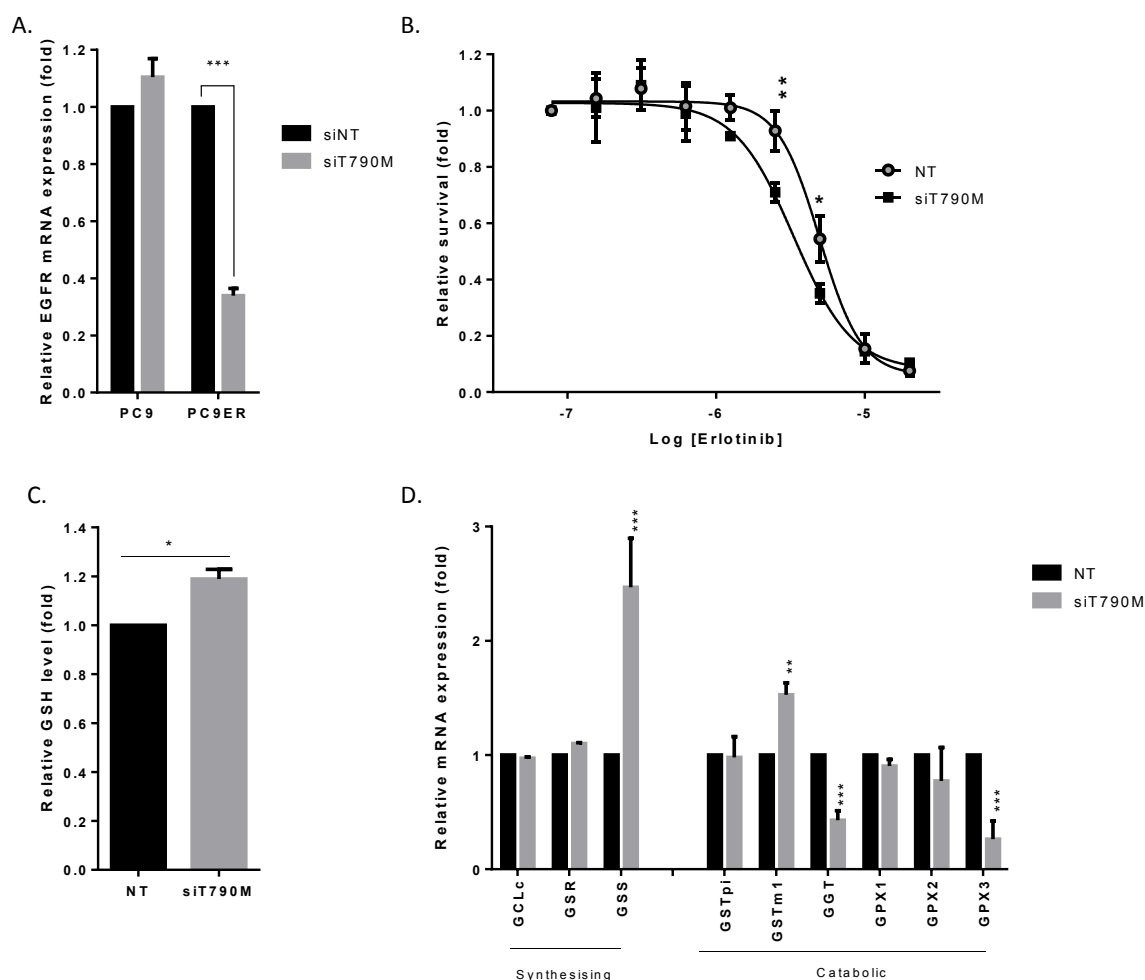


Figure 4.2: siRNA against EGFR T790M increased GSH levels and decreased survival in erlotinib-resistant cell line PC9ER. (A) EGFR mRNA levels were quantified by qPCR in PC9 and PC9ER cells, 48 hours post transfection with siRNA T790M or NT. (B) PC9ER cells were transfected with siRNA against T790M or the NT control. Forty-eight hours post transfection cells were treated with increasing concentrations of erlotinib (48h) prior to fixation and crystal violet staining. The obtained values were normalised against the DMSO control. Statistical analysis was done using two-way ANOVA. (C) GSH levels in PC9ER were quantified by colourimetric assay 48 hours post transfection with siRNA. (D) GSH enzyme mRNA levels were quantified by qPCR 48 hour post transfection with siT790M or siNT. Figures represent the mean of three biological repeats with each replicate done in technical triplicate ($\pm$ SEM). Statistical analysis was done using Student's *t*-test unless otherwise stated. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

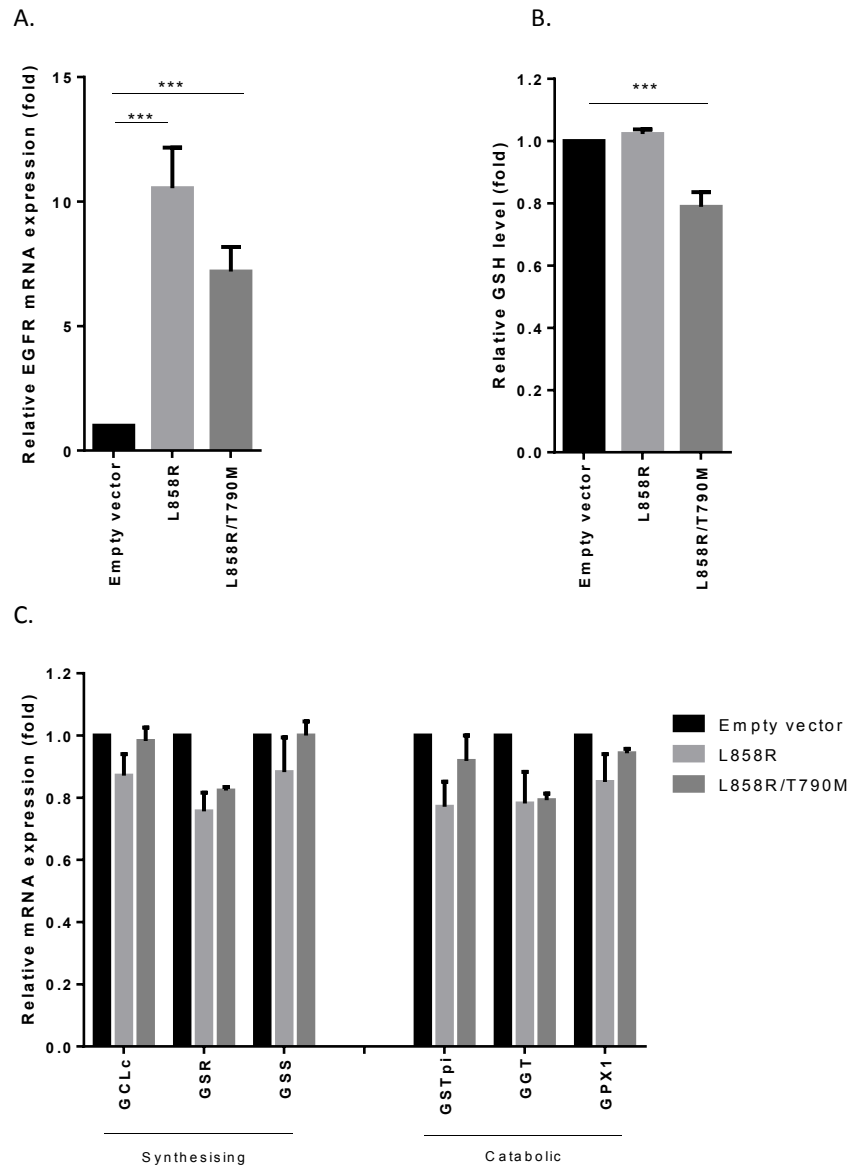


Figure 4.3: HEK293 cells stably transfected with mutated EGFR showed decreased GSH in response to T790M. HEK293 cells were stably transfected with empty vector, EGFR-L858R or EGFR-L858R/T790M followed by the quantification of **(A)** EGFR by qPCR, **(B)** GSH levels by colourimetric assay, **(C)** and GSH metabolic enzyme mRNA levels by qPCR. Figures represent the mean of three biological repeats with each replicate done in technical triplicate ($\pm$ SEM). Statistical analysis was done using Student's *t*-test. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

4.3 Targeting of GSH metabolic enzymes can modulate the response to erlotinib treatment

The change identified in GSH levels between erlotinib-resistant and sensitive cell lines occurred at a transcriptional level. Therefore, it was hypothesised that targeting key enzymes within this pathway may alter erlotinib resistance. Initially, this was done using siRNA to target key enzymes within the synthesis and degradation pathways. The targeting of GSH synthesising enzymes showed a subtle but significant increase in survival of PC9 cells to treatment with erlotinib, without significantly changing survival capability of PC9ER cells (**Figure 4.4A**). Furthermore, the targeting of GSH degrading enzyme significantly increased sensitivity to erlotinib treatment in both PC9 and PC9ER cell lines (**Figure 4.4A**). The knockdown of each of the enzymes was confirmed by qPCR (**Figure 4.4B**). The levels of GSH following knockdown of synthesising and degrading enzymes were quantified by colourimetric assay (**Figure 4.4C**). The targeting of GSH synthesising enzymes significantly decreased GSH levels in both cell lines, with the exception of GSR in PC9ER cells, in which knockdown failed to cause a significant decrease in GSH levels. Furthermore, siRNA-mediated targeting of the degrading enzymes GSTpi and GPX1 significantly increased GSH levels. Taken together this data has highlighted that siRNA-mediated silencing of GSH regulating enzymes is not only capable of modulating GSH levels, but also the response to erlotinib treatment.

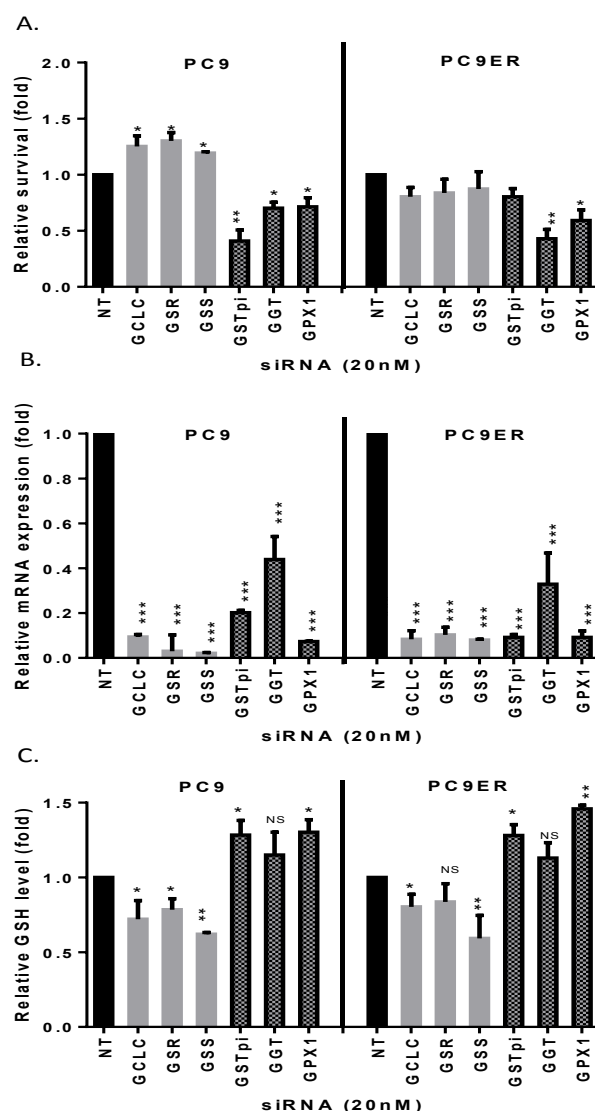


Figure 4.4: Modulation of GSH by siRNA targeting of GSH metabolic enzymes affects the response to erlotinib treatment. (A) PC9 and PC9ER cells were transfected with siRNA targeting GSH synthesizing enzymes (light bars) and catabolic enzymes (dark patterned bars) or NT control (black bars) and cell survival to erlotinib treatment (50 nM) was monitored by crystal violet staining. Data is presented as relative survival to erlotinib normalised to the NT control. (B) siRNA knockdown was confirmed by qPCR. (C) The GSH levels 48 hours post siRNA treatment was determined by colourimetric assay in PC9 and PC9ER. Figures represent the mean of three biological repeats with each replicate done in technical triplicate ($\pm$ SEM). Statistical analysis was done using Student's t-test. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$. I am grateful to Mr William Stokes for carrying out the qPCR arrays in figure B.

To further substantiate these findings and to potentially make our observations clinically relevant we next employed small-molecule inhibitors of GSH synthesizing or catabolising enzymes. Chemical compounds have been designed to regulate GSH levels by targeting enzymes within its metabolic pathway. One such compound is the clinically used diuretic, EA. EA is a potent inhibitor of the GSH degrading enzyme GST. Treatment with EA produced a significant increase in GSH levels in all 4 cell lines (**Figure 4.5A**). This change in GSH was accompanied by increased sensitivity to erlotinib treatment in both PC9ER (**Figure 4.5B**) and H1975 cell lines (**Figure 4.5C**). This was repeated in the PC9 cell line but no significant change was seen following EA treatment (**Appendix figure 8.6**). In addition, treatment with Buthionine sulfoximine (BSO), an inhibitor of the GSH synthesising enzymes GCLc, significantly decreased GSH levels in all four cell lines (**Figure 4.5D**). This decrease in GSH conferred a significant increased resistance to erlotinib in both sensitive cell lines PC9 (**Figure 4.5E**) and H3255 (**Figure 4.5F**). This was repeated in the PC9ER cell line but no significant change was seen following BSO treatment (**Appendix figure 8.6**).

Although this work was predominantly focused on erlotinib resistance it was of interest to see if EA could also sensitise resistant cells to other TKIs used in the clinic to treat patients with EGFR mutant positive lung cancers. Gefitinib, like erlotinib is a first-generation small molecule, reversible EGFR TKI. The T790M mutation confers considerable resistance to gefitinib (**figure 4.6 A and B**) and is accompanied by a decrease in GSH (**Figure 4.6 C**). EA could significantly sensitize both PC9ER (**Figure 4.6D**) and H1975 (**Figure 4.6E**) to gefitinib. This was associated with an increase in GSH levels (**Figure 4.6 F**).

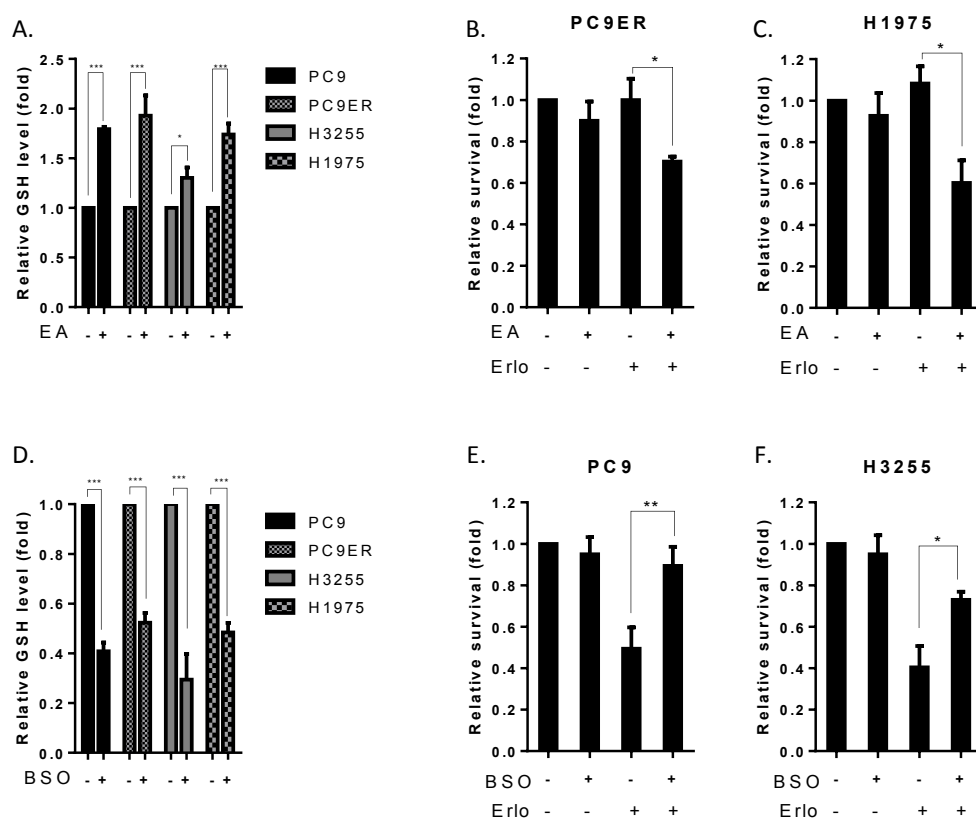


Figure 4.5: Modulation of GSH by EA and BSO treatment effected survival to erlotinib. (A) PC9, PC9ER, H3255 and H1975 cells were treated with EA and GSH levels were subsequently quantified by colourmetric assay. (B) PC9ER and (C) H1975 were treated with EA alone and in combination with Erlotinib for 48 hours and survival were monitored by crystal violet assay. (D) PC9, PC9ER, H3255 and H1975 cells were treated with BSO and GSH levels were subsequently quantified by colourmetric assay. (E) PC9 and (F) H3255 cells were treated with BSO alone and in combination with erlotinib for 48 hours and survival were monitored by crystal violet assay. Data are the relative responsiveness to erlotinib normalised to vehicle (-; DMSO). Figures represent the mean of three biological repeats with each replicate done in technical triplicate ($\pm$ SEM). Statistical analysis was done using Student's *t*-test. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$. I am grateful to Mr William Stokes for his carrying out survival assays shown in B, C, D and E.

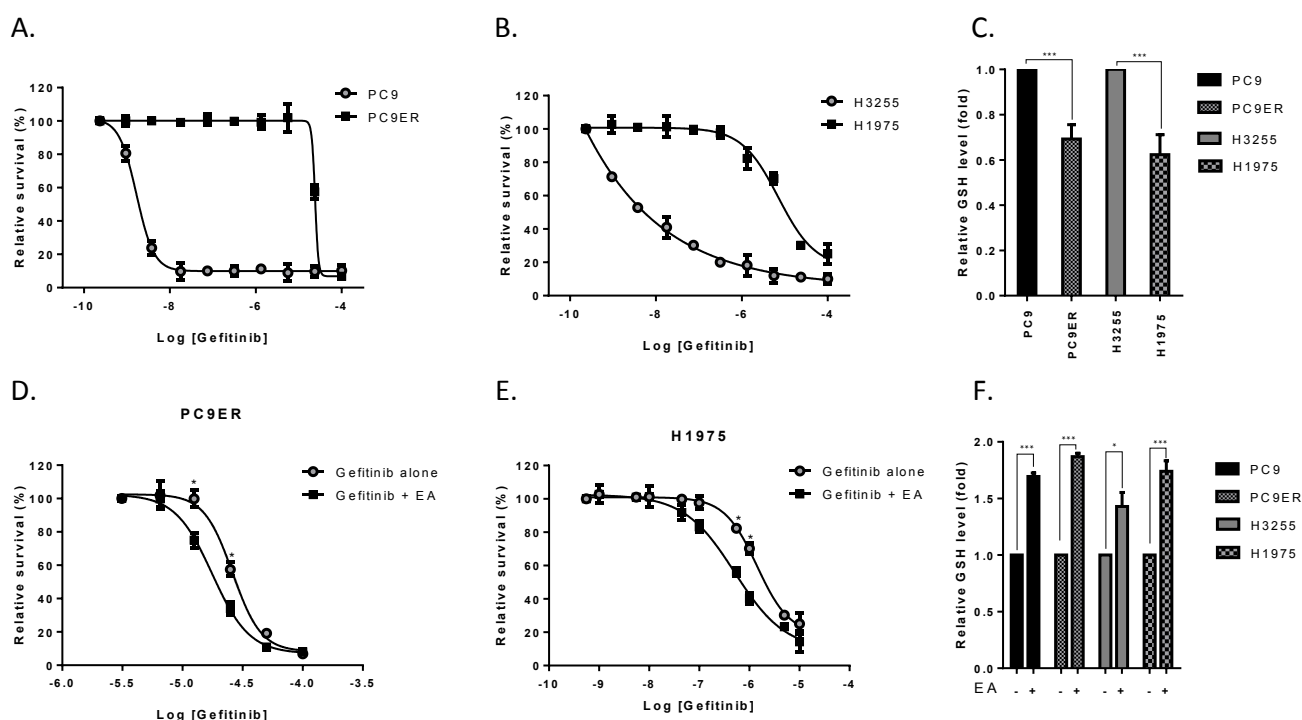


Figure 4.6: Modulation of GSH and survival by treatment with EA increased sensitivity to gefitinib. Cell survival was measured in response to increasing concentrations of gefitinib treatment (48 hours) and quantified by crystal violet staining in **(A)** PC9/PC9ER and **(B)** H3255/H1975. **(C)** PC9, PC9ER, H3255 and H1975 cells were treated with EA and GSH levels were subsequently quantified by colourmetric assay. **(D)** PC9ER and **(E)** H1975 were treated with EA alone and in combination with gefitinib for 48 hours and survival were monitored by crystal violet assay. **(F)** PC9, PC9ER, H3255 and H1975 cells were treated with EA and GSH levels were subsequently quantified by colourmetric assay. Data are the relative responsiveness to Gefitinib normalised to vehicle (-; DMSO). Figures represent the mean of three biological repeats with each replicate done in technical triplicate ($\pm$ SEM). Statistical analysis was done using Student's *t*-test. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

We next examined third generation TKIs that irreversibly bind to the T790M mutated receptor to test whether EA might increase sensitivity to these agents. The most successful of these is AZD9291 (Osimertinib) which inhibits both the EGFR sensitizing mutation, as well as, the resistant T790M (Ward et al., 2013, Cross et al., 2014). AZD9291 has shown promise in clinical trials, but development of resistance occurs through, for example, the occurrence of additional mutations (Eberlein et al., 2015b, Ercan et al., 2015). Using PC9, PC9ER (Figure 4.7A) and H3255 and H1975 cell lines (Figure 4.7B), survival to AZD9291 treatment was quantified to confirm sensitisation of T790M containing cell lines. As previously reported, both PC9ER and H1975 cell lines were highly sensitive to AZD9291 compared to that previously seen for erlotinib and gefitinib (Cross et al., 2014, Eberlein et al., 2015a). The levels of GSH were quantified in all 4 cell lines to confirm that GSH levels were indeed decreased in resistant cell lines (Figure 4.7C). Treatment with EA resulted in a significant increase in the GSH levels (Figure 4.7F), but did not further sensitize the resistant cell lines PC9ER (Figure 4.7D) and H1975 (Figure 4.7 E) to AZD9291.

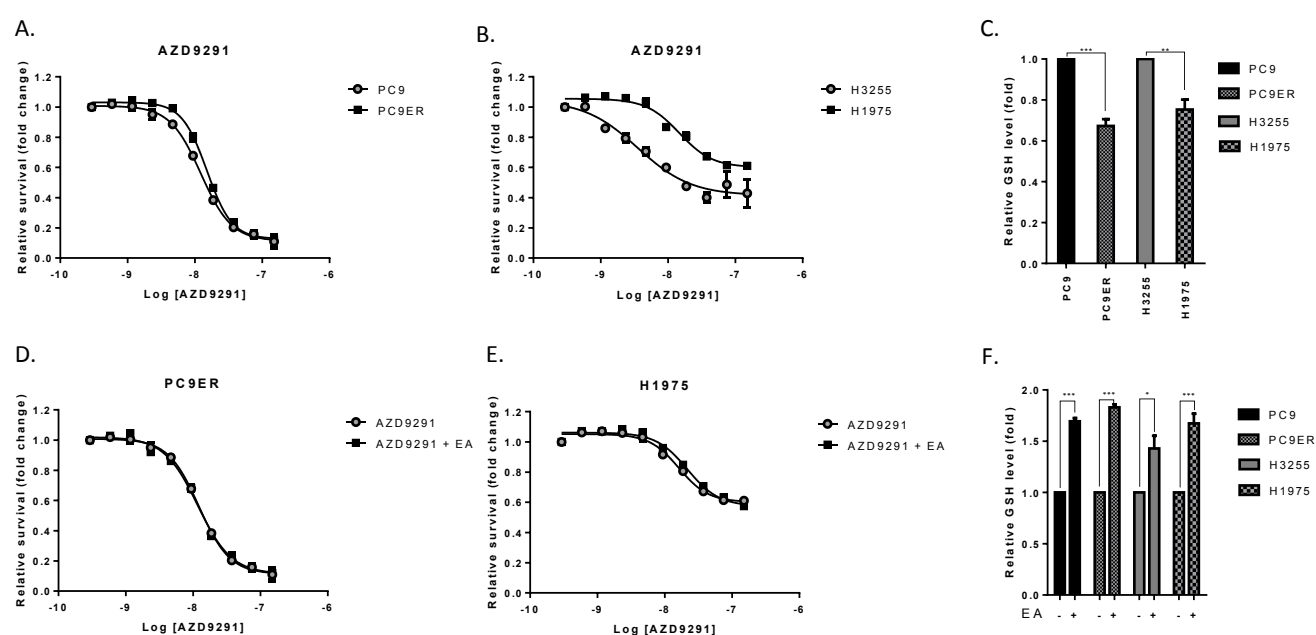


Figure 4.7: Modulation of GSH with GST inhibitor EA did not affect survival to AZD9291. Cell survival was measured in response to increasing concentrations of AZD9291 treatment (48 hours) and quantified by crystal violet staining in (A) PC9 and PC9ER, (B) H3255 and H1975 cell lines. (C) The GSH levels in PC9, PC9ER, H3255 and H1975 cell lines were subsequently quantified by colourimetric assay. (D) PC9ER and (E) H1975 were treated with EA alone and in combination with AZD9291 for 48 hours and their survival were monitored by crystal violet assay. (F) PC9, PC9ER, H3255 and H1975 cells were treated with EA and GSH levels were subsequently quantified by colourimetric assay. Data are the relative responsiveness to AZD9291 normalised to vehicle (-; DMSO). Figures represent the mean of three biological repeats with each replicate done in technical triplicate ($\pm$ SEM). Statistical analysis was done using Student's *t*-test. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

4.4 Systemic administration of Ethacrynic acid re-sensitises PC9ER NSCLC tumours to erlotinib in a mouse xenografts model.

We have shown that modulating levels of GSH can influence the responsiveness to erlotinib treatment *in vitro*. In particular, the treatment of resistant cell lines PC9ER and H1975 with GST inhibitor, EA, significantly sensitized the cells to erlotinib. Hence, it is hypothesised that co-administration of physiologically relevant doses of EA may improve the responsiveness of EGFR TKI-resistant tumours to erlotinib *in vivo*. Therefore, PC9ER cells were injected subcutaneously into the flank of BALB/c *nude* mice and tumours were allowed to form. The mice were then treated daily with erlotinib and EA alone or in combination. The treatment with either compound alone could not prevent exponential growth of the tumours. However, co-administration of the erlotinib and EA significantly inhibited the rate of tumour growth by 60 %, showing tumour volumes of $\leq 300 \text{ mm}^3$ at 25 days, while those treated with either drug alone all had a far more extensive tumour burden (**Fig 4.8A**). The survival of the mice was monitored for 27 days, during which the mice were sacrificed once the tumours had reached 300 mm^3 . Mice which underwent dual treatment with both EA and erlotinib showed an increase in survival (**Figure 4.8B**). Following sacrifice of the mice, the tumours were collected and snap-frozen for quantification of their GSH content. The systemic co-administration of EA and erlotinib resulted in an increase of intra-tumoural GSH levels when compared to those treated with erlotinib alone (**Fig 4.7C**). Taken together, these data suggest that increasing intra-tumoural GSH levels, through inhibition of GST may be a viable strategy for the management of erlotinib-resistant disease in humans.

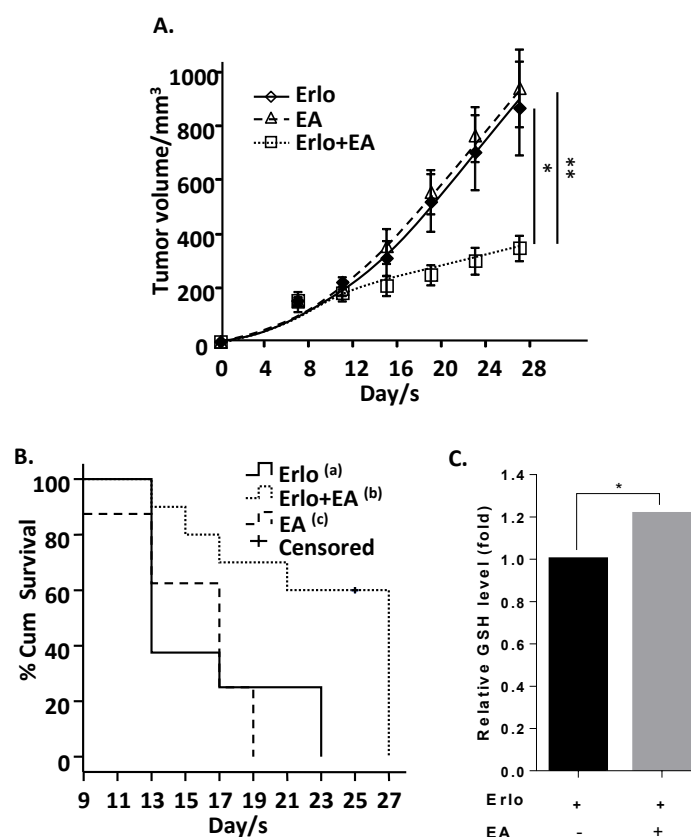


Figure 4.8: Systemic EA administration re-sensitised PC9ER mouse xenografts to erlotinib. Nude mice (n=10/condition) were injected subcutaneously with PC9ER cells. Once tumours had reached 100 mm³ treatments were initiated. Mice randomised into 3 groups (n=10) and treated by intraperitoneal injection of physiologically relevant doses as follows 25 mg kg⁻¹ per day erlotinib/0.5% w/v methylcellulose and/or 6 mg kg⁻¹ per day EA/1% Tween 80 in distilled water. Such treatments were administered daily from day 7 to 26. **(A)** Tumour volume was measured for 27 days and statistical analysis was done using ANOVA. * P ≤ 0.05, ** P ≤ 0.01 and *** P ≤ 0.001. **(B)** Survival was monitored for 27 days, the end-point events occurred when the volumes of tumours had reached 300 mm³. Statistical analysis was done using log-Rank test, P= ab<0.01, P=bc<0.01. **(C)** Intra-tumoral GSH levels were measured *ex vivo* by colorimetric assay following last treatment. Statistical analysis was done using student t-test. * P ≤ 0.05, ** P ≤ 0.01 and *** P ≤ 0.001. *In vivo* studies have been carried out by Hongde Li at the Chinese Academy of Sciences, China.

4.5 mRNA levels of key GSH synthesising enzymes decrease in patients following onset of EGFR TKI-resistance

Biopsies in NSCLC patients following the onset of EGFR TKI-resistance are rare, although the practice is becoming more common. Therefore, the patient numbers in this study are low and represent a proof-of-concept that requires further validation. The mRNA levels of GSH synthesising enzymes were quantified in two patients before (pre-T790M) and after (post-T790M) onset of T790M mediated resistance (**Figure 4.9A**). This indicated a decrease in the expression of GSS, GSR, GCLc and GCLm following the appearance of the T790M mutation. To further validate these changes RNA-Seq was carried out in four unrelated patients with (Patient 1-4) and without (patient 5-8) the presence of the T790M mutation. This revealed that patients with the T790M mutation had a decreased expression of a number of GSH synthesising enzymes (**Figure 4.9 B**). The most prominent change was a decreased level of the GSH synthesising enzyme GCLc in all patients with the T790M mutation. The levels of GCLm, GSR and GSS show decrease in some but not all patients tested. Due to the limited number of patients sampled, this is not conclusive and would require further validation. Taken together our data suggest that probing for the expression of these enzymes in biopsies may aid to predict the responsiveness of patients to erlotinib treatment.

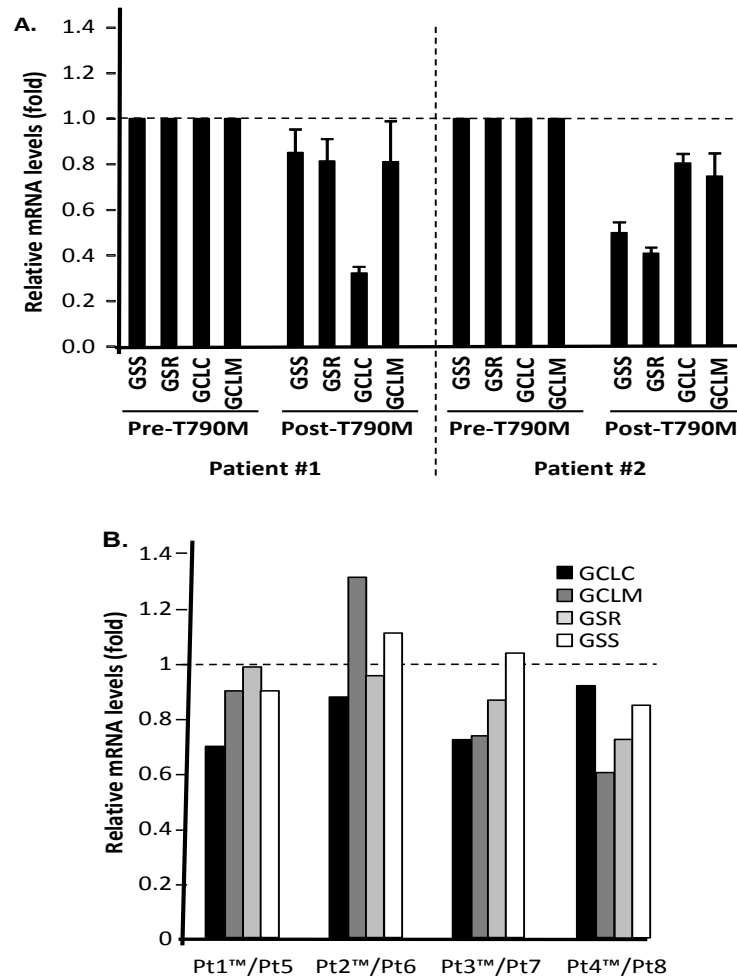


Figure 4.9: mRNA levels of GSH synthesising enzymes are decreased in EGFRm/T790M patient tumours. (A) mRNA levels of GSH metabolic enzymes indicated, were quantified via qPCR in two patients before (pre-T790M) and after (post-T790M) the onset of T790M-mediated resistance. Quantification was carried out in technical triplicate error bars represent SEM. **(B)** RNA-Seq of 4 pairs of unrelated patients Pt 1-4 T790M was absence and in Pt 5-8 the T790M mutation was present. Data in T790M samples are normalised to those in the corresponding non-T790M samples. I would like to thank Elza de Bruin the data in figure A and Guus J.J.E. Heynen for the data in figure B.

4.6 Discussion

Erlotinib and other EGFR TKIs have been very successful in treatment of NSCLC patients with active EGFR. However, development of secondary mutation such as T790M has prevented these inhibitors from significantly increasing the long-term survival of patients. Accumulating evidence suggests that, while EGFR mutations drive alterations in metabolic signatures, the majority of treatments fail to target these changes in clinical settings or *in vivo* models (Makinoshima et al., 2015, Serizawa et al., 2014, Xie et al., 2016). Therefore, we employed ¹H-NMR metabolic profiling of two NSCLC erlotinib-sensitive/resistant cell line pairs in the hope of identifying novel mechanisms of resistance. These NSCLC cell lines were chosen based on three criteria. The first is that both resistant cell lines contain the T790M resistance mutation. Second, PC9 and PC9ER are relatively isogenic, as PC9ER were derived through erlotinib selection of PC9 whereas, H3255/H1975 are genetically unrelated. The final Criterion was that the initial activating mutation was different in each of the cell line pairs; PC9/PC9ER carrying the Δ E746-A750 activating deletion, while H3255/H1975 cells share a L858R point mutation. This would maximise the chances of identifying metabolic changes which occur in both cell lines to be solely dependent on the T790M mutation and independent of additional genetic factors. One of the most significant changes identified was the decrease in GSH levels in erlotinib-resistant cell lines compared to their sensitive counterpart. This pathway is well known to be involved in drug resistance in cancer cells. However, the majority of studies suggest drug resistance is associated with an increase in GSH (Backos et al., 2012, Bracht et al., 2006, Ripple et al., 1993). GSH has been shown to interact directly with certain drug molecules leading to their cellular export through a GST-dependent pathway (Provost et al., 2009, Rappa et al., 1997, Zaman et al., 1995). In addition, GSHs role as an antioxidant molecule decreases the levels of oxidative species, which are often required for the action of these compounds. Interestingly a study by Schwartz and colleagues has shown that cysteine oxidation is a posttranslational modification which can occur on EGFR (Schwartz et al., 2014). This study showed that EGFR-L858R and EGFR-L858R/T790M proteins oxidised by glutathione and H₂O₂, resulted in oxidation exclusively at Cys797 (Schwartz et al., 2014). This is of particular interest as covalent inhibitors are known to target this residue. Glutathiolation resulted in a weaker affinity of reversible quinazoline drugs, Quinazoline covalent inhibitors and pyrimidine inhibitor WZ4002 to both EGFR-L858R and L858R/T790M (Schwartz et al., 2014). Therefore, a decrease in GSH in erlotinib-resistant cell lines required further investigation.

The levels of GSH were initially confirmed to be down regulated by the use of a colourmetric assay (**Figure 4.1 B and D**). This decrease in levels of GSH could be directly attributed to transcriptional changes in enzymes involved in GSH metabolism (**Figure 4.1 C and E**). These changes were confirmed to be a direct consequence of the T790M mutation. The siRNA targeting of EGFR

harbouring the T790M mutation, increased GSH levels (**Figure 4.2C**) and sensitivity to erlotinib (**Figure 4.2B**) as previously reported (Chen et al., 2013). Due to the T790M mutation being a point mutation confirmation of knockdown proved difficult. Comparison of EGFR transcript levels between PC9 and PC9ER cells following treatment with T790M-targeting siRNAs confirmed knockdown of the mutated transcript in PC9ER cells (**Figure 4.2 A**). Other methods that could be used to further confirm selective knockdown such as deep sequencing of cDNA or TaqMan PCR. A further limitation of this experiment was the absence of a wt-control. Although knock-down of the T790M mutated EGFR showed an increase in GSH levels and decreased survival to erlotinib treatment, we cannot discount the possibility that a similar result may be seen from the knockdown of wt-EGFR. Therefore, further experimentation including a wt control is needed to validate this result. It would also be of interest to see the effect of transfection of the T790M mutant EGFR in the PC9 cell line.

To try and further demonstrate direct correlation between the T790M resistance mutation and the associated changes in GSH, HEK-293 cells stably transfected with mutant EGFRs were utilised (**Figure 4.3**). The levels of GSH were decreased in the presence of the T790M mutation (**Figure 4.3B**). However, this did not appear to be regulated at a transcriptional level (**Figure 4.3C**). Therefore, this change may be due to changes in additional mechanisms of GSH regulation such as a change in the availability of precursor molecules cysteine and sulfur amino acids (Lu, 2009). Indeed, while cysteine availability is largely dependent on diet, it can also be dependent on the transport of cysteine into the cell *via* the amino acid transport systems (ASC) (Bannai and Tateishi, 1986).

Comparative analysis revealed that changes in GSH levels could be attributed to transcriptional regulation of GSH metabolising enzymes. Our experiments demonstrated that the inhibition of GSH synthesising enzymes by either RNA interference (**Figure 4.4**), or small molecular compounds (**Figure 4.5**) in sensitive PC9 cells rendered these cells resistant to erlotinib treatment. Conversely, the inhibition of GSH-degrading enzymes sensitised PC9ER cells to erlotinib (**Figure 4.4 and 5.5**). The use of EA co-treatment also sensitised cells to gefitinib, although to a lesser extent to the sensitisation observed in the case of erlotinib (**Figure 4.6**). The survival of all cell lines treated with the third generation inhibitor AZD9291 were not affected by EA co-treatment (**Figure 4.7**). This may be in part due to the fact that AZD9291 is a monoanilino-pyrimidine compound and as such is structurally distinct, it therefore has a pharmacologically differentiated profile from earlier generation of EGFR TKIs (Cross et al., 2014). Alternatively, it could be speculated that a much higher level of changes in GSH is required to see any discernible effect in cell survival to AZD9291.

Finally, the therapeutic benefit of modulation of the GSH pathway was assessed *in vivo*. This study used a physiologically relevant dose of the GST inhibitor, EA, which was systemically administered

into nude mice. This showed a significant reduction in tumour burden associated with a decrease in intra-tumoural concentration of GSH (**Figure 4.8**). EA has a limited toxicity and is widely used as a loop diuretic in the clinic (Somberg and Molnar, 2009). Therefore, this could rapidly be translated into clinical practice. Moreover, EA has previously shown to improve clinical outcome when used in combination with chemotherapies such as alkylating agents, due to EAs role in the prevention of GST-mediated export of these compounds (O'Dwyer et al., 1991, Somberg and Molnar, 2009). Our data therefore suggest that EA may not only improve treatment of NSCLC patients with EGFR activating mutations, but also improve their response to follow-on chemotherapy. It is important to note there are some limitations within our in vivo experiments which need to be addressed. There is a lack of vehicle only control in figure 4.8, it would be advantageous to show vehicle control in any future experiments. Furthermore, as an additional control, PC9 xenografts could have been included, which would have given a clearer idea if this treatment would enhance the effectiveness of EA with erlotinib in single mutants. However, cell experimentation of EA and erlotinib co-treatment in PC9 showed limited change in survival to treatment, when compared to erlotinib alone (**Appendix figure 8.6**).

There are some questions which remain unanswered and require further research including if this decrease in GSH level only occurs downstream of the T790M or if this is a common feature of other erlotinib resistance pathways. It is also important to understand the exact mechanism by which decreased GSH facilitates erlotinib resistance. The treatment of the PC9ER cell line with GST-inhibitor, EA, resulted in increased sensitivity to erlotinib treatment which suggests this is via a GST mediated mechanism. However, this requires further confirmation by experimentation such as using CRISPR GST mutants to identify if there is an increased sensitivity to erlotinib. It would also be of interest to see if CRISPR GST mutants treated with erlotinib with and without EA show any change in survival to erlotinib thereby confirming that this is a GST mediated mechanism.

Overall this data demonstrates that GSH abundance in erlotinib-resistant cell lines is decreased, this appears to be regulated *via* modulation of transcriptional activity of GSH metabolising enzymes. Furthermore, targeting of enzymes within the GSH metabolic pathway with both siRNA and small-molecule compounds could directly influence sensitivity to erlotinib. *In vivo* studies further suggested that decreasing the intracellular GSH in erlotinib-resistant cells may provide a novel therapeutic strategy to improve survival of patients with tumours driven by T790M-EGFR.

Chapter 5.0 Erlotinib-resistant cells have altered regulation of the NRF2 pathway

5.1 Introduction

This thesis has shown that GSH appears to be modulated downstream of EGFR. Indeed, GSH levels are decreased upon expression of the T790M mutant EGFR. A number of previous studies have shown that EGFR is able to regulate the transcription factor Nuclear factor (erythroid-derived 2)-like 2 (NRF2) that is responsible for the transcription of GSH metabolic enzymes (Huo et al., 2014, Yamadori et al., 2012). A study by Yamadori showed that PC9 cells, which contained an activated version of EGFR, have constitutively activated NRF2. Furthermore, they showed that directly targeting EGFR using the inhibitor AG1478, as well as targeting downstream mediators such as MEK1/2 and to a lesser extent PI3K, resulted in a decreased expression of NRF2. In addition, Huo and colleagues have shown that EGFR is able to regulate NRF2 activation and protein stability (Huo et al., 2014). This occurs through nuclear interaction of EGFR with the negative NRF2 regulator, Kelch-like ECH-associated protein 1 (KEAP1) (Huo et al., 2014).

NRF2 is a Cap 'n' collar-basic leucine zipper (CNC-bZIP) transcription factor, known to be able to trans-activate the ARE in target genes such as GCLC (Ohtsuji et al., 2008). KEAP1 has been demonstrated to bind to the Neh 2 region of NRF2, resulting in its ubiquitination and degradation (Itoh et al., 1999b). Indeed, NRF2 binds to KEAP1 via two well defined binding motifs within the Neh2 domain, ETGE with high affinity and DLG with lower affinity (Tong et al., 2006a). The ETGE motif is required for the binding of NRF2 to KEAP1, while the DLG motif positions lysine residues within the Neh2 region of NRF2 to promote ubiquitination (Tong et al., 2006b). NRF2 is sequestered in the cytosol by KEAP1 homodimers that act as an adaptor for cullin-3 (Cul3) dependent ubiquitination of NRF2 (Kobayashi et al., 2004). The poly-ubiquitination of NRF2 results in its degradation via the 26S proteasome (Kobayashi et al., 2004). The overexpression of NRF2, results in its accumulation in the nucleus (Itoh et al., 2010b). However, when co-expressed with KEAP1, NRF2 is localised to the cytosol and the presence of a panel of electrophiles can restore NRF2 nuclear localisation (Itoh et al., 2010b). This highlights the mechanism by which KEAP1 sequesters NRF2 in the cytoplasm until oxidative or electrophilic insult dissociates NRF2 from KEAP1, allowing it to translocate to the nucleus and transcribe target genes (Itoh et al., 2010b). Indeed, oxidative stress or electrophiles modify cysteine residues in KEAP1, resulting in a conformational change and release of NRF2 at the DLG, but not the ETGE motif (Bryan et al., 2013). Therefore, KEAP1 is saturated by NRF2, as it is now unable to be degraded. The newly synthesised NRF2 then accumulates and translocates to the nucleus, where it recruits and heterodimerizes with one of 3 small musculoaponeurotic fibrosarcoma (MAF) proteins which enable it to bind to the AREs (Bryan et al., 2013, Itoh et al., 1997).

Additional binding partners of KEAP1 have since been discovered such as the substrate adapter protein Sequestosome1 (SQSTM1) and partner and localizer of BRCA2 (PALB2) (Komatsu et al., 2010, Lau et al., 2010, Ma et al., 2012). Interestingly, the SQSTM1 gene contains an ARE sequence and can therefore be transcribed by NRF2, thus creating a positive feedback loop (Jain et al., 2010). Jain *et al* went on to suggest that SQSTM1 may be able to displace NRF2 and target KEAP1 for degradation thereby, allowing NRF2 to translocate to the nucleus and transcribe AREs – containing target genes (Jain et al., 2010).

This chapter will examine if the NRF2 is differentially regulated between erlotinib-sensitive and resistant cell lines as a result of the T790M mutation in EGFR. Initially examining the localisation/activation of NRF2 and its interacting partner KEAP1, as well as additional KEAP1 binding partners. Our data shows that the NRF2-KEAP1 pathway is differentially regulated by the presence of the T790M mutation. Furthermore, targeting this pathway with the use of RNA interference is able to modulate not only GSH levels but the response to erlotinib treatment.

5.2 Changes in NRF2 regulation in erlotinib-resistant cell lines

GCLC, GSS and GSR are transcriptional targets of NRF2, a downstream target of EGFR (Huo et al., 2014). Therefore, in order to explain the decrease in the expression of these enzymes in erlotinib-resistant cells, it was hypothesised that NRF2 activity might be impaired in EGFRm/T790M cells. **Figure 5.1 A** summarises the negative regulation of NRF2 by KEAP1 and how KEAP1 itself is negatively regulated by several other proteins including PALB2 and SQSTM1 (**Fig 5.1B**), a process described in detail in the introduction to this thesis (Hast et al., 2013). Analysis of nuclear-cytoplasmic fractions in our cell lines revealed that while NRF2 or KEAP1 localisation remained unchanged between PC9 and PC9ER cells, H3255 cells showed higher nuclear expression of NRF2 compared to H1975 (**Figure 5.2 A and C**). This was accompanied by an increase in cytoplasmic KEAP1 expression in H1975 compared to H3255 cells (**Figure 5.2 A and D**). Although these results alone may explain the changes identified in the GSH pathway between H3255 and H1975, they do not account for the changes identified in PC9 and PC9ER cells. However, PALB2 levels were lower in PC9ER compared to PC9 cells (**Figure 5.2 B and F**). In addition, SQSTM1, a known target for NRF2 mediated transcription, was down-regulated in both the erlotinib-resistant cell lines compared to their sensitive counterparts (**Figure 5.2 B and E**). Collectively, these data suggest that reduced NRF2 activity may lead to the observed decreased transcription of GSH synthesizing enzymes seen in the erlotinib-resistant as opposed to sensitive cell line pairs.

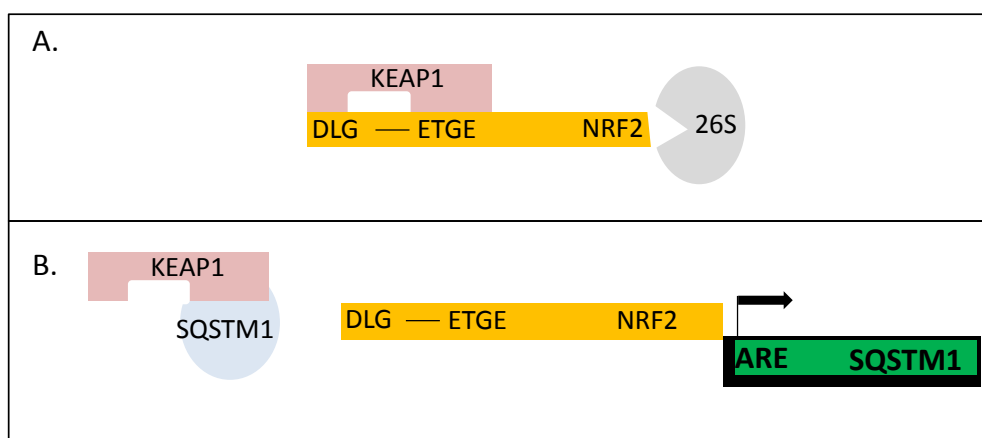


Figure 5.1: NRF2 regulation via KEAP1 mediated pathway. (A) In the cytoplasm KEAP1 is able to bind to NRF2 via two binding domains consisting of the amino acid sequences DLG and ETGE. KEAP1 binding results in poly-ubiquitination via the cul3 E3 ligase and is consequently degraded by the 26S proteasome. **(B)** NRF2 binding to KEAP1 may be counteracted by KEAP1 binding to additional binding partners such as SQSTM1. When NRF2 is not bound to KEAP1 it is able to translocate to the nucleus. NRF2 heterodimerizes with small Maf proteins which facilitate its ability to bind to the Antioxidant Response Element (ARE) in the promoter region of NRF2-regulated genes. These genes include SQSTM1 creating a positive feedback loop.

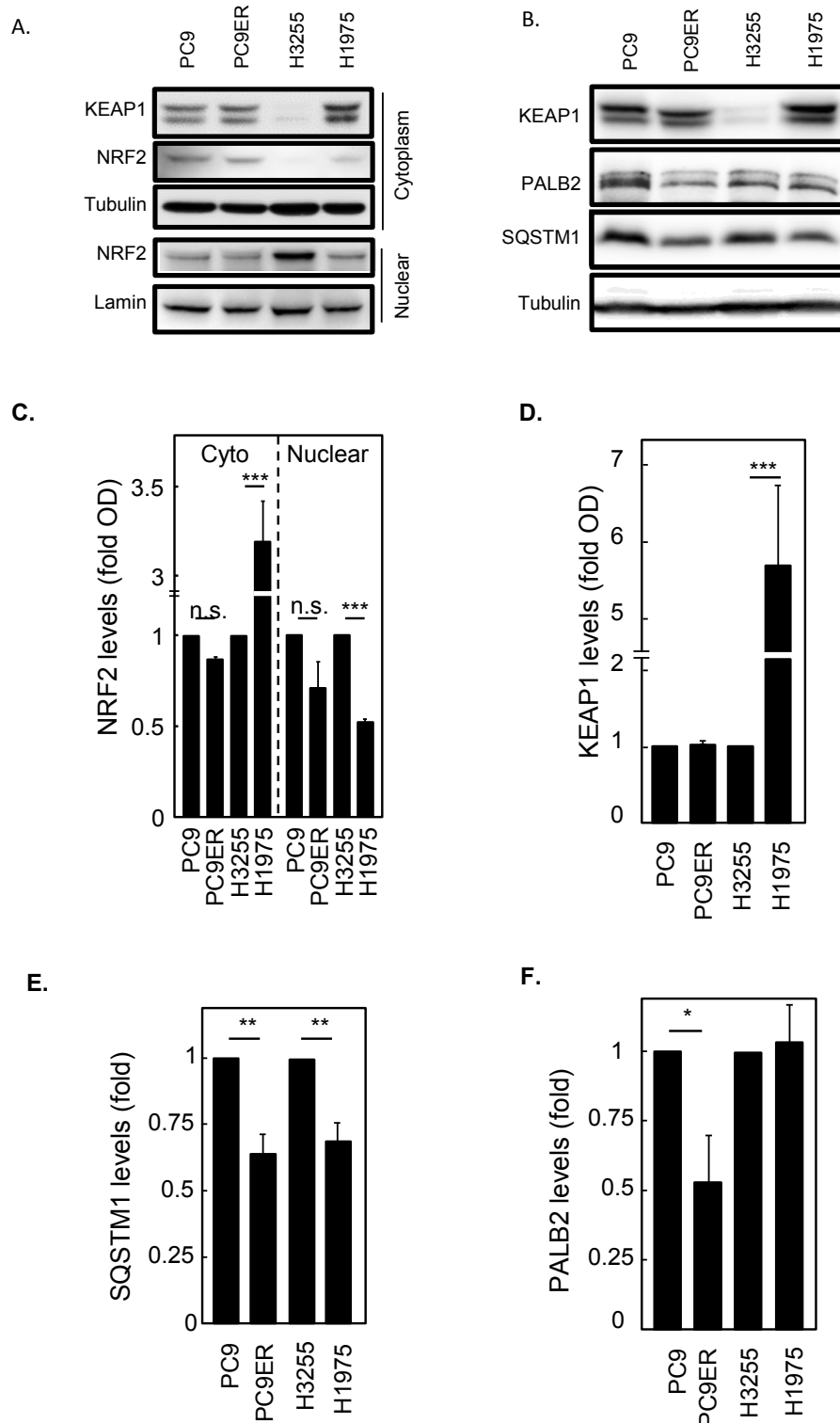


Figure 5.2: NRF2 signalling is altered in erlotinib-resistant cells. (A, B) Sub-cellular fractionation followed by SDS-PAGE/Western blotting of PC9, PC9ER, H3255 and H1975. Detection of Lamin and Tubulin was used as loading controls for nuclear and cytoplasmic fractions, respectively. Data shown are representative of three biological repeats. Western blots were quantified using the optical densitometry function in ImageJ and the results obtained for (C) NRF2, (D) KEAP1, (E) SQSTM1 and (F) PALB2 normalised to the corresponding control cell line and plotted. For PC9ER, PC9 cells were used as control while for H1975, H3255 were used for normalisation. Statistical analysis; t-test. ***; $p \leq 0.001$, ** $P \leq 0.01$ and * $p \leq 0.05$. I am grateful to Dr Rajat Roy for his carrying out SDS-PAGE/Western blotting in figures A and B.

Our results suggest that mutant EGFR containing T790M is associated with reduced NRF2-mediated transcription of GSH enzymes levels. To test this hypothesis more directly, the active (L858R) or active/resistant (L858R/T790M) EGFR mutants were over-expressed in the HEK293 cells that have low endogenous expression of EGFR (**Figure 5.3 A**), and downstream changes in the NRF2 pathway were investigated. The levels of GSH were decreased in cells expressing the T790M EGFR mutation compared to the single L858R mutant or empty vector control (**Figure 5.3 B**). This change in expression was associated with reduced PALB2 and SQSTM1 expression (**Figure 5.3 C**). Furthermore, siRNAs targeting T790M-mutated EGFR in PC9ER cells were used to independently validate the direct role of the resistance mutation in regulating the NRF2 pathway. To confirm specificity of the T790M siRNA, mRNA levels of total EGFR was accessed in both PC9 and PC9ER cell lines (**Figure 5.3 D**). A significant decrease in the levels of EGFR mRNA was observed exclusively in PC9ER cells, suggesting specific silencing of the T790M-mutated EGFR (**Figure 5.3D**). The GSH levels in PC9ER cells showed a significant increase following siRNA-mediated knockdown of T790M with two independent oligonucleotide sequences (**Figure 5.3 E**). Furthermore, this was accompanied by an increase in SQSTM1, PALB2 and NRF2 expression (**Figure 5.3 F**). Our results indicate that lower GSH levels in erlotinib-resistant EGFR mutant NSCLC cells are a direct consequence of the T790M mutation, which is accompanied by impairment of NRF2 activity.

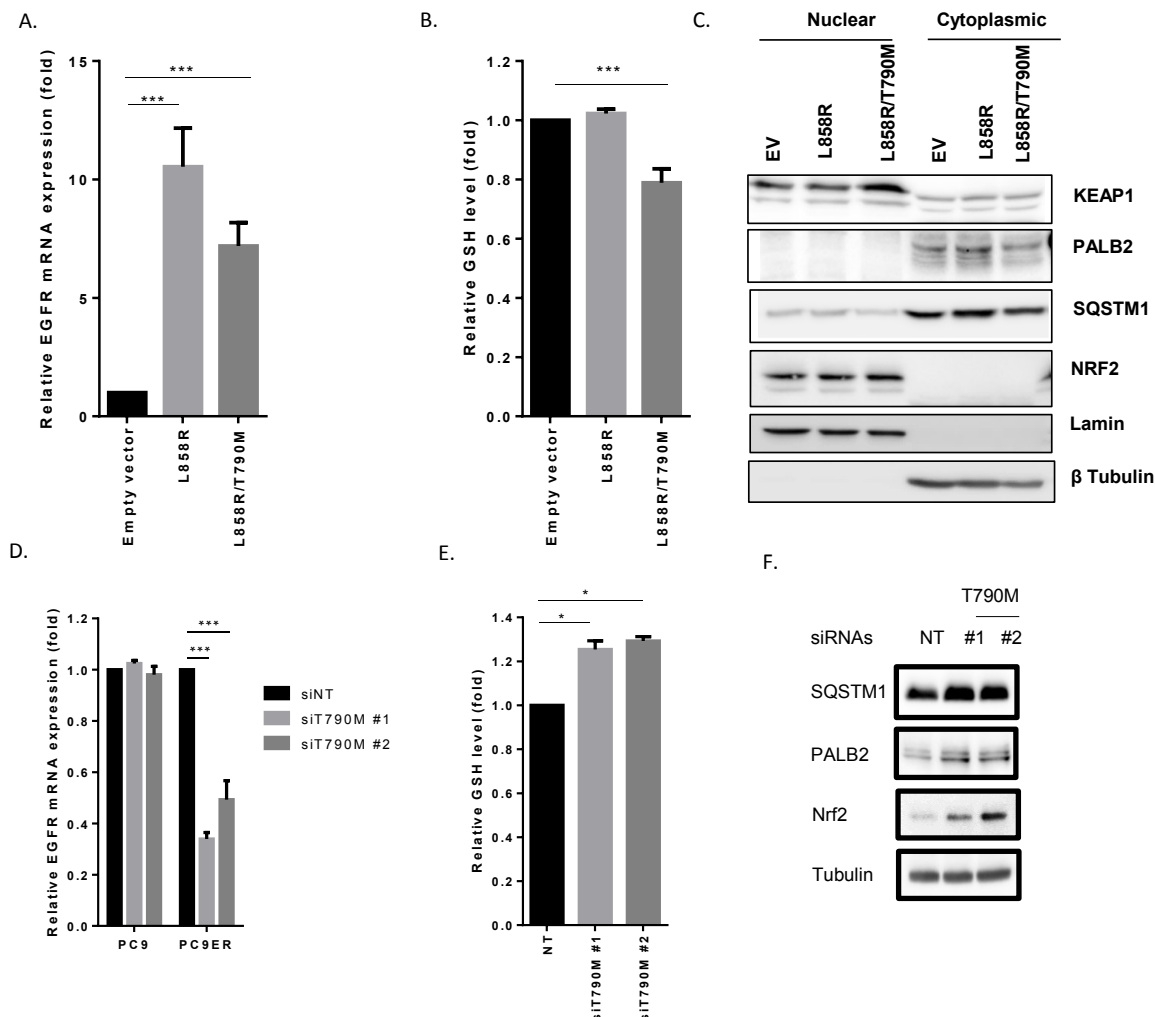


Figure 5.3 The T790M mutation directly affects NRF2 regulation. HEK293 cells were stably transfected with empty vector, EGFR-L858R or EGFR-L858R/T790M (48 hours). **(A)** EGFR levels were quantified by qPCR. **(B)** GSH levels were quantified by colourimetric assay. **(C)** Sub-cellular fractions from HEK293 cells stably transfected with L858R, L858R/T790M or empty vector control cells were analysed by SDS-PAGE/Western blotting for the indicated proteins. Detection of Lamin and Tubulin were used as loading controls for nuclear fractions cytoplasmic fractions, respectively. PC9 and PC9ER cells were transfected with 2 independent siRNA oligos against the siT790M #1 and #2 or NT control (48 hours) and **(D)** EGFR levels were quantified by qPCR. PC9ER cells were transfected with siT790M #1 and #2 or NT control (48 hours) and **(E)** GSH levels were quantified by colourimetric assay. **(F)** Protein lysates were analysed 48 hours post transfection via SDS-PAGE/Western blotting for SQSTM1, PALB2 and NRF2. Detection of Tubulin was used as loading controls. Western blots are representative of 3 biological repeats. qPCR and GSH colourimetric assays are shown as a mean of 3 biological repeats carried out in technical triplicate. Statistical analysis; t-test. ***, $p \leq 0.001$, ** $P \leq 0.01$ and * $p \leq 0.05$. I am grateful to Dr Rajat Roy for carrying out SDS-PAGE/Western blotting shown in figures C and F.

5.3 Targeting of the NRF2 pathway modulates the response to erlotinib treatment

The NRF2 pathway is known to regulate GSH levels through transcriptional regulation of GSH metabolic enzymes and the results presented suggest this pathway to be modulated in response to the T790M mutation. It was therefore hypothesised that targeting this pathway would modulate GSH levels and by virtue erlotinib resistance, as we have shown that increasing GSH levels leads to an increase in sensitivity to erlotinib treatment (Chapter 4). This hypothesis was tested via the direct targeting of NRF2 using siRNAs, in the PC9/PC9ER cell line pair. We were unable to compare the effects of these siRNAs in the other cell line pair due to our lack of ability to transfect the H3255 cells. NRF2 silencing resulted in an increased resistance to erlotinib in the PC9 cell line (**Figure 5.4 A**), whilst no significant effect was observed in PC9ER cells (**Figure 5.4B**). This was accompanied by a significant decrease in levels of GSH in PC9 and to a lesser extent in PC9ER (**Figure 5.4C**). These results emphasised the importance of NRF2 in the regulation of GSH as well as erlotinib resistance. We then further investigated the role of other key proteins involved in NRF2 regulation. Silencing of KEAP1 using siRNA in PC9ER cells resulted in a significant increase in sensitivity to erlotinib and to a lesser extent in the PC9 cells (**Figure 5.4E and F**). This was accompanied by a significant increase in GSH levels (**Figure 5.4G**). Furthermore, siRNA targeting of SQSTM1, resulted in an increased level of erlotinib resistance in PC9 cells (**Figure 5.5A**), although it did not alter resistance in PC9ER cells (**Figure 5.5B**). As expected, silencing SQSTM1 was associated with a decrease in GSH levels (**Figure 5.5C**). All knockdowns were confirmed by qPCR (**Figure 5.4D, 5.4H and 5.5D**). Overall these data indicate that the NRF2 pathway could regulate GSH metabolism and also response to erlotinib treatment in NSCLC cell lines.

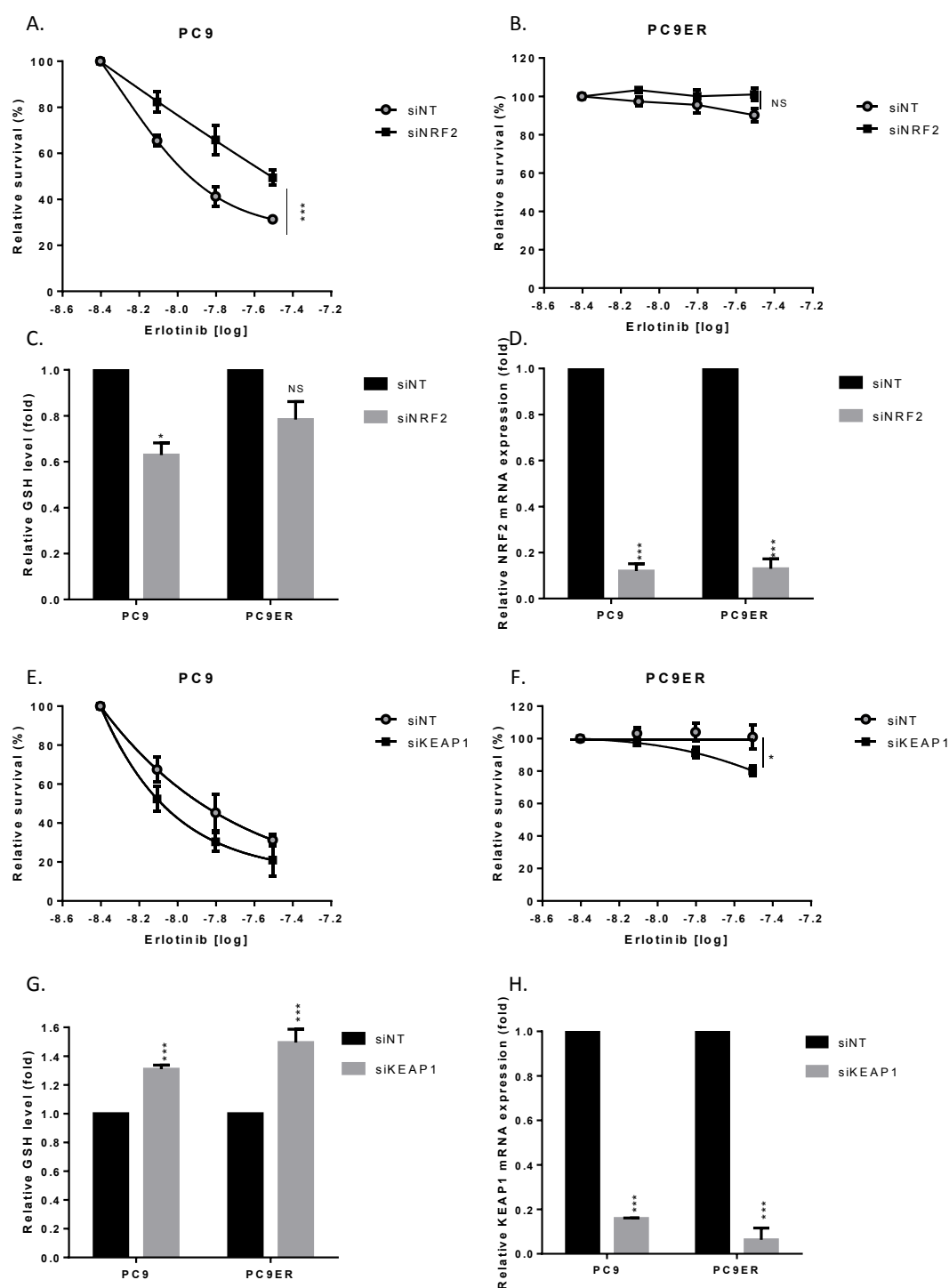


Figure 5.4: Targeting of the NRF2 pathway alters response to erlotinib treatment. (A) PC9 and (B) PC9ER cells were transfected with siRNA against NRF2 or NT control. Cells were treated with a range of concentrations of erlotinib close to the IC₅₀ (PC9). Statistical analysis was done using two-way ANOVA. (C) GSH levels were quantified by colourimetric assay 48 hours post transfection. (D) qPCR quantification of NRF2 levels 48 hours post transfection. (E) PC9 and (F) PC9ER cells were transfected with siRNA against KEAP1 or NT control. Cells were treated with a range of concentrations of erlotinib close to the IC₅₀ (PC9). Statistical analysis was done using two-way ANOVA. (G) GSH levels were quantified by colourimetric assay 48 hours post transfection. (H) qPCR quantification of KEAP1 levels 48 hours post transfection. Figures represent three biological repeats with error bars showing $\pm$ SEM. Statistical analysis were done using Student's *t*-test unless otherwise stated. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

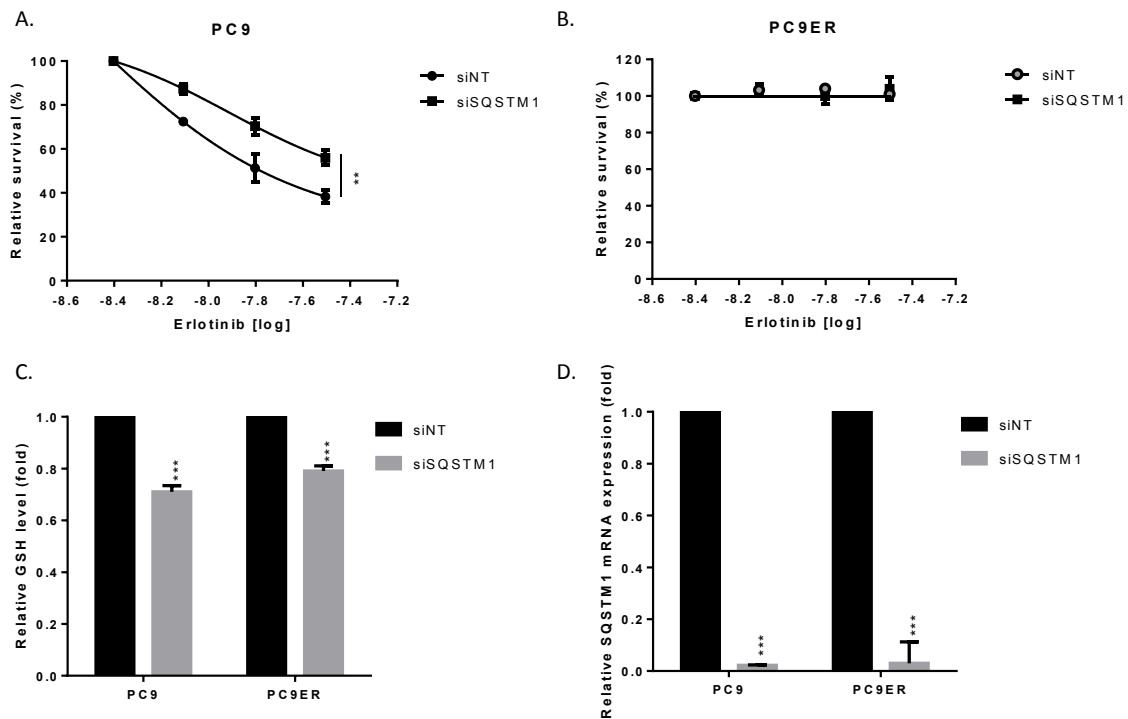


Figure 5.5: siRNA depletion of SQSTM1 increased sensitivity to erlotinib. (A) PC9 and (B) PC9ER cells were transfected with siRNA against SQSTM1 or NT control. Cells were treated with a range of concentrations of erlotinib close to the IC₅₀ (PC9). Statistical analysis was done using two-way ANOVA. (C) GSH levels were quantified by colourimetric assay 48 hours post transfection. (D) qPCR quantification of SQSTM1 levels 48 hours post transfection. Figures represent three biological repeats with error bars showing $\pm$ SEM. Statistical analysis were done using Student's *t*-test unless otherwise stated. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

5.4 Discussion

The importance of GSH in erlotinib resistance has been highlighted in Chapter 4, whereby erlotinib-resistant cell lines have been shown to have a decrease in levels of GSH via direct transcriptional regulation of GSH-metabolising enzymes. Furthermore, targeting of GSH-catabolising enzymes by either siRNA or small molecular inhibitors sensitised resistant cells to erlotinib treatment. NRF2 is a transcription factor which binds to the ARE of target genes such as GSH synthesising enzymes GCLC and GST (Gorrini et al., 2013). NRF2 in turn is tightly regulated by its ubiquitin ligase KEAP1, targeting NRF2 for proteasome-mediated degradation (Kobayashi et al., 2004). This pathway is modulated in erlotinib-resistant cell lines (**Figure 5.2**). Indeed, erlotinib-resistant H1975 cells show a decrease in the nuclear localisation of NRF2 when compared to H3255 cells. This was accompanied by a decrease in cytoplasmic KEAP1. However, the localisation of NRF2 and KEAP1 appears unchanged in the PC9/PC9ER cell lines. Nevertheless, there was a decrease in expression of SQSTM1 in PC9ER compared to PC9 cells. SQSTM1 contains an ARE sequence within its promoter region and, therefore, its transcription is regulated by NRF2 (Jain et al., 2010). In addition, we have previously shown that other NRF2 target genes containing ARE sequences, such as the GSH synthesizing enzyme GCLC, showed decreased expression in erlotinib-resistant PC9ER cells when compared to the sensitive PC9 cells (**Chapter 4**). Taken together, these data suggest a decrease in NRF2 activity. The identification of the exact mechanisms leading to changes in NRF2 activation downstream of the T790M mutation requires further research. A number of studies have previously used Chromatin Immunoprecipitation (ChIP) and luciferase assays to profile NRF2 activity (Chorley et al., 2012, Kwak et al., 2002). Therefore, we suggest utilisation of these techniques would allow us to identify the extent of changes in NRF2 activity between sensitive and resistant cell lines. As no change in NRF2 localisation occurs in the PC9/PC9ER cell line pair, we suggest that there are additional pathways of NRF2 regulation at play. This may be through NRF2 interaction with small Maf proteins or post-translational events. These proteins are DNA-binding, leucine zipper-containing transcription factors, which act as homodimers or heterodimers (Itoh et al., 1997). Maf G, Maf K and Maf F proteins have all shown to be vital to NRF2 transcription of GSH metabolic enzymes (Katsuoka et al., 2005). Monitoring expression of Maf F, G and H proteins in the sensitive versus resistant cell lines could offer clues to how NRF2 activity is regulated in these cells. In addition to this, assays such as electrophoretic mobility shift assay (EMSA) will enable us to identify if Maf proteins are bound to NRF2 at ARE sites of the target genes.

The change in regulation of NRF2 regulation appears to be a direct consequence of the T790M mutation. This was confirmed in both siRNA targeting of the T790M mutation in PC9ER cells, and the stable transfection of EGFR active (L858R) and EGFR active resistant (L858R/T790M) in the HEK293

cell line (**Figure 5.3**). The presence of the T790M mutation conferred a decrease in the expression of KEAP1 binding partners SQSTM1 and PALB2. This was accompanied by a decrease in GSH. Furthermore, the absence of the T790M mutation in the PC9ER cell line following siRNA targeting resulted in an increase in NRF2. This increase in NRF2 may be responsible for the associated increase in GSH.

Using siRNAs, we were able to show that direct targeting of NRF2 increased resistance to erlotinib treatment in the PC9 cell line (**Figure 5.4 A**). This increased resistance was associated with a significant decrease in GSH (**Figure 5.4 C**). This highlights the importance of NRF2 in erlotinib resistance within NSCLC cell lines. In addition, siRNA-mediated targeting of the negative NRF2 regulator, KEAP1, decreased survival to erlotinib treatment (**Figure 5.4 E and F**). This change was associated with an increase in GSH (**Figure 5.4 G**); therefore suggesting that KEAP1 has a role in erlotinib resistance, possibly through a GSH mediated pathway. The KEAP1 binding partner, SQSTM1, is able to bind to KEAP1 by its STGE motif (Jain et al., 2010). Indeed SQSTM1 is able to displace NRF2, thus inhibiting its ubiquitination and degradation, thereby, driving NRF2-dependent transcription (Hast et al., 2013). Hence, SQSTM1 is a negative regulator of KEAP1. Decreasing SQSTM1 levels using siRNAs results in an increase in survival (**Figure 5.5A**), which is accompanied by a decrease in GSH levels (**Figure 5.5C**). This is possibly due to the absence of SQSTM1-mediated competition for KEAP1 binding sites. Thereby, resulting in an increase in NRF2 binding to KEAP1, ultimately exacerbating its degradation. NRF2 degradation would result in a reduction in the transcription of GSH metabolic enzymes; this would explain the decrease in GSH seen following SQSTM1 inhibition (**Figure 5.5 C**).

A number of genes are downstream targets of NRF2, in addition to GSH synthesising enzymes genes, including several ATP-dependent multidrug-resistant drug efflux pumps (Meijerman et al., 2008). Therefore, by altering NRF2 expression either by the direct (NRF2) or indirect (KEAP1/SQSTM1) modulation of the NRF2 regulatory processes may affect alternative pathways such as those involving ATP-dependent multidrug-resistant drug efflux pumps. However, as the nuclear levels of NRF2 are lower in H1975 compared to that of H3255, this would suggest that the levels of ATP-dependent multidrug-resistant drug efflux pumps are also reduced. This implies that the resistance to erlotinib may be independent of NRF2-related changes in multidrug-resistant drug efflux pumps. However, this would require further experimental confirmation.

Taken together our data has underlined the role of NRF2 in the modulation of GSH synthesising enzymes in NSCLC cell lines. Furthermore, through its regulation of GSH levels it is able to modulate the response to erlotinib treatment. This emphasises the importance of GSH regulation in erlotinib-

resistant NSCLC cell lines. However, although NRF2 has been suggested to be an interacting partner of EGFR, the mechanism by which the T790M confers a change in its regulation requires further investigation. Furthermore, as we show no change in the nuclear localisation of NRF2 between PC9 and PC9ER, methods such as luciferase reporter assays and CHIP are required to validate the proposed reduced activity of NRF2 in erlotinib-resistant cell lines.

Chapter 6.0 Reverse Phase Protein Array
identification of signalling changes in response to
the T790M mutation

6.1 Introduction

Our preceding data has shown that T790M mutant EGFR leads to reduced GSH levels via an NRF2-mediated pathway. However, the signalling mechanism coupling EGFR to NRF2 remains to be elucidated. Moreover, the regulation of protein expression and function can occur at many levels including transcription, mRNA stability, translational and post-translational modifications. High-throughput technologies have been used to assess DNA and mRNA levels for many years. However, high-throughput protein quantification technologies are a far more recent concept. RPPA is a sensitive, high-throughput functional proteomics technology (Akbari et al., 2014). It allows the relative quantification of expression of total and phosphorylated proteins and can therefore be used to assess the activation/functionality of a given protein/pathway (Creighton and Huang, 2015). This technology allows for the quantification of multiple samples simultaneously, with a small amount of protein required per sample (Akbari et al., 2014). The basic outline of this assay is described in **figure 6.1**. The intensity of each spot is proportional to the concentration of the protein. Relative quantification comparisons of protein concentration can be made between multiple samples using total protein markers such as fast green (Loebke et al., 2007) or Sypro Ruby (Berggren et al., 2002). The most prominent limitation of this technique is that it can only be used to quantify the expression of known proteins. RPPA requires antibody binding to the protein to produce a signal for quantification. As such, antibody specificity and availability are limiting factors (Liotta et al., 2003). To help circumvent this limitation, a number of online resources have become available including validation protocols and published lists of validated RPPA antibodies currently in use (Akbari et al., 2014). These efforts have enabled RPPA to demonstrate potential clinical applications (Pawelczak et al., 2001, Zha et al., 2004).

A number of studies have utilised the RPPA platform to identify signalling changes in several cancers including NSCLC. Indeed, Ummanni *et al.*, previously utilised RPPA in the study of 85 NSCLC cell lines (Ummanni et al., 2014). This identified cells with sensitivity to anti-EGFR drugs based on increased levels of EGFR phosphorylation, rather than increased abundance of EGFR itself. In addition, they showed a correlation of erlotinib sensitivity with a reduction in the expression of proteins involved within the PI3K/AKT/p70S6K signalling pathway (Ummanni et al., 2014). A further study by Byers *et al.*, utilised the RPPA platform to analyse NSCLC cell lines and identified an inverse correlation between markers of sensitivity to docetaxel and erlotinib (Byers et al., 2008). Indeed, markers such as TSC2, LKB1 and AMPK were associated with docetaxel sensitivity and resistance to erlotinib.

The majority of published studies have focused on cells which are sensitive to drug treatments, with an aim to discover novel biomarkers. This study was particularly focussed on identifying targets differentially expressed between cells expressing activated EGFR with or without the additional T790M mutation. We hoped to highlight signalling changes in response to the T790M mutation, which could be targeted to improve the efficiency of erlotinib treatment and reveal more information about the signalling molecules coupling EGFR to NRF2.

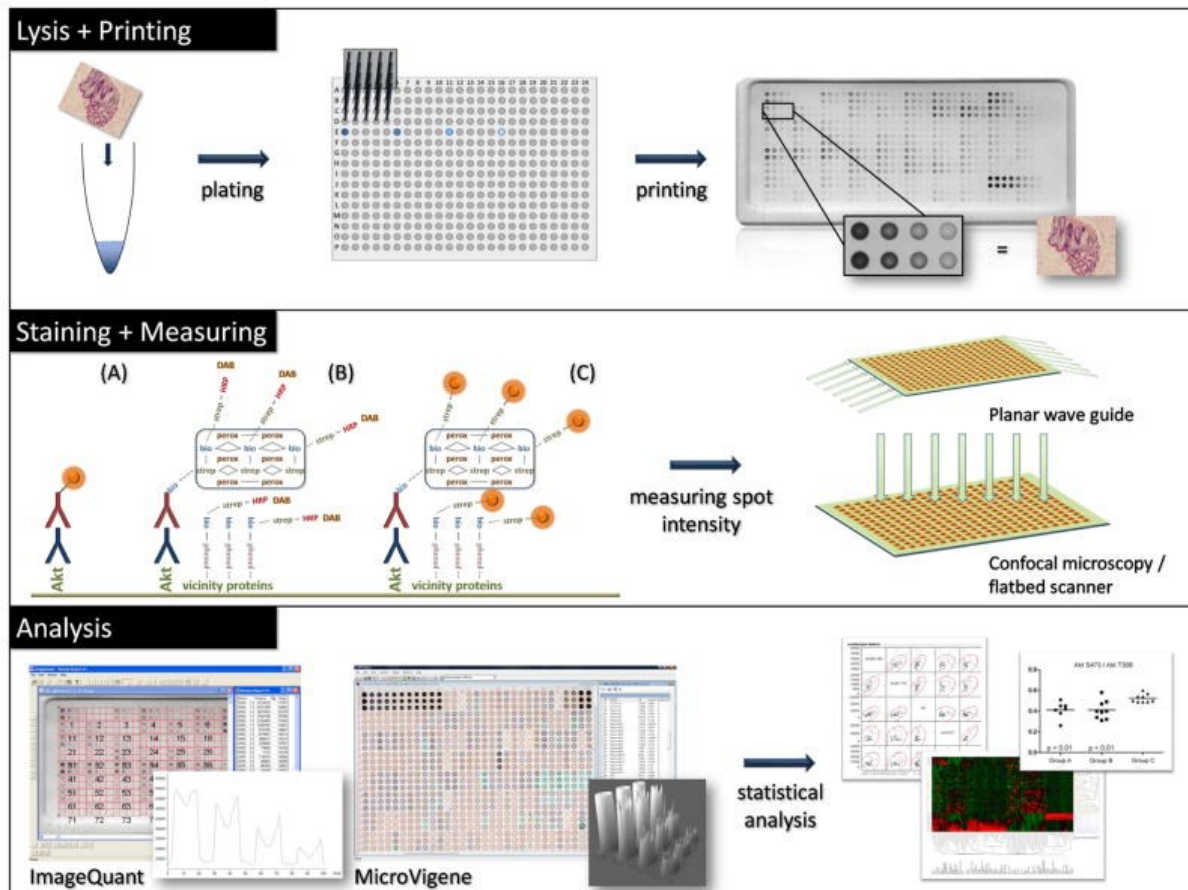


Figure 6.1: Reverse Phase Protein Array protocol outline. Lysis and printing: RPPAs are constructed by preparing the samples in a microtiter plates. Samples are prepared in serial dilutions prior to being deposited via a robotic arrayer onto nitrocellulose coated slides. Each spot within an array represents a protein composition of the same sample at the same time of stabilisation. **Staining and measuring:** A single array slide is stained with one validated antibody that has been shown to be specific for the protein of interest. Detection solutions can be fluorescent, chemiluminescent or chromogenic. Planar waveguide is an example of fluorescent spot analysis which can be seen above. However, fluorescent spot analysis is also commonly analysed via confocal laser scanning. **Analysis:** A number of spot finding software have been developed. These programs convert pixel density for each of the spots into a numerical value. Histograms of the spots morphology can also be viewed to ensure the signal is uniform across the indicated spot. The quantities of pixel intensity can then be normalised to appropriate controls, statistical and bioinformatics analysis can then be performed (Taken from (Mueller et al., 2010)).

6.2 Epidermal growth factor (EGF) stimulation of PC9 and PC9ER cell lines

RPPA was utilised to identify changes in response to the T790M mutation by comparing the relatively isogenic cell line pair of PC9 and PC9ER. The resistant PC9ER cells were derived from the PC9 cells by dose escalation of erlotinib. PC9 and PC9ER cells were stimulated with EGF, to activate EGFR signalling pathways and changes in the phosphorylation or total levels of protein studied over a period of time. Time points up to 4 hours, post EGF stimulation. Data showed a significant increase in phosphorylation of ERK and AKT in both PC9 and PC9ER cell lines with no significant difference between PC9 and PC9ER (**Figure 6.2A-C**).

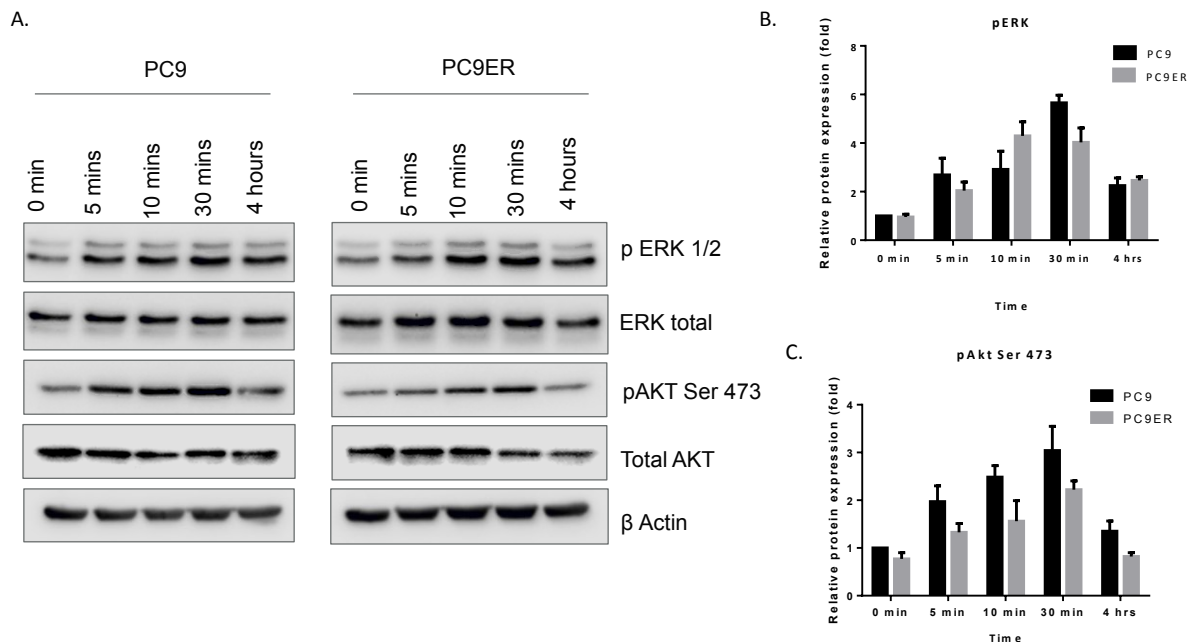


Figure 6.2: Confirmation of stimulation of PC9 and PC9ER with EGF (10ng/ml). (A) PC9 and PC9ER cells were stimulated with 10 ng/ml of EGF for a time course of 0, 5, 10, 30 min and 4 hours followed by SDS-PAGE/Western blotting for relevant proteins. β Actin was used as a loading control, figure is representative of 3 biological repeats (B) Quantification of phosphorylation of ERK normalised to total ERK levels. (C) Quantification of phosphorylation of AKT at Serine 473 normalised to total AKT. Figures B and C show the mean of three biological repeats ($\pm$ SEM). Statistical analysis was done using Student's *t*-test. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

Protein lysates from PC9 and PC9ER cell lines stimulated with EGF across a time course were run through RPPA to identify changes in 94 proteins simultaneously (**Appendix Table 8.1**). The levels of ERK and AKT phosphorylation were quantified and showed a similar pattern of expression to that seen earlier (**Figure 6.3**). However, the levels of phosphorylation appear to peak at an earlier time point (10 minute), than that seen via western blots (30 mins), although, this was not significant (**Figure 6.3**).

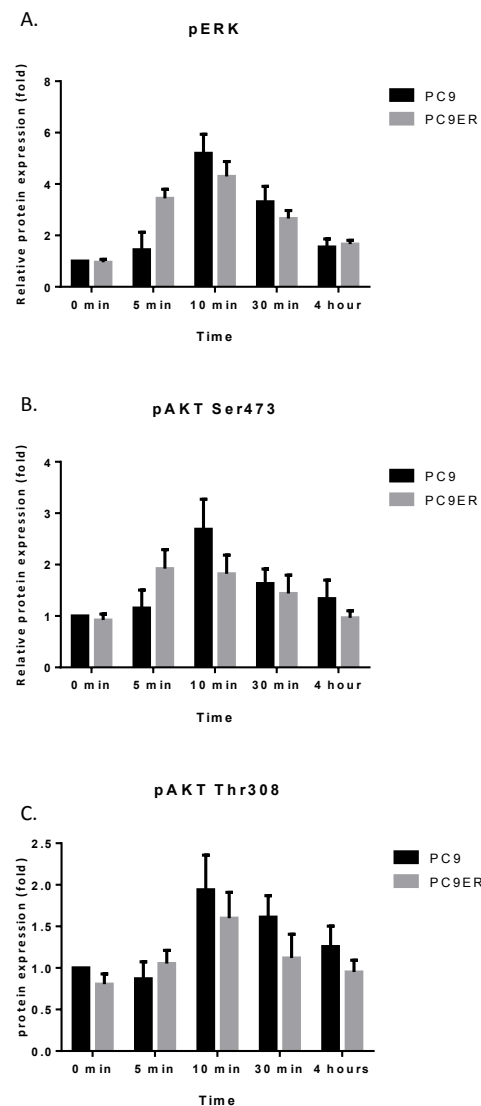


Figure 6.3: RPPA confirmation of activation of ERK and AKT following stimulation with EGF. PC9 and PC9ER cells were stimulated with EGF for 0, 5, 10, 30 mins and 4 hours. The levels of phosphorylation were quantified and normalised to total levels of protein for **(A)** ERK **(B)** AKT Serine 473 **(C)** AKT Threonine 308. Figures represent the mean of three biological repeats with each done in technical triplicate ($\pm$) SEM. Statistical analysis was done using student's t-test. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$. I am grateful to Kenneth Macleod for his help with this RPPA study.

6.3 Increased activation of beta oxidation pathway in PC9ER cell line

RPPA is commonly used as an indicator of alterations within pathways rather than the identification of changes in specific proteins. This RPPA study identified a number of pathways which may be altered in response to erlotinib. One of which was the beta oxidation pathway. Beta oxidation is the catabolic process by which fatty acid molecules are broken down within the mitochondria to generate acetyl-CoA, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH₂). Changes in cellular energetics are a commonly noted hallmark of cancer (Hanahan and Weinberg, 2011). The pathway of beta oxidation is outlined in **Figure 6.4**.

LKB1 also known as serine/threonine kinase 11 (STK11) is an upstream kinase of AMPK. LKB1 when in complex with STRAD and MO25, phosphorylates AMPK at Threonine 172 (Hawley et al., 2003). There is a significant increase in protein levels of LKB1 at the 30 minute time point (**Figure 6.5 A**). However, as LKB1 needs to be in complex with STRAD and MO25 for its action, this information alone does not signify an increase in beta oxidation. The level of phosphorylated AMPK at threonine 172, were increased in PC9ER compared to PC9 however, only at the 0 minute time point (**Figure 6.5 B**). This suggests that the change seen at the 30 minute time point does not directly affect the phosphorylation status AMPK. However, due to the variation seen between repeats this needs further validation. Unfortunately, we were unable to validate the level of phosphorylation of AMPK via western blot using the same antibody employed in the RPPA study. This may suggest the antibody is not specific or the level of protein is not high enough to be detected with this method, as no signal was detected via western blot despite several rounds of optimisation. However, we were able to quantify the amount of total AMPK, which indicated a significant increase in PC9ER compared to PC9 (**Figure 6.6**).

Taken together these data indicate an increase in the levels of LKB1 and AMPK upstream of beta-oxidation. An increase in the phosphorylation of AMPK may lead to an increase in the phosphorylation of its downstream target ACC. ACC is key in the regulation of both beta-oxidation and fatty acid synthesis (Berg JM, 2002). When ACC is phosphorylated it is inactivated allowing acetyl-carnitine to be formed. In the absence of this phosphorylation malonyl CoA is formed and fatty acid synthesis proceeds (Berg JM, 2002). Here, we show that ACC phosphorylation was higher in PC9ER at the 0 mins, 5 mins and 30 mins time points (**Figure 6.7**).

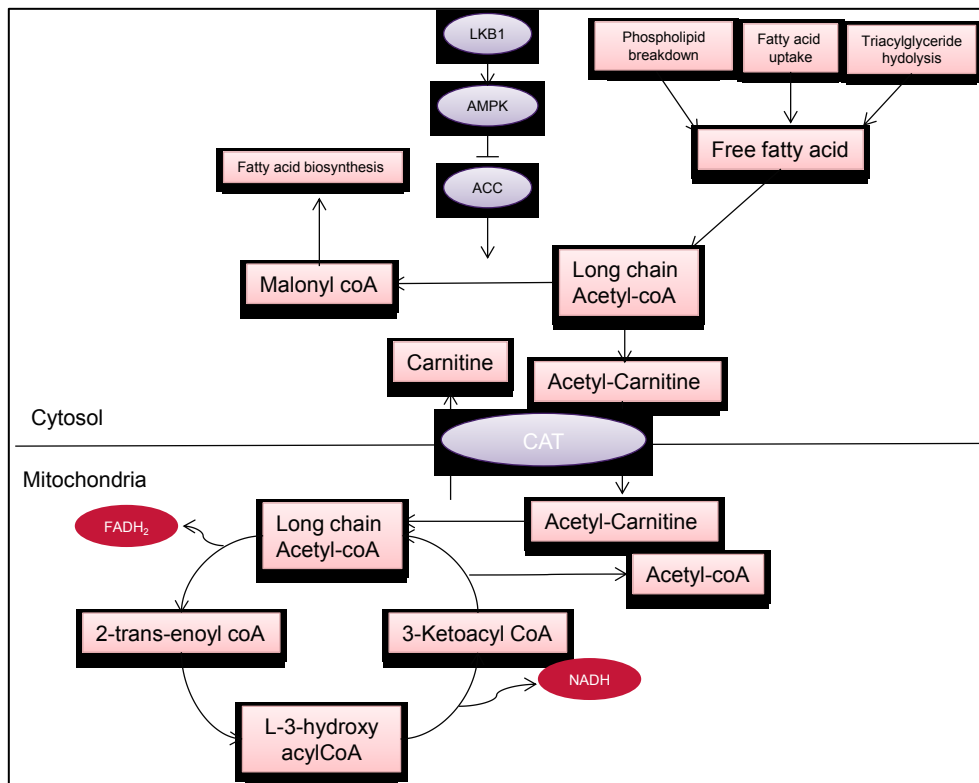


Figure 6.4: Schematic diagram of the beta oxidation pathway. Free fatty acid gained from phospholipid degradation, fatty acid uptake or triacylglyceride hydrolysis is converted to long chain Acetyl-CoA. At this stage beta oxidation or fatty acid synthesis occurs dependent on the phosphorylation status of Acetyl-CoA carboxylase (ACC). Adenosine monophosphate (AMP) binds to adenosine monophosphate-activated protein kinase (AMPK) causing a conformational change, enabling its phosphorylation by Liver kinase B1 (LKB1)/ STE20-Related Kinase Adaptor (STRAD)/ mouse protein 25 (MO25). The activated AMPK phosphorylates and inactivates ACC thereby allowing the formation of acetyl-carnitine as oppose to malonyl-CoA, which is required for beta oxidation and fatty acid synthesis respectively. Acetyl-carnitine is then transported across the mitochondrial membrane by carnitine acetyltransferases (CAT) also known as carnitine palmitoyltransferase I (CPT1) which can itself be inhibited by malonyl CoA. In the mitochondria the long chain acyl-CoA molecules are broken down to acetyl-CoA. The number of acetyl-CoA produced is dependent on the length of the fatty acid being oxidised. The four main enzymes are; acyl-CoA dehydrogenase, enoyl-CoA hydratase, hydroxyacyl-CoA dehydrogenase, and ketoacyl-CoA thiolase. At the end of each beta oxidation cycle two new molecules are formed acetyl CoA and acetyl CoA which is reduced by 2 carbons. In addition NADH and FADH₂ are produced which are then used by the electron transport chain.

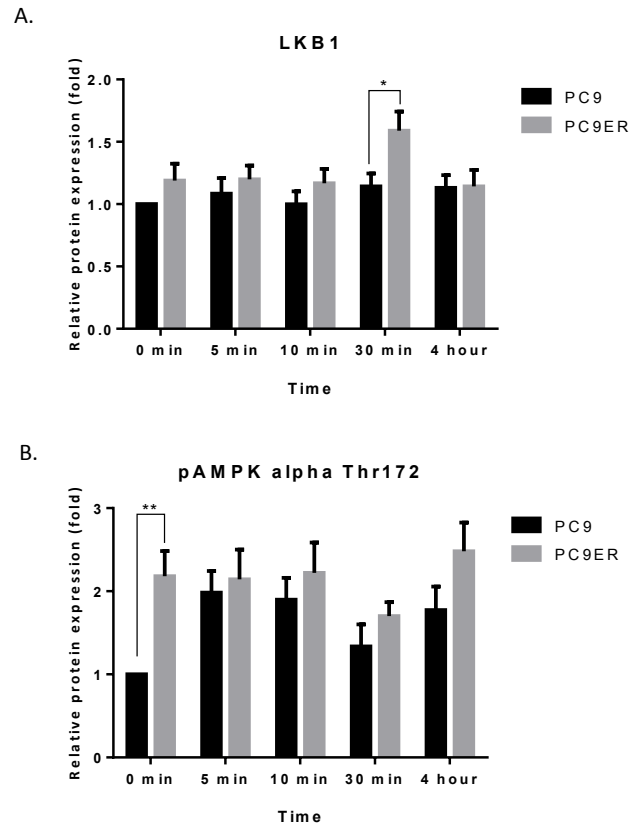


Figure 6.5: RPPA analysis identified increased activation and abundance of inhibitors of fatty acid synthesis in PC9ER. PC9 and PC9ER cells were stimulated with EGF for 0, 5, 10, 30 min and 4 hours and the levels of proteins were quantified. **(A)** Total LKB1 were quantified and normalised to total protein amount identified by fast green staining. **(B)** Phosphorylation of AMPK at threonine 172 was normalised to total AMPK. Figures represent the mean of three biological repeats with each done in technical triplicate ($\pm$) SEM. Statistical analysis was done using student's t-test. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$. I am grateful to Kenneth Macleod for his help with this RPPA study.

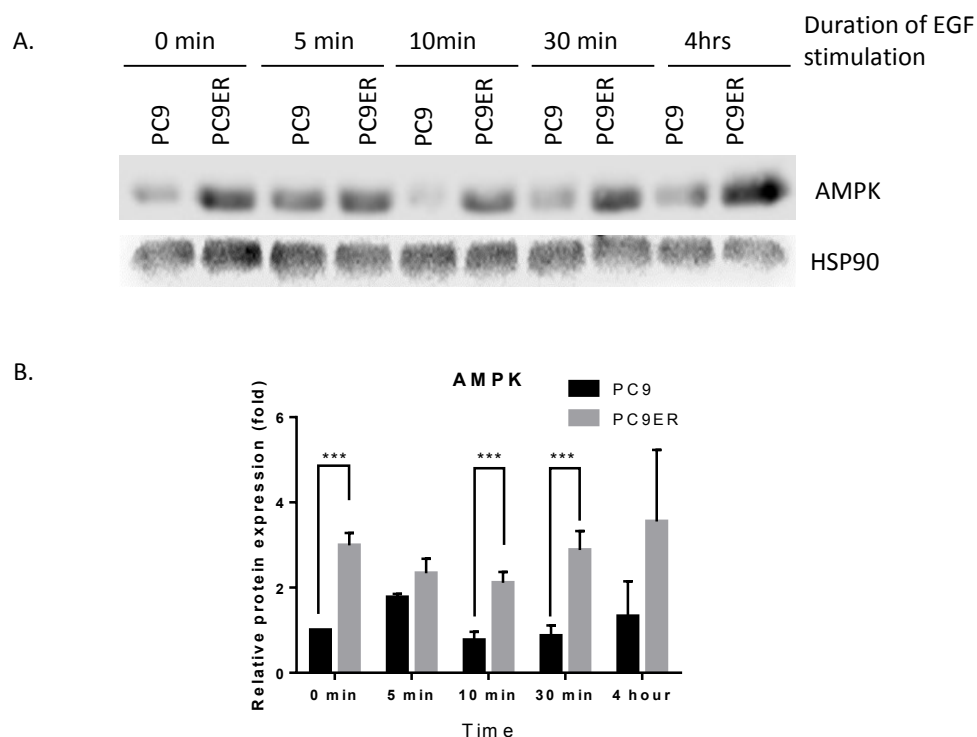


Figure 6.6 PC9ER have higher expression of AMPK than PC9. (A) PC9 and PC9ER cells were stimulated with 10 ng/ml of EGF for a time course of 0, 5, 10, 30 min and 4 hours followed by SDS-PAGE/Western blotting for relevant proteins. Heat shock protein 90 (HSP90) was used as a loading control. Figure is a representative of three biological repeats. (B) Quantification of AMPK normalised to HSP90 shown as the mean of three independent biological repeats ($\pm$) SEM. Statistical analysis was done using student's t-test. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$. I am grateful to Chun Hang Lau for his contributions to SDS PAGE / Western blotting under my supervision.

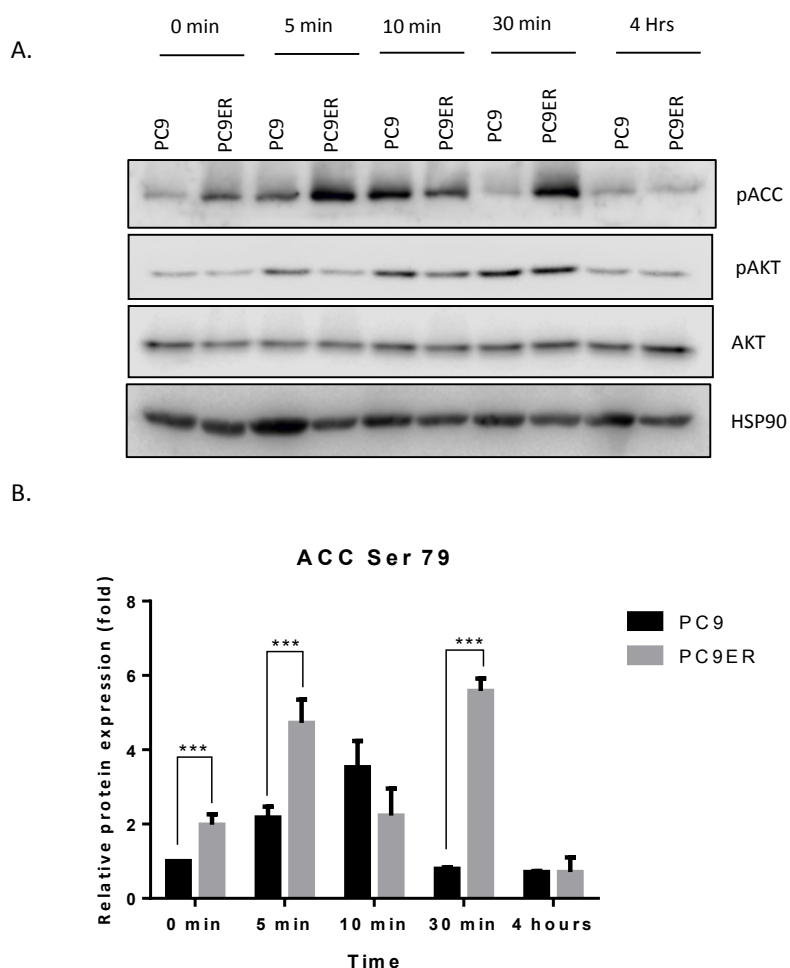


Figure 6.7: PC9ER have an increase in the inactivating phosphorylation of ACC in PC9ER compared to PC9. (A) PC9 and PC9ER cells were stimulated with 10 ng/ml of EGF for a time course of 0, 5, 10, 30 min and 4 hours followed by SDS-PAGE/Western blotting for relevant proteins. HSP90 was used as a loading control. Figure is a representative of three biological repeats. **(B)** Quantification of ACC phosphorylation at serine 79 normalised to HSP90 shown as the mean of three independent biological repeats ($\pm$) SEM. Statistical analysis was done using student's t-test. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

Taken together these data suggest an increase in beta-oxidation. Analysis of metabolites from PC9, PC9ER, H3255 and H1975, using mass-spectrometry, also revealed an increase in the levels of metabolites involved within the beta-oxidation, further validating these findings. The mass-spectrometry data indicated an increase in acetyl CoA in resistant cell lines, although this was only significant between H3255 and H1975 (**Figure 6.8 A**). This was accompanied by an increase in a number of acetylcarnitines, which were significantly higher in resistant cell lines (**Figure 6.8 B, C, D, E, F, G, H and I**). In addition, an increase in the levels of a number of long-chain fatty acids was observed within both the resistant cell lines (**Table 6.1**).

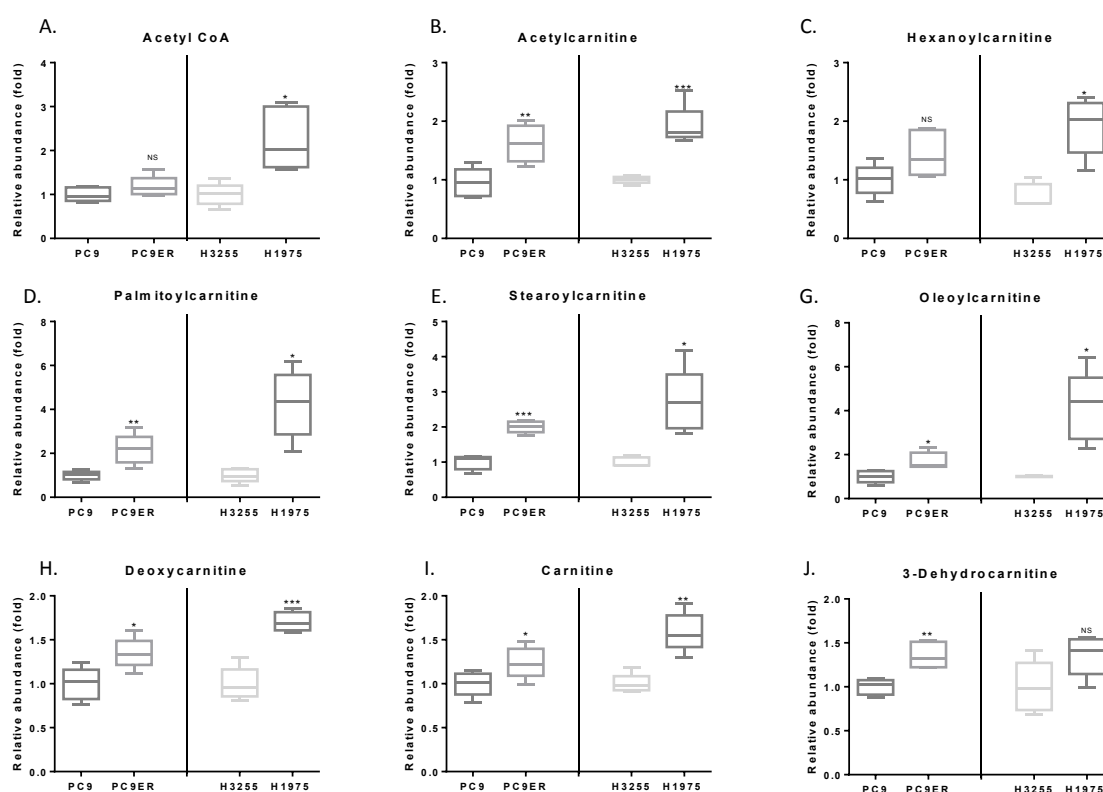


Figure 6.8: Mass spectrometry identified increase in metabolites in the beta oxidation pathway. Mass spectrometry analysis of metabolites in the beta oxidation pathway (A) acetylcarnitine, (B) hexanoylcarnitine, (C) palmitoylcarnitine, (D) stearoylcarnitine, (E) oleoylcarnitine, (F) deoxycarnitine, (G) carnitine and (H) 3-dehydrocarnitine. Resistant cell lines PC9ER and H1975 were each normalised to their sensitive counterpart PC9 and H3255 respectively. All MS data are the average of five biological repeats. Statistical analysis was done using Student's t-test: * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$. Mass spectrometry analysis was carried out by Metabolon (Durham, US).

Sub Pathway	Biochemical Name	Platform	Fold of Change	
			H1975 Res	PC9ER Res
			H3255 Sens	PC9 Sens
Long Chain Fatty Acid	myristoleate (14:1n5)	LC/MS neg	2.81	1.26
	pentadecanoate (15:0)	GC/MS	1.1	1.28
	margarate (17:0)	LC/MS neg	1.78	1.14
	10-heptadecenoate (17:1n7)	LC/MS neg	2.78	1.07
	stearate (18:0)	LC/MS neg	1.9	1.08
	oleate (18:1n9)	GC/MS	1.01	1.3
	13-octadecenoate (18:1n5)	GC/MS	0.92	1.14
	nonadecanoate (19:0)	LC/MS neg	1.15	1.07
	10-nonadecenoate (19:1n9)	LC/MS neg	1.73	1.23
	arachidate (20:0)	LC/MS neg	1.89	1.1
	eicosenoate (20:1n9 or 11)	LC/MS neg	1.22	1.07
	behenate (22:0)	GC/MS	1.09	1.24
	erucate (22:1n9)	LC/MS neg	2.02	1.27
	lignocerate (24:0)	GC/MS	1.55	1.4
	hexacosanoate (26:0)	GC/MS	0.57	1.97

Table 6.1: Mass spectrometry identified an increase in long chain fatty acid abundance in erlotinib resistance compared to erlotinib sensitive cell line. Metabolites from PC9, PC9ER, H3255 and H1975 were quantified using mass spectroscopy and shown as fold change where PC9ER and H1975 were normalised to PC9 and H3255 respectively. Mass spectrometry analysis was carried out by Metabolon (Durham, US).

This study revealed an increase in beta-oxidation in PC9ER cell line compared to PC9 cells. Next, we investigated the response to Erlotinib upon perturbation of the beta-oxidation pathway using small molecule inhibitors. However, Dorsomorphin a potent, selective, reversible ATP-competitive inhibitor of AMPK, showed only minor sensitisation when used in combination with erlotinib in cell survival assays (**Figure 6.9 A and B**). In addition, etomoxir, an irreversible inhibitor of carnitine palmitoyltransferase-1 (CPT-1), resulting in the formation of acylcarnitines, also failed to cause any change in sensitivity to erlotinib in either PC9 or PC9ER cell lines (**Figure 6.9 C and D**).

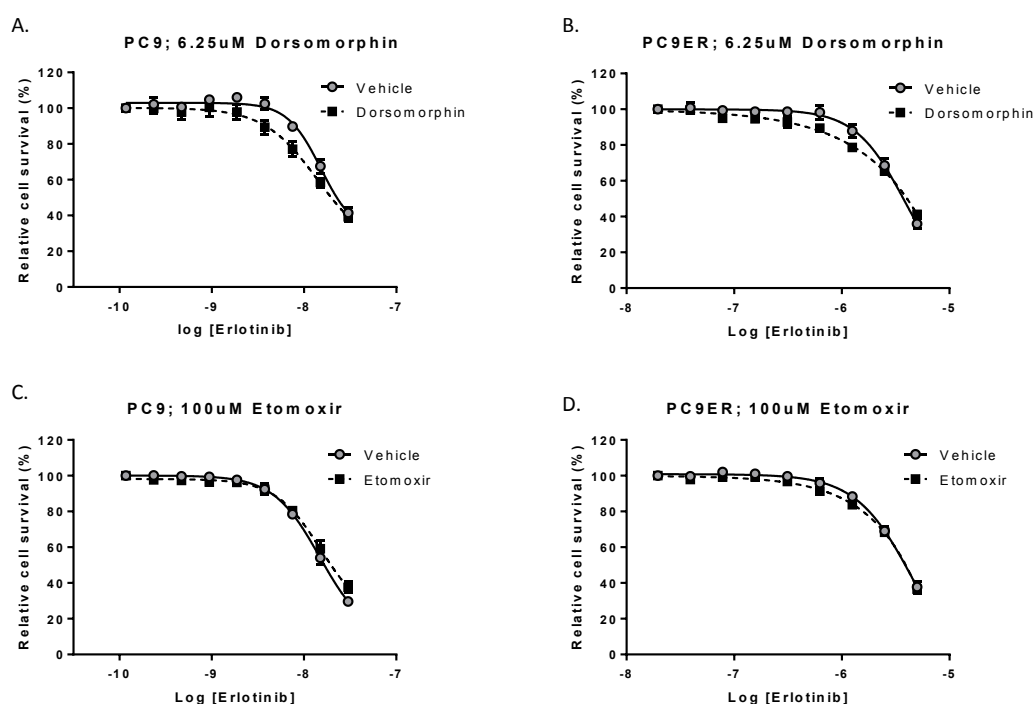


Figure 6.9 Modulation of beta oxidation pathway does not affect sensitivity to erlotinib. Cells were treated with (A, B) dorsomorphin (6.25 μ M), (C, D) etomoxir (100 μ M) or vehicle control for 24 hours prior to 48 hours treatment with erlotinib or vehicle control. Survival was monitored by crystal violet assay. Data are the relative responsiveness to erlotinib normalised to vehicle (-; DMSO). Figures represent the mean of three biological repeats with each replicate done in technical triplicate ($\pm$ SEM).

6.4 Increased activation of Cell cycle checkpoint proteins in PC9ER compared to PC9 cells.

In addition to the beta-oxidation pathway, RPPA analysis also identified changes in the expression of a number of cell cycle checkpoint proteins. Ataxia telangiectasia mutated kinase (ATM) and ataxia telangiectasia and rad3-related kinase (ATR) are related kinases that are involved in cell cycle checkpoints and DNA damage response (Kastan and Lim, 2000). There are a number of substrates of ATM/ATR including p53, checkpoint kinase 1 (ChK1), checkpoint kinase 2 (ChK2) and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) (Zhao and Piwnicka-Worms, 2001, Smith et al., 2010, Tibbetts et al., 1999, Kastan and Lim, 2000). RPPA analysis, using a pan-phospho Ser/Thr antibody that specifically recognises substrates of ATM/ATR, revealed higher activity of these kinases in the erlotinib-resistant cells, independent of EGF stimulation (**Figure 6.10 A**). This change was then validated via quantification of phosphorylation of known substrates. The levels of total P53 (**Figure 6.10 B**) and phosphorylation at serine 15 (**Figure 6.10 C**) were both higher in PC9ER compared to PC9 cells. ChK1 activation and nuclear localisation requires phosphorylation by ATM/ATR at serine 317 and 345, respectively. Levels of phosphorylation of CHK1 at serine 345 were significantly higher in PC9ER compared to PC9 following 0, 5, 10 and 30 minutes of stimulation with EGF (**Figure 6.10 D**). The levels of Chk1 phosphorylation in PC9ER remain unchanged, regardless of stimulation with EGF. However, in the PC9 cell line, the levels of ChK1 increased following 4 hour stimulation with EGF (**Figure 6.10 D**). Chk1 phosphorylates cdc25C at serine 216, which allows Cdc25 to bind 14-3-3 and target it for proteolytic degradation (Donzelli and Draetta, 2003). No significant changes in phosphorylation of cdc25C at serine 216 were observed (**Figure 6.10 E**). This may be due to large variations seen in technical and biological repeats and therefore, requires further validation. Cyclin dependent kinases (CDKs1-11) are classified depending on the cell cycle phase in which they exert their effects. CDK1 is able to stimulate M-phase initiation and progression (Nigg, 2001). A critical step in the activation of CDK1 is CDK1 de-phosphorylation at threonine 14 and tyrosine 15 (Wells et al., 1999a). Total levels of CDK1 increased in PC9ER cells (**Figure 6.10 F**), however, this was coupled with an increase in phosphorylation at Tyrosine 15 (**Figure 6.10 G**). This suggested that despite an increase in total levels of CDK1, there was no increase in the activation levels of this kinase. The protein levels of CDK2 showed an increase following stimulation with EGF for 30 minutes (**Figure 6.10 H**). However, due to lack of an appropriate antibody, we were unable to verify its phosphorylation status in this study.

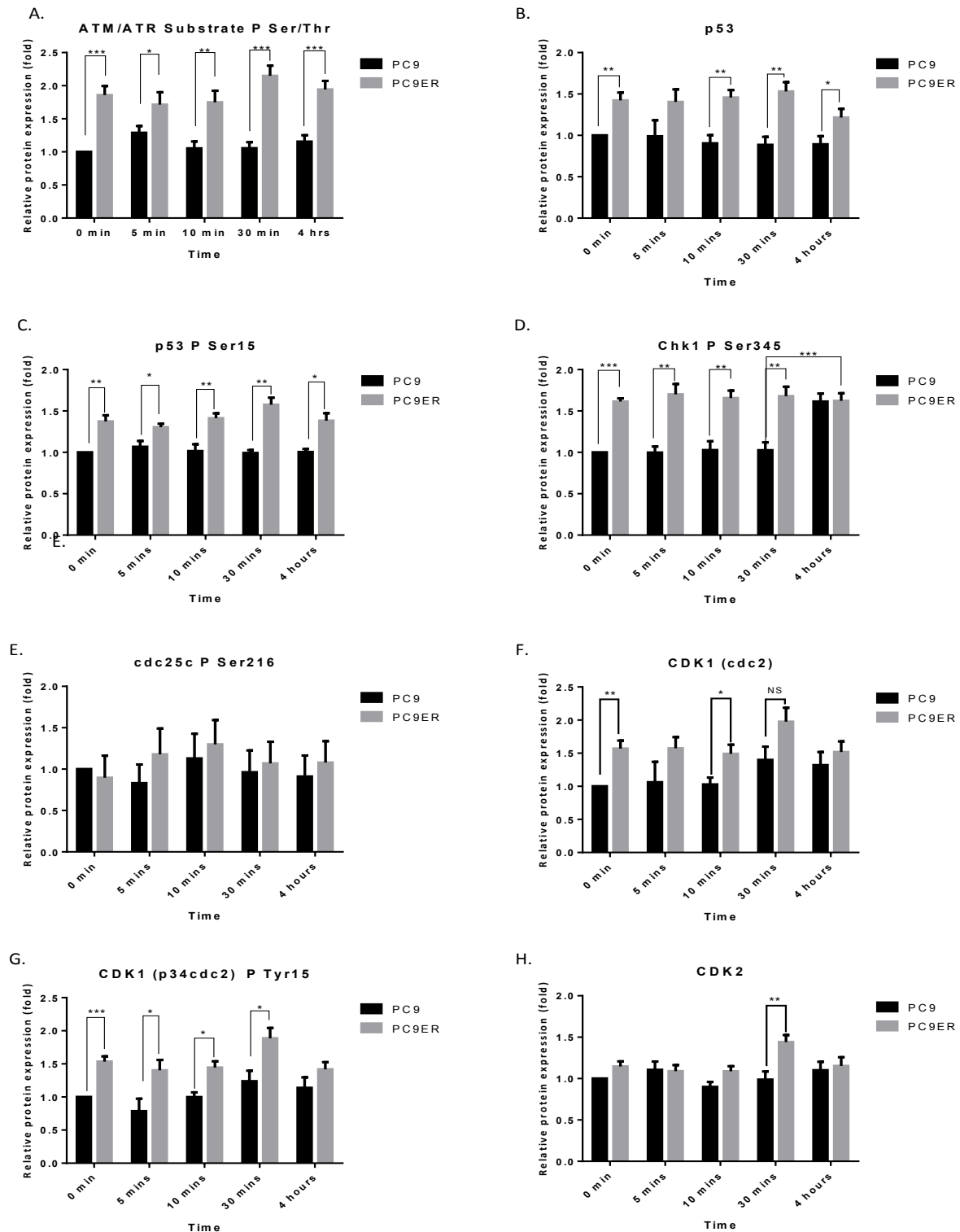


Figure 6.10: Increase in ATM/ATR substrate activation. PC9 and PC9ER cells were stimulated with EGF for 0, 5, 10, 30 mins and 4 hours. The levels of either total or phosphorylated protein were then quantified by RPPA. **(A)** pATM/ATR substrate **(B)** total p53 **(C)** p-p53 ser15*, **(D)** pChk1 Serine 345*, **(E)** pcdc25c serine 216, **(F)** total CDK1 **(G)** phosphorylated CDK1 at tyrosine 15 **(H)** total CDK2. The levels of each of the proteins were normalised against total protein quantified via fastgreen with the exception of those labelled (*) where phosphorylation levels are normalised to that of the specific protein. Figures represent the mean of three biological repeats with each done in technical triplicate ($\pm$) SEM. Statistical analysis was done using student's t-test. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$. I am grateful to Kenneth Macleod for his help with this RPPA study.

Taken together these RPPA data suggest an increase in the levels of a number of cell cycle checkpoint proteins, with an emphasis on those which are regulated by ATM/ATR. Western blotting was used to further validate these changes, as well as, upstream and downstream regulators of this pathway. ATR protein levels were found to be elevated in PC9ER cells (0min, 10 min and 30 minute of EGF stimulation) (**Figure 6.11, repeat shown in appendix figure 8.7**). Phosphorylation of ATR has been implicated in its activation (Vauzour et al., 2007, Smith et al., 2010). Here, ATR phosphorylation at serine 428 of un-stimulated PC9ER cells was significantly higher compared to PC9 (**Figure 6.11**). Furthermore, there was a significant increase in expression of the downstream protein CHK1 independently of EGF stimulation (**Figure 6.11**). However, we were unable to confirm the levels of CHK1 phosphorylation via western blot. The levels of total P53 show little change between PC9 and PC9ER cells. However, there is an increase in the levels of P53 phosphorylation at Serine 15 following stimulation with EGF for 0, 5 and 10 mins.

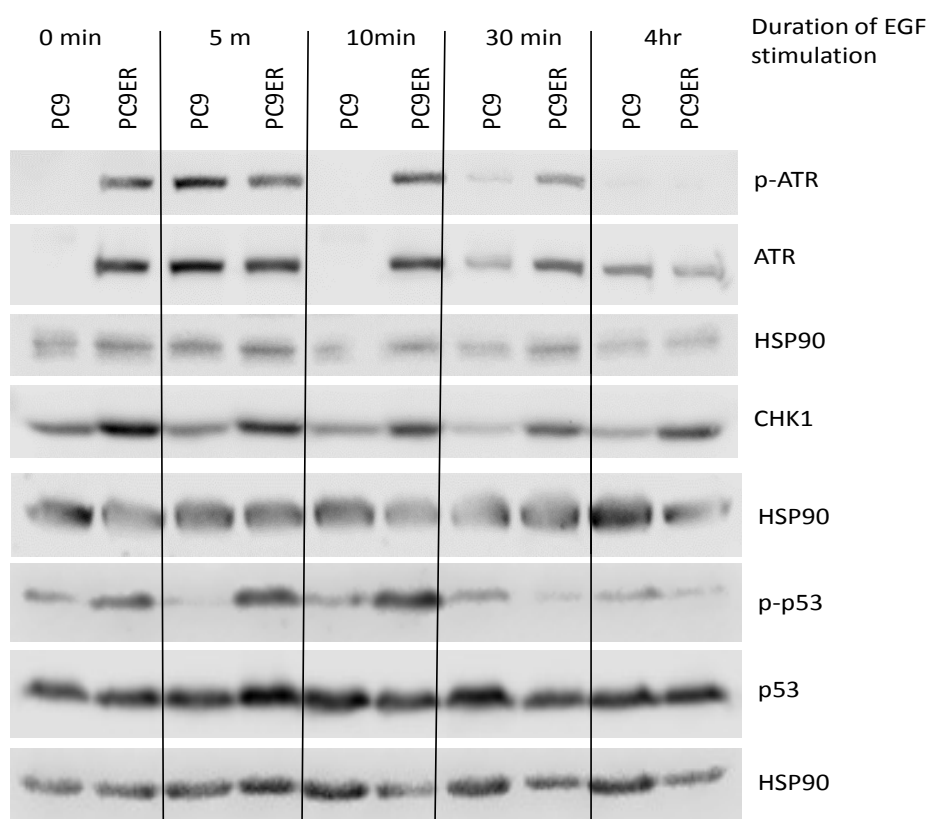


Figure 6.11: Expression of cell cycle checkpoint proteins in PC9 and PC9ER following EGF stimulation. PC9 and PC9ER cells were stimulated with 10 ng/ml of EGF for a time course of 0, 5, 10, 30 min and 4 hours followed by SDS-PAGE/Western blotting for relevant proteins. HSP90 was used as a loading control. Figure is a representative of three biological repeats. I am grateful to Chun Hang Lau for his contributions to SDS PAGE / Western blotting under my supervision.

The cell cycle is a highly controlled process which is inhibited upon DNA damage in response to drug toxicity, ionising radiation or by-products of metabolism. We propose that the increase in cell cycle checkpoint proteins may be a result of increased ROS as a consequence of decreased level of GSH in the resistant cell lines (Chapter 4, figure 4.1). Indeed, the levels of Nitric oxide (NO) were compared between PC9 and PC9ER cells and showed to be higher in PC9ER cells (**Figure 6.12**).

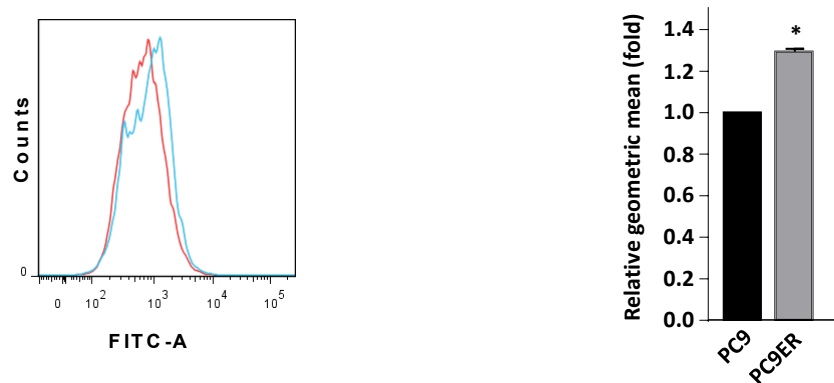


Figure 6.12 Increase in NO levels in PC9ER compared to PC9. The levels of nitric oxide in PC9 and PC9ER were compared by FACS using the DAF-FM probe, **(A)** FACS profile and **(B)** Fold changes in the geometric mean of the signal are shown. Figures represent the mean of three biological repeats with each replicate done in technical triplicate ($\pm$ SEM).

As both RPPA and western blot data indicated an increase in the levels of ATM/ATR substrates, it was of interest to identify if inhibition of these substrates affected sensitivity to erlotinib treatment. Three inhibitors were utilised in this study VX970, LY2603618 and MK1775. The ATR inhibitor VX970 did not significantly affect PC9 cells response to erlotinib treatment (**Figure 6.13 A**). However, there is a small but significant increase in sensitivity to erlotinib in PC9ER (**Figure 6.13 B**). It is important to note that treatment with VX970 alone induced a significant amount of death in both PC9 and PC9ER cells (**Figure 6.13 C**).

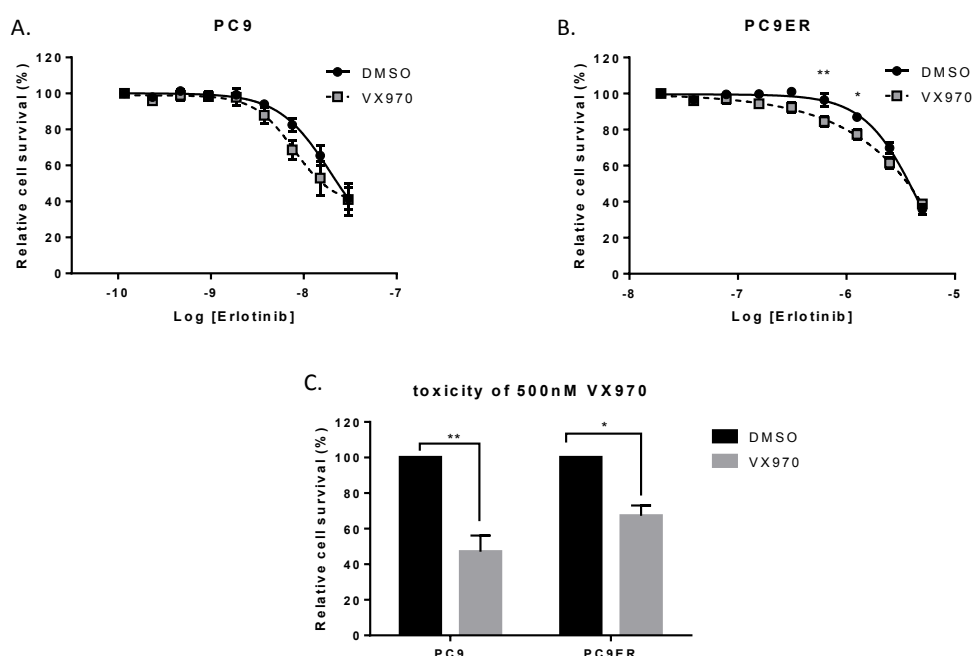


Figure 6.13 Inhibition of ATR by VX970 in both PC9 and PC9ER increased sensitivity to erlotinib. (A) PC9 and (B) PC9ER cells were treated with VX970 (500 nM) 24 hours prior to treatment with erlotinib for 48 hours. Survival was normalised to vehicle control or VX970 alone respectively. (C) The toxicity of VX970 was monitored by treatment of PC9 and PC9ER with VX970 (500 nM) or Vehicle control. Survival was monitored by crystal violet assay. Figures represent the mean of three biological repeats with each replicate done in technical triplicate ($\pm$ SEM) Statistics: two-way ANOVA for (A-C). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. I am grateful to Chun Hang Lau for his contribution to survival and toxicity assays under my supervision.

CHK1 previously showed higher expression in PC9ER when compared to PC9 (**Figure 6.11-6.12**). Therefore, we hypothesised that CHK1 inhibition may affect sensitisation to erlotinib. LY2603618 is a potent and selective small molecular inhibitor of CHK1. LY2603618 has been shown to enhance efficiency of nucleoside analogues, such as gemcitabine (Doi et al., 2015). The inhibition of CHK1 via LY2603618 did not affect sensitivity to erlotinib in either PC9 or PC9ER cells (**Figure 6.14 A and B**).

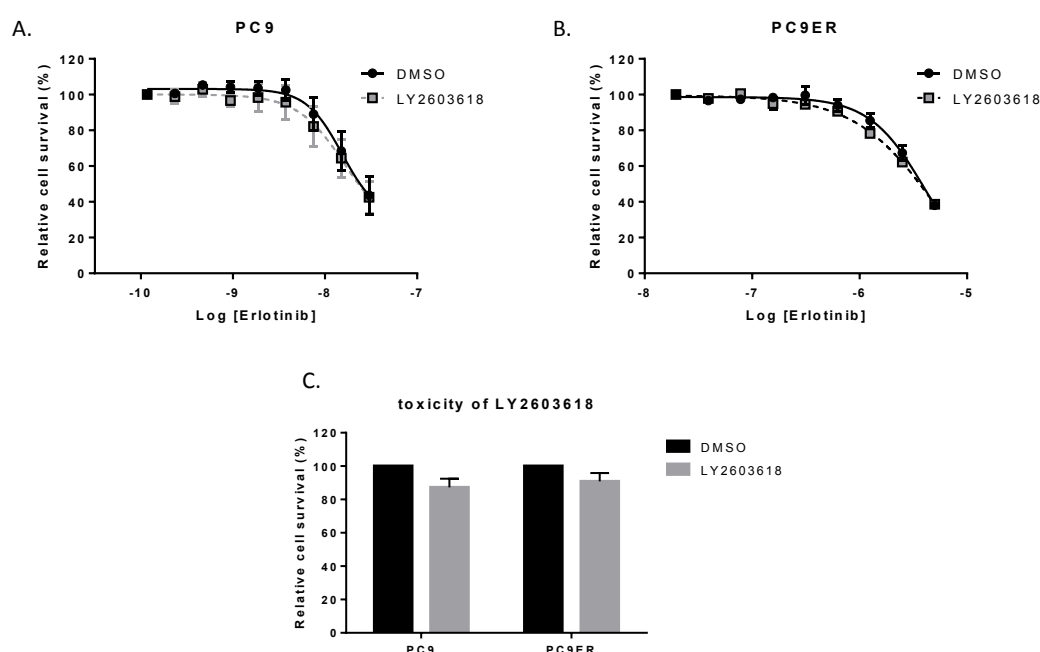


Figure 6.14 Inhibition of ATR by LY2603618 in both PC9 and PC9ER increased sensitivity to erlotinib. (A) PC9 and **(B)** PC9ER cells were treated with LY2603618 (625 nM) 24 hours prior to treatment with erlotinib for 48 hours. Survival was normalised to vehicle control or LY2603618 alone respectively. **(C)** The toxicity of LY2603618 was monitored by treatment of PC9 and PC9ER with LY2603618 (625 nM) or Vehicle control. Survival was monitored by crystal violet assay. Figures represent the mean of three biological repeats with each replicate done in technical triplicate ($\pm$ SEM) Statistics: two-way ANOVA for (A-C). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. I am grateful to Chun Hang Lau for his contribution to survival and toxicity assays under my supervision.

In addition, the Inhibition of WEE1 activity was carried out using the ATP-competitive, reversible inhibitor MK1775. The inhibition of WEE1 led to an increase in sensitisation to erlotinib treatment in both PC9 (**Figure 6.15 A**) and PC9ER (**Figure 6.15 B**). Treatment with MK1775 induced significant death in the PC9 cell line, but not PC9ER cells (**Figure 6.15 C and D**).

The fact that direct inhibition of CHK1 did not affect sensitivity to erlotinib but knockdown of its downstream target WEE1 showed a significant increase in sensitivity, suggests that a parallel pathway may be at play. Indeed Hirai *et al.*, have shown that MK1775 also targets YES a member of the Src family of RTKs (Hirai et al., 2009). Therefore, for further validation, YES and WEE1 were silenced by siRNA targeting and sensitisation was assessed following treatment with erlotinib (**Figure 6.15 E and F**). Confirmation of knockdown was obtained following western blotting (**Figure 6.15 G, H**). Treatment with siRNAs targeting YES did not affect the sensitivity to erlotinib treatment in PC9ER cells (**Figure 6.15 E**). However, siRNA targeting of WEE1 showed a significant increase in sensitivity to erlotinib treatment (**Figure 6.15 F**). We can therefore confirm that the response to MK1775 was as a result of its action on WEE1.

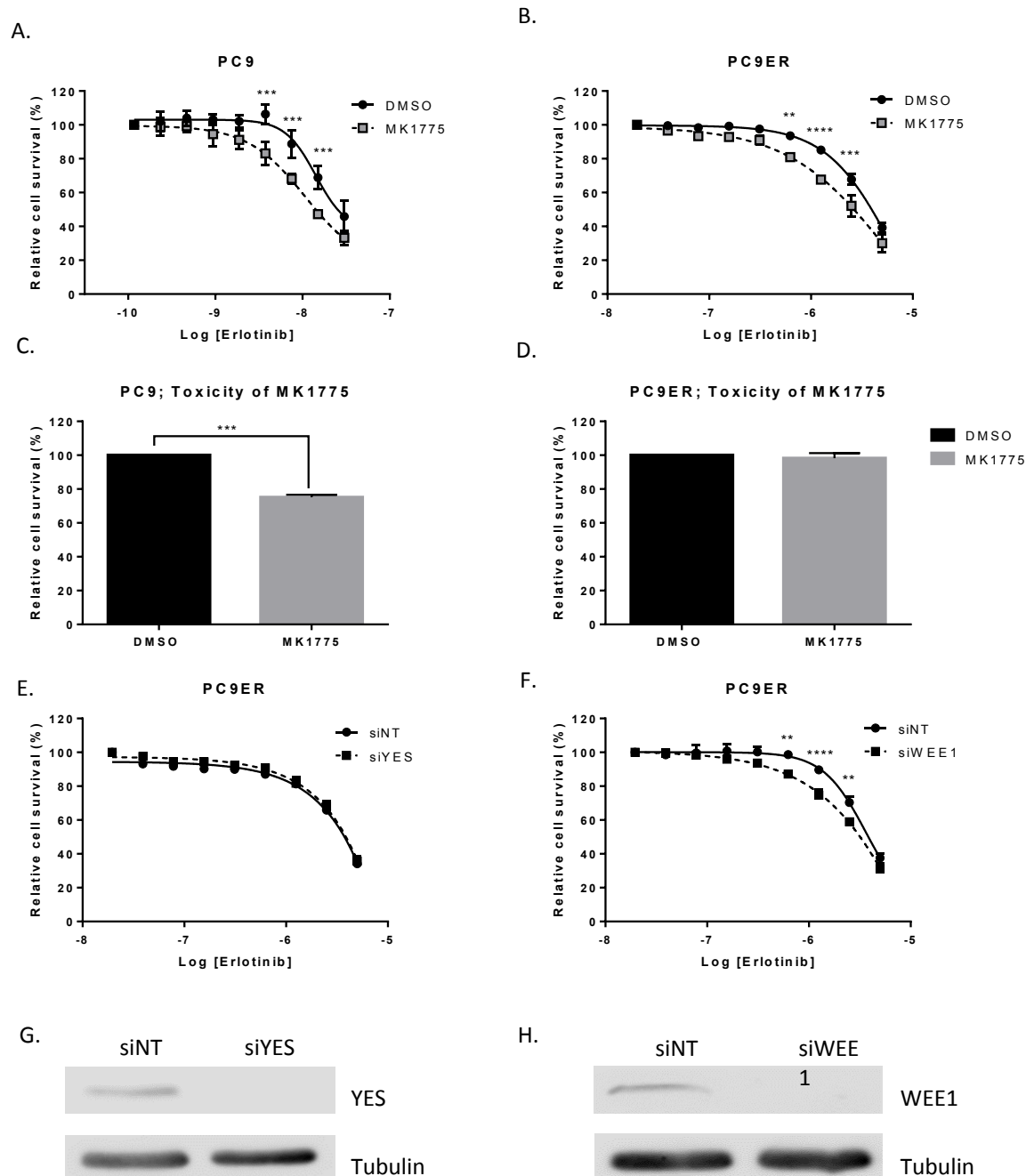


Figure 6.15 Inhibition of WEE1 increased sensitivity to erlotinib treatment. (A) PC9 and (B) PC9ER cells were treated with MK1775 (156 nM) 24 hours prior to treatment with erlotinib for 48 hours. Survival was normalised to vehicle control or MK1775 alone respectively. The toxicity of MK1775 was monitored by treatment of (C) PC9 and (D) PC9ER with MK1775 (156 nM) or Vehicle control. Survival was monitored by crystal violet assay. PC9ER cells were targeted using siRNA against (E) YES or (F) WEE1 or NT control for 24 hours prior survival was monitored after 48 hours by crystal violet assay. Data are the relative responsiveness to erlotinib normalised to vehicle (-; DMSO). PC9ER were transfected with siRNA against (G) YES or (H) WEE1 or NT control for 48 hours prior to western blotting. Figures represent the mean of three biological repeats with each replicate done in technical triplicate ($\pm$ SEM) Statistics: two-way ANOVA for (A-C). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. I am grateful to Chun Hang Lau for his contribution to survival and toxicity assays under my supervision.

6.5 Discussion

The T790M mutated EGFR has been shown to have enhanced kinase activity due to an increased affinity of the receptor for ATP, therefore competing out the binding for reversible inhibitors (Yoshikawa et al., 2012, Yun et al., 2007). This has led to the development of new covalent drugs such as afatinib and AZD9291. However, in the clinical setting, these agents have thus far either failed to show improvement in the resistant patients or have shown that continual treatment leads to additional mutations that overcome inhibition (Miller et al., 2012b, Kim et al., 2015). Therefore, we suggest that decreased affinity of EGFR for ATP competitor drugs is not the singular reason for resistance in the T790M background. So far, this thesis has highlighted a decrease in the abundance of GSH and GSSG in cells with the T790M-EGFR resistance mutation, which we propose is due to a change in the regulation of NRF2. The mechanism by which T790M-mutated EGFR confers this change remained unknown. Therefore, using RPPA we hoped to identify signalling changes which occur at a protein level in response to the T790M mutation.

The RPPA platform was used to analyse the expression profiles of PC9 and PC9ER cells stimulated with or without EGF. This showed that despite constitutive activation of the EGFR, treatment with EGF further activated this pathway (**Figure 6.2 and 6.3**). Furthermore, both Western blotting and RPPA indicated that there was no significant difference in the phosphorylation of either AKT or ERK between PC9 and PC9ER cells. This was surprising as previous studies by Yamadori *et al.*, showed that EGFR signals to NRF2 through a MEK-1/2, and to a lesser extent AKT-dependent, pathway (Yamadori et al., 2012). However, as no significant change was noted it is unlikely to be the mechanism that explains the decrease in GSH levels in erlotinib-resistant cell lines.

RPPA was run using 94 different antibodies; each protein expression was quantified and run through DAVID bioinformatics analysis to reveal signalling pathways which were altered between our cell line pairs. This unveiled two key pathways which were altered between the erlotinib-sensitive and resistant cell lines, PC9 and PC9ER, respectively. The first discussed in this thesis was the beta-oxidation pathway. There was a significant increase in the levels of both LKB1 and phosphorylation of AMPK in PC9ER compared to PC9 cells. Although, the change in LKB1 levels only occurred at the 30 minute time point, whereas, phosphorylation of AMPK showed a significant increase at the 0 min of stimulation (**Figure 6.5**). LKB1 is active when in complex with MO25 and STRAD (Hawley et al., 2003, Boudeau et al., 2004). However, in the absence of studies such as co-IP, we could not confirm an increase in complex formation. Phosphorylation of LKB1 has also been suggested to play a role in its regulation. Indeed, ATM is able to phosphorylate LKB1 at threonine 366, this phosphorylation is known to increase LKB1s catalytic activity (Sapkota et al., 2002, Baas et al., 2003). We have shown an

increase in ATR in PC9ER cell lines and we therefore propose that the increase in LKB1 may be through an ATR-regulated mechanism. This requires further investigation.

The increase in AMPK activation in PC9ER compared to PC9 cells at the 0 minute may suggest that under starved conditions PC9ER cells are better adapted to induce beta-oxidation for energy production. Furthermore, the levels of total AMPK were also increased at all-time points following EGF stimulation. However, this was only significant for the 0, 10 and 30 mins time-points (**Figure 6.6**). The switch between beta-oxidation and fatty acid synthesis is largely dependent on the phosphorylation status of ACC (Mihaylova and Shaw, 2011). Phosphorylation of ACC allows for the formation of acetyl-carnitine, which is required for beta oxidation. Indeed we have shown an increase in the phosphorylation of ACC in the resistant cell line PC9ER when compared to its sensitive counterpart (**Figure 6.7**). Using mass-spectrometry we were able to confirm an increase in a number of acetyl-carnitines in both resistant cell lines compared to their sensitive counterparts (**Figure 6.8**). This was accompanied by an increase in the levels of a number of long-chain fatty acids (**Table 6.1**). These data strongly suggest an increase in the levels of beta-oxidation in cells containing the T790M-mutated EGFR. Despite this, further clarification is required ideally through the direct quantification of beta oxidation with the use of assays such as metabolic flux. This would be required to confirm there is an increase in this pathway, through the quantification of turnover of key molecules.

An increase in beta-oxidation has been noted in prostate cancer, where it has been shown to be the dominant bioenergetics pathway (Liu, 2006). A number of inhibitors have been developed to modulate beta-oxidation activity. Dorsomorphin and etomoxir are two such molecules used in this study. Treatment with Dorsomorphin is expected to result in decreased beta-oxidation through its inhibition of AMPK. Despite the increase previously seen in the levels of AMPK in PC9ER compared to PC9 cells, treatment with Dorsomorphin caused no significant change in sensitivity to erlotinib (**Figure 6.9 A and B**). Etomoxir is a reversible inhibitor of CPT1, thereby preventing the formation of acyl carnitines required for transport of fatty acyl chains into the intermembrane space of the mitochondria. Because of this, CPT1 has been highlighted as a possible target in the treatment of prostate cancer (Schlaepfer et al., 2014). Interestingly Zaugg *et al.*, previously identified CPT1 as a potential oncogene. They showed that expression of CPT1 in cancer cells promoted beta-oxidation and ATP production, tumour growth, as well as rescue from metabolic stress and resistance to mTOR complex 1 inhibitors (Zaugg et al., 2011). However, treatment of both PC9 and PC9ER cells did not affect sensitivity to erlotinib treatment (**Figure 6.9 C and D**). Therefore, although our data strongly suggest that beta-oxidation is elevated in cells resistant to erlotinib, we were unable to improve

response to this drug with compounds targeting this pathway. This suggests that although there is an increase in beta-oxidation in resistant cell lines this may be a consequence of, rather than a cause of, resistance. However, we did not have biomarker measurements in our study to confirm that these drugs efficiently inhibited their targets in our cell lines. Hence further studies should be carried out to confirm our results.

Key components of cell cycle checkpoint were shown to be significantly increased in PC9ER compared to PC9 cells (**Figure 6.10**). However, it is worth noting the expression of ATR and p-ATR has shown an unusual pattern of expression. Therefore, a repeat blot is shown in the **appendix figure 8.7**. This may be a result of incomplete solubilisation of the sample in RIPA buffer and therefore needs further investigation.

Higher levels of ROS, such as NOS, have frequently been implicated in activation of cell cycle checkpoints downstream of ATM/ATR (Shackelford et al., 2000). Indeed, NOS levels were increased in PC9ER compared to PC9 cells (**Figure 6.12**). This may be a result of the decreased levels of GSH metabolites discussed in chapter 4. Up-regulation of cell cycle checkpoint proteins has frequently been implicated in drug resistance, primarily with drugs which induce DNA damage (Eich et al., 2013). No studies have thus far linked erlotinib resistance directly to cell cycle checkpoint proteins. Nevertheless, we have shown that inhibition of ATR via VX790 treatment enhanced response to erlotinib in both PC9 and PC9ER cells (**Figure 6.13**). VX790 is the first clinical ATR inhibitor. It has been shown to sensitise lung cancer cell lines to platinum-based DNA-damaging chemotherapy *in vitro* (Hall et al., 2014). In addition, *in vivo* studies showed that treatment with VX970 blocked ATR activity and enhanced the efficiency of Cisplatin (Hall et al., 2014). Next, we tested whether inhibition of components downstream of ATR, such as Chk1, was able to alter sensitivity to erlotinib. LY2603618, was the first selective Chk1 inhibitor to enter clinical cancer trial. Its use results in impaired DNA synthesis, elevated H2A.X phosphorylation, as well as, premature entry into mitosis (King et al., 2014). Chk1 inhibition did not affect survival to erlotinib treatment in either PC9 or PC9ER cell lines (**Figure 6.14**). However, the inhibition of WEE1, a downstream kinase of Chk1, using the small-molecule inhibitor MK1775 as well as siRNA-mediated silencing of WEE1, showed a strong sensitisation to erlotinib treatment in both PC9 and PC9ER cells (**Figure 6.15**). Indeed, there are several ongoing clinical trials studying the effects of MK1775 for combination treatment with radiotherapy and chemotherapy (clinicaltrials.gov, 2016b, Clinicaltrials.gov, 2016e, Clinicaltrials.gov, 2016d). We suggest WEE1 may be a possible target for enhancing the efficacy of erlotinib treatment in NSCLC. A possible explanation as to why inhibition of WEE1 resulted in a change in sensitivity, while that of CHK1 did not may lie in the regulation of WEE1 itself. Indeed, WEE1 has been shown to

be phosphorylated by a number of kinases through its multiple phosphorylation sites including Y90, Y103, Y110, Y295, Y362 and S642 that regulate its activity (Murakami et al., 1999, Katayama et al., 2005, Lee et al., 2001, Rothblum-Oviatt et al., 2001, Stanford and Ruderman, 2005). Therefore, inhibition of CHK1 may not be sufficient to completely prevent WEE1 activation.

Overall these data have shown that there is indeed an increase in beta-oxidation in resistant cell lines PC9ER and H1975 compared to their sensitive counterparts PC9 and H3255, respectively. However, approaches such as metabolic flux analysis could be used to further confirm this finding. As treatment with inhibitors of both AMPK and CPT-1 did not affect sensitivity to erlotinib, we propose this may be a marker of resistance rather than a cause. In order to confirm this, the expression of key molecules within this pathway should be analysed in NSCLC patient prior to and after acquiring the T790M mutation. In regards to cell cycle checkpoint proteins, there appears to be an increase in the levels of a number of proteins downstream of ATM/ATR. Furthermore, targeting WEE1 specifically led to significant sensitisation to erlotinib treatment. Therefore, we suggest that combination therapy with erlotinib and the WEE1 inhibitor, MK1175, may hold promise in the treatment of erlotinib-resistant NSCLC. This clearly requires further research both *in vitro* and *in vivo*. *In vitro* experiments are required to understand the mechanism by which depletion of WEE1 enhances the effects of erlotinib. In addition, testing in a larger panel of cell lines is also needed.

Chapter 7.0 Discussion and Future Directions

7.1 General discussion

Lung cancer is the second most common cancer in the UK, and 85-90% of these fall into the NSCLC category (Jemal et al., 2011). In western countries around 10% of NSCLC patients have been identified to have activating mutations within their EGFR. This increases to 30-50 % in Asian countries (Sharma et al., 2007). The use of EGFR inhibitors have been shown to work well within this patient subset. However, resistance quickly develops so progression-free survival is around 6-9 months (Ganjoo and Wakelee, 2007). In around 50 % of cases, this is due to the acquisition of an additional T790M mutation within the mutant activated EGFR (Sequist et al., 2011, Yu et al., 2013). The mechanism of resistance due to the T790M mutation was originally thought to be due to changes in the affinity of erlotinib to the dually mutated EGFR (Yun et al., 2008). However, emerging data has suggested that the T790M mutation might drive erlotinib resistance through additional mechanisms (Miller et al., 2012a, Li et al., 2008). Here, we have used ¹H-NMR, mass spectrometry and RPPA to gain further insight into the mechanisms underlying T790M-mediated erlotinib resistance. Using these techniques to compare erlotinib-sensitive and T790M mutant resistant cell lines, we identified a number of differences that are discussed below. We expect that some of these might lead to new therapies to reverse T790M-mediated erlotinib resistance.

7.1.1 Modulation by siRNA of phosphatidylcholine metabolism in erlotinib-resistant cells does not affect resistance to erlotinib treatment

¹H-NMR identified a number of metabolites that were altered our erlotinib-sensitive and resistant NSCLC cell lines. Phosphocholine and choline which are involved in the phosphatidylcholine pathway (Gibellini and Smith, 2010), were both shown to be increased in erlotinib-resistant cell lines. This pathway is responsible for producing the majority of phospholipid species present within the eukaryotic membranes and, therefore, has a significant role in the structure and function of this compartment (Gibellini and Smith, 2010). Using siRNAs to target enzymes controlling this pathway (i.e. synthesizing or degrading enzymes), we attempted to determine whether modulation of the expression of these enzymes might directly influence erlotinib sensitivity/resistance in our NSCLC cell lines. However, targeting this pathway had no impact on erlotinib resistance in T790M-expressing cells nor induce resistance in erlotinib sensitive cells. Whilst this suggests that increased phosphocholine and choline seen in T790M-expressing cells might simply be a bystander phenomenon rather than a driver of resistance, our study had some limitations. For example, we did not show that targeting these enzymes resulted in a change in the production or degradation of phosphatidylcholine. Analysis

of the phosphatidylcholine pathway could be achieved via the use of assays such as metabolic flux. The use of mass-spectrometry highlighted the possibility that the changes identified were metabolites involved in phosphatidylcholine degradation, rather than formation. However, an additional layer of complexity in this study was the degradation pathways reliance on phospholipase enzymes. There are a number of phospholipase enzymes which have the ability to degrade phosphatidylcholine. Therefore, although no change is seen following silencing of a single phospholipase enzyme, we cannot confirm that phosphatidylcholine metabolism has no effect on erlotinib resistance in T790M expressing cells. Due to the importance of the phosphatidylcholine pathway in the generation of not only phospholipids, but also signalling molecules, it is unsurprising that there are a large number of compensatory mechanisms in place. Therefore, we propose that this pathway may prove difficult to modulate for therapeutic purposes.

7.1.2 Acquisition of the T790M mutation lowers GSH levels in association with decreased mRNA expression of GSH synthesizing enzymes

The levels of GSH and GSSG were found to be decreased in both our resistant cell lines when compared to their sensitive counterparts. This change in GSH was unusual as the majority of studies have indicated an increase in GSH to accompany drug resistance (Corti et al., 2010, Spitz et al., 1993). Interestingly, we were able to reveal that mRNA expression levels of key enzymes controlling the production of GSH were reduced, whilst the levels of degrading enzymes were variably modulated in T790M EGFR mutant expressing NSCLC cells as compared to their single activated mutant counterparts. The most prominent changes were the decrease in the mRNA for the synthesising enzymes GSS, but also GCLC and GSR.

7.1.3 Inhibition of GST enhances the response to erlotinib treatment in cells contain the T790M mutation

Using siRNA, we were able to show that targeting of this pathway modulated the response to erlotinib treatment. Indeed, targeting of GSH degrading enzymes increased the sensitivity of erlotinib-resistant cell lines to erlotinib treatment. This was further supported by the use of the molecular inhibitor of GST, EA that increased response to erlotinib treatment. EA is loop diuretic that is frequently used in the clinic for the treatment of conditions such as high blood pressure and is therefore ideal for use *in vivo*. Furthermore, nude mice subcutaneously injected with PC9ER cells treated with a combination of EA and erlotinib showed a significant reduction in tumour burden when compared to treatment with erlotinib alone. Quantification of intracellular

levels of GSH in these tumours showed a significant increase in this metabolite in EA-treated tumours. This highlighted that erlotinib and EA may be the basis for a combination therapy targeting erlotinib-resistant NSCLC. This was supported by NSCLC patient data, which showed a significant reduction in the levels of GSH synthesising enzymes in patients bearing the T790M mutation. Despite this convincing data to support that GSH has a role in erlotinib resistance, the mechanism by which the T790M mutation confers this change required further investigation.

7.1.3.4 Inhibition of NRF2 activity and decrease in GSH levels are direct consequences of acquisition of the T790M-EGFR mutation

NRF2 is a master regulator of the antioxidant responses (Gorrini et al., 2013). It is able to bind the promoter region of ARE target genes such as GCLC and GSS (Huo et al., 2014). As such it is able to regulate GSH levels. Due to the importance of the antioxidant response, there are many levels of control in the regulation of NRF2 activity (Bryan et al., 2013). However, the best investigated is that of the KEAP1-NRF2 complex (Kansanen et al., 2013). The localisation of NRF2 is key to its action, KEAP1 is able to bind NRF2 and hold it in the cytosol, where it is ubiquitinated and degraded. We have shown there is a significant difference in the localisation of NRF2 and KEAP1 in H1975 compared to H3255 cells. However, we were unable to show a significant change in PC9 and PC9ER cells. However, SQSTM1 did show a significant down-regulation in the resistant cell lines H1975 and PC9ER, compared to their sensitive counterparts. SQSTM1 contains an ARE sequence within its promoter region and its expression is therefore regulated by NRF2 (Jain et al., 2010). We have previously shown a decrease in the expression of a number of NRF2 target genes in resistant cell lines when compared to their sensitive counterparts (Chapter 4.0). Therefore, we suggest that there is a decrease in NRF2 activity, independent of changes in NRF2 localisation. MAF proteins play a key role in NRF2 regulation. Indeed, heterodimerization of MAF proteins with NRF2 is required for NRF2 to bind to the ARE therefore, their expression affects the transcription of NRF2 target genes (Bryan et al., 2013, Itoh et al., 1997). We thereby suggest that NRF2 activity may be altered through MAF-mediated regulation of NRF2 activity.

In order to identify if the changes in NRF2 activity were a direct response to the T790M mutation, we utilised siRNAs against the T790M mutation in the PC9ER cell line. We also used HEK293 cells stably transfected with both the EGFR active and active/resistant mutated receptor. We were able to confirm that EGFR T790M confers a change in the NRF2 pathway. Indeed, HEK293 cells stably transfected with EGFR T790M, showed higher cytoplasmic

expression of KEAP1 and decreased expression of KEAP1 binding partners SQSTM1 and PALB2. This finding was supported by siRNA down-regulation of T790M in PC9ER cells, resulting in an increase in NRF2 protein expression and increased expression levels of KEAP1 binding partners SQSTM1 and PALB2. This finding supports previously published data showing EGFR to have a role in NRF2 regulation (Huo et al., 2014, Yamadori et al., 2012). Furthermore, we have shown that the T790M mutation directly affects the NRF2 signalling pathway.

Also, we have shown that targeting of the NRF2 pathway modulates the response to erlotinib treatment, with corresponding changes in GSH levels. The targeting of NRF2 significantly increased resistance in PC9 cell line and was accompanied by a decrease in GSH levels. Conversely, the targeting of KEAP1 and SQSTM1 resulted in an increase in sensitivity to erlotinib treatment and increased GSH levels. This highlights the role of NRF2 in the regulation of GSH levels and erlotinib resistance.

7.1.3.5 Inhibition of WEE1 decreases survival to erlotinib treatment

Cell cycle checkpoint proteins were identified to be significantly up regulated in the PC9ER cell line when compared to its sensitive counterpart, PC9. Indeed, RPPA analysis identified substrates of ATM/ATR to be significantly up regulated in PC9ER cells. ATM and ATR are known to phosphorylate downstream substrates including P53 and Chk1 (Sancar et al., 2004, Canman et al., 1998). Phosphorylation of these proteins was increased in PC9ER compared to PC9 cells regardless of EGF stimulation. Furthermore, Western blot analysis went on to show a significant increase in ATR at both the total and phosphorylation levels. To identify if this pathway has a role in erlotinib resistance, we used the ATR inhibitor, VX970. Treatment with this molecule increased the response to erlotinib treatment in both PC9 and PC9ER cell lines. However, treatment with VX970 alone induced a significant amount of death in both cell lines, which prevented definite conclusions to be drawn on the validity of this approach as an erlotinib-sensitising strategy. In contrast, Chk1 inhibition did not affect survival to erlotinib treatment. However, inhibition of WEE1 via both siRNA-mediated silencing and the molecular inhibitor, MK1775, significantly increases response to erlotinib treatment in both PC9 and PC9ER cells. There are several ongoing clinical trials studying the effects of MK1775 for combination treatment with radiotherapy or chemotherapy (clinicaltrials.gov, 2016b, Clinicaltrials.gov, 2016e, Clinicaltrials.gov, 2016d). Currently there are no ongoing trials for MK1775 with TKIs, such as erlotinib. However, co-treatment in the NSCLC cell lines, PC9 and PC9ER has shown

promise. Further validation is needed in order to identify if these findings can be generalised to additional tumour cell line pairs.

7.1.6 Increased beta-oxidation in erlotinib-resistant cell lines

This thesis has identified an increase in the levels of beta-oxidation in erlotinib-resistant cell lines compared to their sensitive counterparts. This increase was initially identified through RPPA analysis, which showed an increase in both LKB1 and AMPK in resistant cells. The total levels of LKB1 were increased in PC9ER compared to PC9. However, its phosphorylation of AMPK requires interaction with its binding partners STRAD and MO25 (Hawley et al., 2003). LKB1 is regulated by a number of proteins including HSP90 and Cdc37, which aid in the stability and assembly of the LKB1-STRAD-MO25 complex (Boudeau et al., 2003). The increased protein expression of LKB1 in PC9ER compared to PC9 correlated with an increase in the levels of phosphorylation of AMPK at threonine 172. However, phosphorylation of AMPK did not follow the same pattern as that seen for LKB1. Indeed, PC9ER had the highest levels of phosphorylated AMPK at the 0 time-point. This may suggest that PC9ER cells may be better primed to induce beta-oxidation in low energy levels. AMPK activation results in the phosphorylation of ACC at serine 79, which is significantly increased in PC9ER cells. ACC is responsible for the change between fatty acid synthesis and beta-oxidation. ACC when phosphorylated is inactivated and allows for the formation of acetyl-carnitine; whereas the absence of this phosphorylation results in the formation of Malonyl-CoA required for fatty acid synthesis (Berg JM, 2002). Our increase in ACC phosphorylation is accompanied by increase in a number of acetyl-carnitines identified by mass-spectrometry. Taken together, these data supports our initial finding suggesting an increase in beta-oxidation in erlotinib-resistant cell lines, which could be further validated by assays such as metabolic flux.

7.1.3 Targeting of beta-oxidation does not affect resistance to erlotinib treatment

Our data strongly suggested an increase in beta-oxidation in the resistant cell lines. However, further validation of this would require metabolic flux analysis. Due to the time restraints and cost involved, we initially sought to confirm if modulation of this pathway results in an altered response to erlotinib treatment. Dorsomorphin and etomoxir are the two inhibitors which were utilised within this study. PC9 and PC9ER cells were treated with erlotinib in the presence or absence of these inhibitors and the response to erlotinib was accessed by crystal violet assays. Dorsomorphin is a potent, selective, reversible ATP-competitive inhibitor of AMPK. Whereas

Etomoxir, is an irreversible inhibitor of CPT-1. CPT-1 is responsible for catalysing the transfer of the acyl group of a long-chain fatty acyl-CoA from coenzyme A to carnitine, resulting in the formation of acetylcarnitines. However, treatment with either of these inhibitors failed to show a synergistic effect to erlotinib treatment. Therefore, we suggest beta-oxidation may be a response to erlotinib resistance but not one of its causes. This change in beta-oxidation may instead occur as a result of alterations within other signalling pathways such as the cell cycle checkpoint proteins (i.e. ATR) that we have found to be modified in our resistant cell lines. However, further research is needed to confirm this.

7.2 Further directions

A number of novel findings came out of this thesis. However, several questions remain to be addressed. Therefore, the main points of future research based on this project are outlined in the following sections.

7.2.1 How does the T790M mutation confer a change in GSH metabolism?

The levels of GSH are lower in erlotinib-resistant cell lines. Furthermore, this change occurs as a consequence of modifications of the transcription of GSH-synthesising enzymes. NRF2 is known to be a key regulator of GSH metabolism enzymes (Ramos-Gomez et al., 2001, McMahon et al., 2001) and we have found changes to the activity of this transcription factor to be involved. Indeed, siRNA targeting of NRF2 and KEAP1 does modulate the response to erlotinib and GSH levels within the PC9ER cell line. The activity of NRF2 was suggested to be altered regardless of the absence of change being consistently seen in NRF2 localisation. Therefore, another explanation must exist for changes in NRF2 activity. To assess this we could employ luciferase reporter assays that would confirm changes to the overall NRF2 activity. In addition Chromatin Immunoprecipitation (ChIP)-sequencing (seq) could be used to identify specific changes to the binding of NRF2 to GSH-synthesising genes in resistant versus sensitive cells. If indeed we confirm a change in the activity of NRF2 we then need to elucidate the underlying causes for this. The most prominent regulatory pathway other than NRF2s interaction with its negative regulator KEAP1, is the need for NRF2 to bind to MAF proteins in the nucleus (Bryan et al., 2013, Itoh et al., 1997). This could be achieved via co-IP of NRF2 and then probing for interaction with MAF proteins in both PC9 and PC9ER. Furthermore, the use of electrophoretic mobility shift assay (EMSA) would enable to assess if NRF2 is bound to MAF proteins at ARE binding sites.

7.2.2 How does the GSH impact on the response to erlotinib?

The majority of studies have suggested that GSH is increased in cells with drug resistance. However, we have shown that decreased GSH appears to facilitate drug resistance to erlotinib treatment. The mechanism by which GSH achieves this change in erlotinib resistance is not currently understood and therefore requires further research. GSH has previously been reported to interact directly with certain drug compounds (David-Cordonnier et al., 2003). The ability of GSH to interact with drug compounds results in their cellular export via a GST dependent pathway (Provost et al., 2009, Rappa et al., 1997, Zaman et al., 1995). In addition GSH-conjugates have shown to bind directly to MDR 1 which facilitates their export out of the cell (Loe et al., 1997, Priebe et al., 1998). Indeed, a number of GSH-erlotinib conjugates have been identified (Li et al., 2010b). Therefore, changes in the conjugates formed in our cell lines may be a possible mechanism by which GSH modulates response to erlotinib. The presence and/or nature of such conjugates in our cell lines would need to be investigated. Finally, a study by Schwartz and colleagues identified a reduced affinity to multiple EGFR inhibitors following direct oxidation of EGFR by GSH. Therefore, it would also be of interest to identify if there is a difference in the oxidation of EGFR between resistant and sensitive cell lines.

7.2.3 Are GSH levels decreased in patients following onset of EGFR TKI-resistance?

This study provided an indication that the expression of GSH synthesising enzymes is lower in tumours which are resistant to erlotinib. However this data only provided a proof of concept and requires further validation with a higher number of patient samples. However, second biopsies in NSCLC patients following onset of EGFR TKI-resistance are rare and this may therefore prove difficult. This practice is becoming more common and therefore, this study may become a possibility in the future. In addition to the quantification of enzyme levels, the direct quantification of intratumoural GSH would also prove useful. This would enable adequate stratification of patients to maximise the chances of combinational treatments with erlotinib being beneficial.

7.2.4 Can EA be used in combination with erlotinib to improve response to erlotinib in clinical settings?

EA is a loop diuretic that is currently used in the clinic for the treatment of high blood pressure (Cannon et al., 1965, Ledingham and Bayliss, 1965). This thesis has highlighted that EA increases the efficiency of erlotinib in cells containing the T790M mutation *in vitro* and *in vivo*. Therefore,

the next logical step is to assess whether these effects can be translated in patients who are resistant to erlotinib. For this, clinical trials to evaluate safety and effectiveness of this new combination would need to be initiated and the pathway for this is outlined below.

Table 7.1 Brief overview of clinical trials

	Phase I	Phase II	Phase III	FDA Approval	Phase IV
Number of patients	15 – 30	Fewer than 100	100s to 1000s		
Purpose	Determine safety and dosage	Evaluate effectiveness and identify any side effects	Confirm effectiveness and monitor adverse reactions for long term use		Studies the long term effects and cost effectiveness

7.3.5 Could increase in beta-oxidation be used as a clinical marker of resistance to erlotinib?

Beta-oxidation is increased in erlotinib-resistant cell lines. However; its inhibition did not appear to affect survival to erlotinib treatment *in vitro*. This suggested that changes in beta-oxidation are the result of erlotinib resistance and not one of its causes. The mechanism by which erlotinib resistance confers this change remains relatively unknown. The levels of beta-oxidation have previously been shown to be significantly affected by expression of EGFR (Yang et al., 2010). However, how the T790M could affect this pathway requires further research. Initially, this could be done via co-IP of EGFR and proteins involved within this pathway such as AMPK. AMPK has been shown to be modulated in response to erlotinib resistance and, therefore, provides an ideal candidate for these studies. In addition to this, Tandem affinity purification (TAP)-TAG may prove a useful technique to address this as it would allow the identification of unknown mutant EGFR interactors. TAP-TAG involves the creation of a fusion protein with a TAP-tag at one end (Burckstummer et al., 2006). The plasmid is transfected into cells, lysates from which are then used in a two-step purification process. This two-step purification process is designed to remove non-specific interaction. The eluate of this purification ideally contains only proteins that interact with the protein of interest, in our case EGFR with or without the T790M mutation.

7.2.6 Mechanism of altered WEE1 expression in response to the T790M mutation

The inhibition of WEE1 has shown to increase the sensitivity of PC9 and PC9ER cell lines to treatment with erlotinib. However, the mechanism of how this occurs remains unknown. Indeed, the levels of ATR and Chk1 have shown to be higher in PC9ER compared to PC9. Therefore, validation of WEE1 phosphorylation status would be a vital information going forward. This may propose novel therapeutic strategies out of our research as the inhibition of WEE1 has previously been used in combinational studies with chemotherapeutic treatments (clinicaltrials.gov, 2016b, Clinicaltrials.gov, 2015) where inhibition of WEE1 is thought to thrust cancer cells into mitotic catastrophe (De Witt Hamer et al., 2011).

7.2.7 Is the effect of WEE1 inhibition on erlotinib sensitivity dependent on the T790M mutation?

Although inhibition of WEE1 via MK1775 treatment has shown an increase in the sensitivity of PC9ER to erlotinib treatment, we do not know if this is an artefact of the PC9/PC9ER cell lines background. Therefore, this study needs to be extended to additional cell lines, possibly including H3255 and H1975. Also, to see if changes to WEE1 are in direct response to the T790M mutation, we can use our HEK293 cell lines expressing the mutated T790M-mutated EGFR as well as siRNAs targeting the T790M mutation in resistant cell lines and assess how this modulates WEE1 activity.

7.3 Final Conclusion

This work aimed to identify novel therapeutic strategies to tackle erlotinib resistance in the clinic. Indeed, we have found that GSH degrading enzymes may be novel therapeutic targets and also identified a compound, ethacrynic acid, which could be combined with erlotinib for clinical use. However, further mechanistic studies are needed to understand how the T790M mutation on EGFR modifies GSH metabolism to induce resistance to first-generation EGFR TKIs. We have also identified WEE1 as a possible additional therapeutic target to revert resistance and the existence of compounds against this kinase currently used in the clinic may accelerate future translation of our findings. However, further research is needed to confirm the widespread applicability of our results. Despite the need for further research, we have gained invaluable insight into signalling and metabolic pathways associated with erlotinib resistance and may in the future participate to the translation of our findings to the clinic.

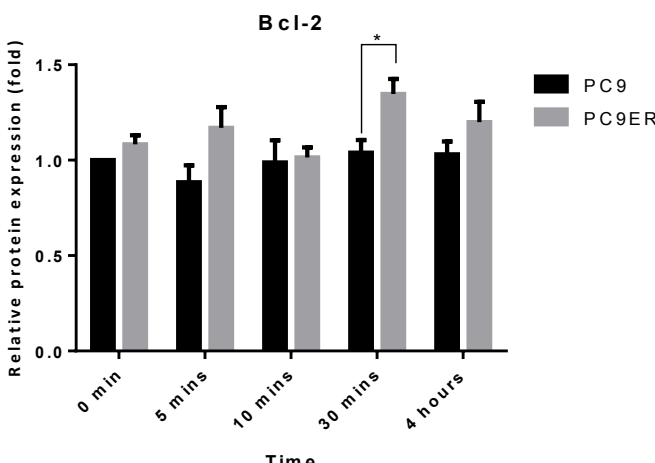
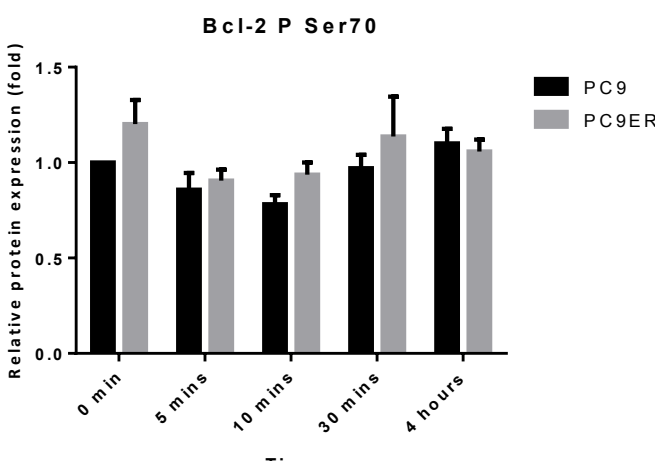
Chapter 8.0 Appendix

Appendix table 8.1: RPPA antibodies. PC9 and PC9ER cells were stimulated with EGF over a time course of 0, 5, 10, 30 mins and four hours. Lysates were then run through RPPA analysis to analyse the expression profiles of the following antibodies. Each experiment was carried out in biological and technical triplicate.

Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
4E-BP1 P Ser65	4E-BP1 is a translation repressor protein, which inhibits cap-dependent translation via its ability to bind to eIF4E a translation initiation factor (Pause et al., 1994). Its Hyperphosphorylation disrupts this interaction and results in activation of cap-dependent translation (Pause et al., 1994).	Cell Signaling Technologies	9451	rabbit	4E-BP1 P Ser65 Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC9 (fold)</th><th>PC9ER (fold)</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.25*</td></tr><tr><td>5 mins</td><td>1.35</td><td>1.35</td></tr><tr><td>10 mins</td><td>1.65</td><td>1.65</td></tr><tr><td>30 mins</td><td>1.75</td><td>2.6**</td></tr><tr><td>4 hours</td><td>1.7</td><td>1.4**</td></tr></tbody></table>	Time	PC9 (fold)	PC9ER (fold)	0 min	1.0	1.25*	5 mins	1.35	1.35	10 mins	1.65	1.65	30 mins	1.75	2.6**	4 hours	1.7	1.4**
Time	PC9 (fold)	PC9ER (fold)																					
0 min	1.0	1.25*																					
5 mins	1.35	1.35																					
10 mins	1.65	1.65																					
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4 hours	1.7	1.4**																					

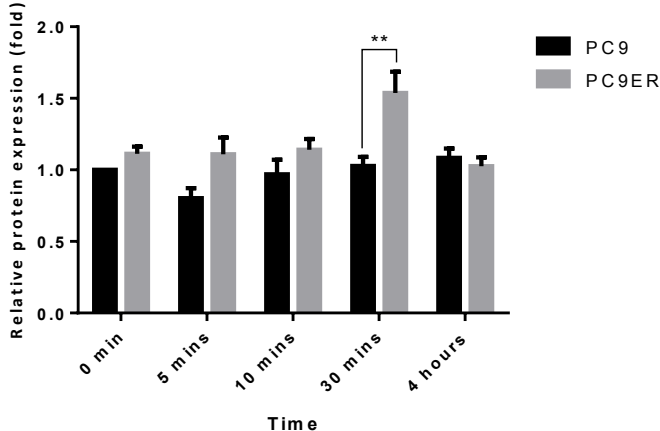
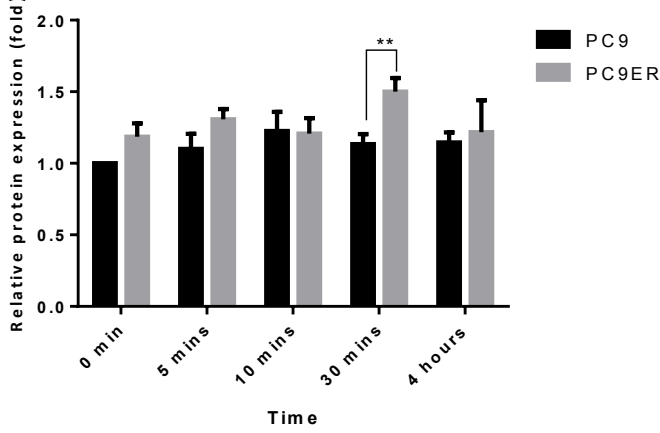
Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
4E-BP1 P Thr37,Thr46		Cell Signaling Technologies	2855	rabbit	4E-BP1 P Thr37,Thr46 Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.2</td></tr><tr><td>5 mins</td><td>1.3</td><td>1.2</td></tr><tr><td>10 mins</td><td>1.2</td><td>1.3</td></tr><tr><td>30 mins</td><td>1.2</td><td>1.8</td></tr><tr><td>4 hours</td><td>1.3</td><td>1.1</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.2	5 mins	1.3	1.2	10 mins	1.2	1.3	30 mins	1.2	1.8	4 hours	1.3	1.1
Time	PC9	PC9ER																					
0 min	1.0	1.2																					
5 mins	1.3	1.2																					
10 mins	1.2	1.3																					
30 mins	1.2	1.8																					
4 hours	1.3	1.1																					
AMPK alpha P Thr172	Activated AMPK modulates the metabolic switch from an anabolic to catabolic state (Luo et al., 2005). AMPK is phosphorylated and activated by LKB1-STRAD-MO25 complex (Hawley et al., 2003, Hawley et al., 1996, Lizcano et al., 2004).	Cell Signaling Technologies	2535	rabbit	AMPK alpha P Thr172 Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>2.2</td></tr><tr><td>5 mins</td><td>2.0</td><td>2.2</td></tr><tr><td>10 mins</td><td>1.9</td><td>2.2</td></tr><tr><td>30 mins</td><td>1.4</td><td>1.7</td></tr><tr><td>4 hours</td><td>1.8</td><td>2.5</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	2.2	5 mins	2.0	2.2	10 mins	1.9	2.2	30 mins	1.4	1.7	4 hours	1.8	2.5
Time	PC9	PC9ER																					
0 min	1.0	2.2																					
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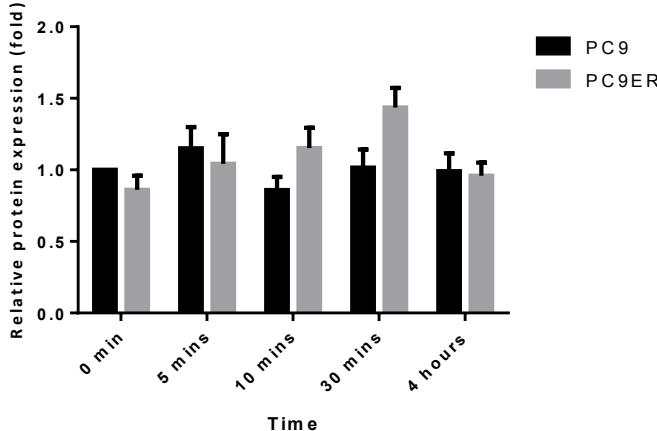
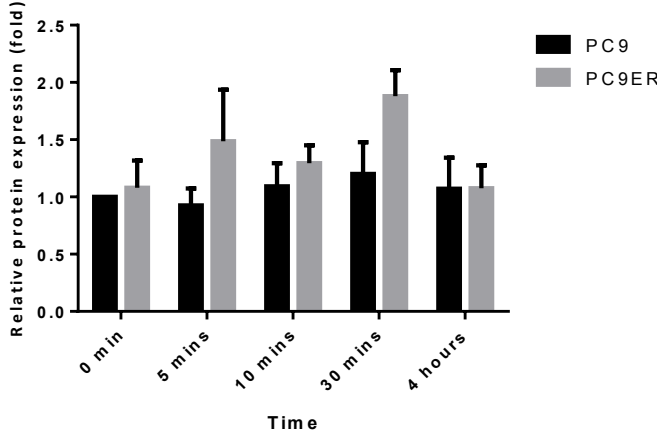
Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
ATM/ATR Substrate P Ser/Thr	Ataxia telangiectasia mutated kinase (ATM) and ataxia telangiectasia and Rad3-related kinase (ATR) are related kinases that regulate cell cycle checkpoints and DNA repair (Kastan and Lim, 2000). CST has developed antibody that recognizes phosphorylated substrates which contain and are phosphorylated on serine or threonine in the S*/T*Q motif.	Cell Signaling Technologies	2851	rabbit	ATM/ATR Substrate P Ser/Thr Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>~1.9</td></tr><tr><td>5 min</td><td>~1.3</td><td>~1.7</td></tr><tr><td>10 min</td><td>~1.1</td><td>~1.8</td></tr><tr><td>30 min</td><td>~1.1</td><td>~2.2</td></tr><tr><td>4 hrs</td><td>~1.2</td><td>~2.0</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	~1.9	5 min	~1.3	~1.7	10 min	~1.1	~1.8	30 min	~1.1	~2.2	4 hrs	~1.2	~2.0
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10 min	~1.1	~1.8																					
30 min	~1.1	~2.2																					
4 hrs	~1.2	~2.0																					
BAX	In cellular stress conditions Bcl-2-associated X protein (BAX) translocates to the mitochondrion membrane, this results in release of cytochrome c and triggers apoptosis (Jurgensmeier et al., 1998). Its action is repressed by binding to the repressor of apoptosis B-cell lymphoma 2 (Bcl-2) (Wei et al., 2001).	Epitomics	1063	rabbit	BAX Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>~1.4</td></tr><tr><td>5 mins</td><td>~1.1</td><td>~1.4</td></tr><tr><td>10 mins</td><td>~1.0</td><td>~1.4</td></tr><tr><td>30 mins</td><td>~1.1</td><td>~1.9</td></tr><tr><td>4 hours</td><td>~1.3</td><td>~1.5</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	~1.4	5 mins	~1.1	~1.4	10 mins	~1.0	~1.4	30 mins	~1.1	~1.9	4 hours	~1.3	~1.5
Time	PC9	PC9ER																					
0 min	1.0	~1.4																					
5 mins	~1.1	~1.4																					
10 mins	~1.0	~1.4																					
30 mins	~1.1	~1.9																					
4 hours	~1.3	~1.5																					

Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
Bcl-2	Bcl-2 has a role in survival through inhibition of cytochrome C release from the mitochondria via inhibition of BAX in response to various stimuli (Murphy et al., 2000). Phosphorylation at serine 70 in the flexible loop domain has been shown to be required for enhancement of Bcl-2 anti-apoptotic function (Ruvolo et al., 2001).	Epitomics	1017-1	rabbit	 Bcl-2 Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC9 (fold)</th><th>PC9ER (fold)</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.1</td></tr><tr><td>5 mins</td><td>0.9</td><td>1.2</td></tr><tr><td>10 mins</td><td>1.0</td><td>1.0</td></tr><tr><td>30 mins</td><td>1.05</td><td>1.35*</td></tr><tr><td>4 hours</td><td>1.05</td><td>1.2</td></tr></tbody></table>	Time	PC9 (fold)	PC9ER (fold)	0 min	1.0	1.1	5 mins	0.9	1.2	10 mins	1.0	1.0	30 mins	1.05	1.35*	4 hours	1.05	1.2
Time		PC9 (fold)	PC9ER (fold)																				
0 min	1.0	1.1																					
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Bcl-2 P Ser70	Cell Signaling Technologies	2827	rabbit	 Bcl-2 P Ser70 Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC9 (fold)</th><th>PC9ER (fold)</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.2</td></tr><tr><td>5 mins</td><td>0.9</td><td>0.9</td></tr><tr><td>10 mins</td><td>0.8</td><td>0.95</td></tr><tr><td>30 mins</td><td>1.0</td><td>1.15</td></tr><tr><td>4 hours</td><td>1.1</td><td>1.05</td></tr></tbody></table>	Time	PC9 (fold)	PC9ER (fold)	0 min	1.0	1.2	5 mins	0.9	0.9	10 mins	0.8	0.95	30 mins	1.0	1.15	4 hours	1.1	1.05	
Time	PC9 (fold)	PC9ER (fold)																					
0 min	1.0	1.2																					
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30 mins	1.0	1.15																					
4 hours	1.1	1.05																					

Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
Bcl-x	Bcl-x, is a member of the Bcl-2 protein family. It is involved in the inhibition of cell death, or apoptosis (Yang et al., 1995, Adams and Cory, 1998).	Epitomics	1018	rabbit	Bcl-x <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>0.95</td></tr><tr><td>5 mins</td><td>0.9</td><td>0.9</td></tr><tr><td>10 mins</td><td>0.95</td><td>0.9</td></tr><tr><td>30 mins</td><td>1.05</td><td>1.2</td></tr><tr><td>4 hours</td><td>1.2</td><td>1.05</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	0.95	5 mins	0.9	0.9	10 mins	0.95	0.9	30 mins	1.05	1.2	4 hours	1.2	1.05
Time	PC9	PC9ER																					
0 min	1.0	0.95																					
5 mins	0.9	0.9																					
10 mins	0.95	0.9																					
30 mins	1.05	1.2																					
4 hours	1.2	1.05																					
beta-Catenin	β -Catenin is a key downstream effector in the Wnt signalling pathway (Cadigan and Nusse, 1997). Has been suggested to have a role in tumorigenesis (Polakis, 1999) and embryonic development (Funayama et al., 1995). It is phosphorylated at Threonine 41 and serine 45 by GSK-3 β which results in its destabilization (Yost et al., 1996).	Cell Signaling Technologies	9562	rabbit	beta-Catenin <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.3</td></tr><tr><td>5 mins</td><td>0.8</td><td>1.3</td></tr><tr><td>10 mins</td><td>1.0</td><td>1.4</td></tr><tr><td>30 mins</td><td>1.2</td><td>1.7</td></tr><tr><td>4 hours</td><td>1.1</td><td>1.35</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.3	5 mins	0.8	1.3	10 mins	1.0	1.4	30 mins	1.2	1.7	4 hours	1.1	1.35
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Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
beta-Catenin P Thr41,Ser45		Cell Signaling Technologies	9565	rabbit	beta-Catenin P Thr41,Ser45 Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.55***</td></tr><tr><td>5 mins</td><td>0.9</td><td>1.2***</td></tr><tr><td>10 mins</td><td>1.1</td><td>1.15</td></tr><tr><td>30 mins</td><td>1.05</td><td>1.5**</td></tr><tr><td>4 hours</td><td>1.15</td><td>1.35</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.55***	5 mins	0.9	1.2***	10 mins	1.1	1.15	30 mins	1.05	1.5**	4 hours	1.15	1.35
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0 min	1.0	1.55***																					
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10 mins	1.1	1.15																					
30 mins	1.05	1.5**																					
4 hours	1.15	1.35																					
BID	BH3 Interacting Domain Death Agonist (BID), a pro-apoptotic member of the Bcl-2 family (Gross et al., 1999). BID protein interacts with BAX leading to insertion of BAX into the mitochondrial membrane(Eskes et al., 2000).	Epitomics	1008	rabbit	Bid Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.35</td></tr><tr><td>5 mins</td><td>1.3</td><td>1.45</td></tr><tr><td>10 mins</td><td>1.1</td><td>1.4</td></tr><tr><td>30 mins</td><td>1.2</td><td>2.0***</td></tr><tr><td>4 hours</td><td>1.55</td><td>1.6</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.35	5 mins	1.3	1.45	10 mins	1.1	1.4	30 mins	1.2	2.0***	4 hours	1.55	1.6
Time	PC9	PC9ER																					
0 min	1.0	1.35																					
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Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
Bim	BIM a pro-apoptotic member of the BCL-2 protein family, which induces Bax/Bak-oligomerization on mitochondria membrane (Gross et al., 1999).	Epitomics	1036	rabbit	BIM  Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) Significance: ** (p < 0.01) at 30 mins <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.1</td></tr><tr><td>5 mins</td><td>0.8</td><td>1.1</td></tr><tr><td>10 mins</td><td>1.0</td><td>1.1</td></tr><tr><td>30 mins</td><td>1.0</td><td>1.5</td></tr><tr><td>4 hours</td><td>1.1</td><td>1.0</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.1	5 mins	0.8	1.1	10 mins	1.0	1.1	30 mins	1.0	1.5	4 hours	1.1	1.0
Time	PC9	PC9ER																					
0 min	1.0	1.1																					
5 mins	0.8	1.1																					
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30 mins	1.0	1.5																					
4 hours	1.1	1.0																					
Calmodulin	Calmodulin is a small protein which mediates many cellular effects such as apoptosis (Persechini et al., 1989). It is able to bind to four Ca ²⁺ ions, resulting in conformational changes (Finn et al., 1995). It can then complex with and activate a number of enzymes e.g. nitric oxide synthase (Weissman et al., 2002).	Calbiochem		mouse IgG1	Calmodulin  Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) Significance: ** (p < 0.01) at 30 mins <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.2</td></tr><tr><td>5 mins</td><td>1.1</td><td>1.3</td></tr><tr><td>10 mins</td><td>1.2</td><td>1.2</td></tr><tr><td>30 mins</td><td>1.1</td><td>1.5</td></tr><tr><td>4 hours</td><td>1.1</td><td>1.2</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.2	5 mins	1.1	1.3	10 mins	1.2	1.2	30 mins	1.1	1.5	4 hours	1.1	1.2
Time	PC9	PC9ER																					
0 min	1.0	1.2																					
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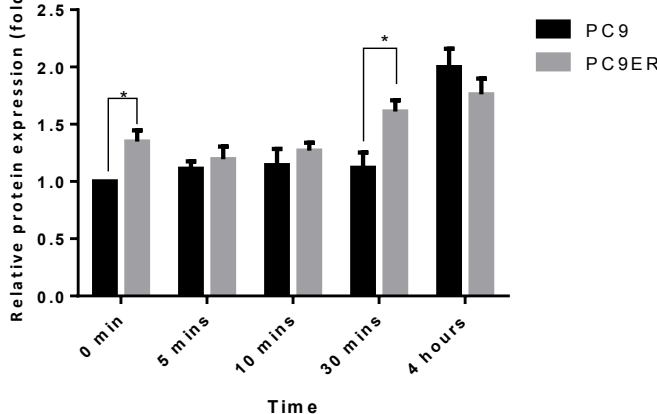
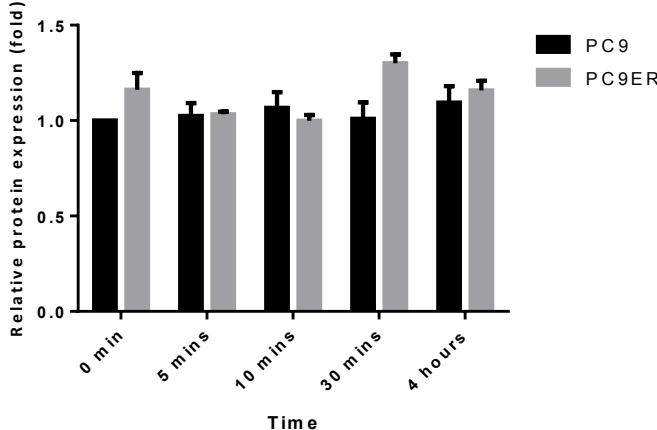
Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
Calpain2	Calpain2 is a calcium-dependent thiol proteinase, which is regulated by a number of mechanism including its subcellular localisation, phosphorylation by EGFR-ERK signalling cascade as well as endogenous inhibitor calpastatin (Perrin and Huttenlocher, 2002). It has a role in migration by modifications of substrates involved in cell adhesion and cytoskeletal arrangement (Perrin and Huttenlocher, 2002).	Cell Signaling Technologies	2539	rabbit	Calpain2 <table><caption>Calpain2 Relative protein expression (fold)</caption><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>0.9</td></tr><tr><td>5 mins</td><td>1.2</td><td>1.1</td></tr><tr><td>10 mins</td><td>0.9</td><td>1.2</td></tr><tr><td>30 mins</td><td>1.0</td><td>1.5</td></tr><tr><td>4 hours</td><td>1.0</td><td>1.0</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	0.9	5 mins	1.2	1.1	10 mins	0.9	1.2	30 mins	1.0	1.5	4 hours	1.0	1.0
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4 hours	1.0	1.0																					
CamKII alpha (22B1) P Thr286	CaMKII is an important member of the calcium/calmodulin-activated protein kinase family, which has a role in neural synaptic stimulation and T cell receptor signalling (Hughes et al., 2001, Barria et al., 1997). Phosphorylation at threonine 286 results in its constitutive activation (Barkai et al., 2000)	Abcam	ab2724	mouse IgG1	Cam KII alpha (22B1) P Thr286 <table><caption>Cam KII alpha (22B1) P Thr286 Relative protein expression (fold)</caption><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.1</td></tr><tr><td>5 mins</td><td>1.0</td><td>1.5</td></tr><tr><td>10 mins</td><td>1.1</td><td>1.3</td></tr><tr><td>30 mins</td><td>1.2</td><td>1.9</td></tr><tr><td>4 hours</td><td>1.1</td><td>1.1</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.1	5 mins	1.0	1.5	10 mins	1.1	1.3	30 mins	1.2	1.9	4 hours	1.1	1.1
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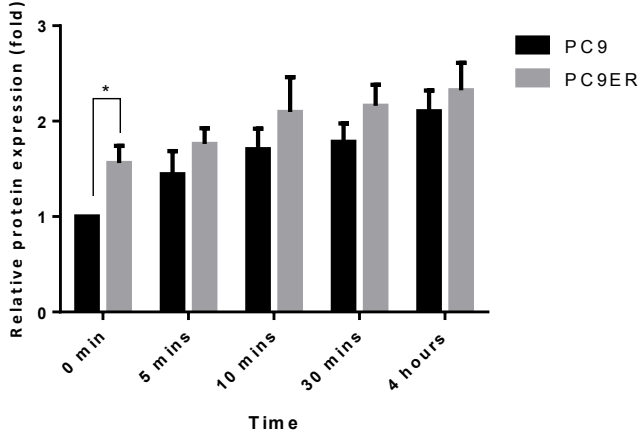
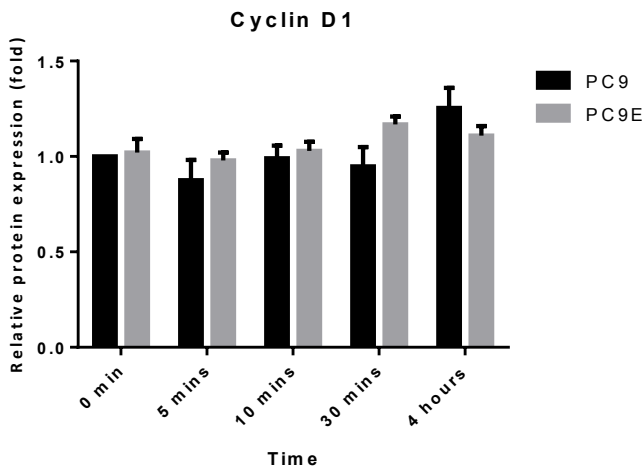
Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
CamKII P Thr286		Cell Signaling Technologies	3361	rabbit	Cam KII P Thr286 Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC9 (fold)</th><th>PC9ER (fold)</th></tr></thead><tbody><tr><td>0 min</td><td>0.8</td><td>1.2</td></tr><tr><td>5 mins</td><td>0.85</td><td>1.15</td></tr><tr><td>10 mins</td><td>1.1</td><td>1.0</td></tr><tr><td>30 mins</td><td>1.1</td><td>1.55</td></tr><tr><td>4 hours</td><td>1.4</td><td>1.35</td></tr></tbody></table>	Time	PC9 (fold)	PC9ER (fold)	0 min	0.8	1.2	5 mins	0.85	1.15	10 mins	1.1	1.0	30 mins	1.1	1.55	4 hours	1.4	1.35
Time	PC9 (fold)	PC9ER (fold)																					
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5 mins	0.85	1.15																					
10 mins	1.1	1.0																					
30 mins	1.1	1.55																					
4 hours	1.4	1.35																					
Caspase 3	Caspase-3 is involved in the caspase cascade, the sequential activation of caspases plays a central role in the execution-phase of cell apoptosis (Slee et al., 1999).	Cell Signaling Technologies	9662	rabbit	Caspase 3 Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC9 (fold)</th><th>PC9ER (fold)</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.35</td></tr><tr><td>5 mins</td><td>1.3</td><td>1.45</td></tr><tr><td>10 mins</td><td>1.05</td><td>1.2</td></tr><tr><td>30 mins</td><td>1.15</td><td>1.6</td></tr><tr><td>4 hours</td><td>1.35</td><td>1.25</td></tr></tbody></table>	Time	PC9 (fold)	PC9ER (fold)	0 min	1.0	1.35	5 mins	1.3	1.45	10 mins	1.05	1.2	30 mins	1.15	1.6	4 hours	1.35	1.25
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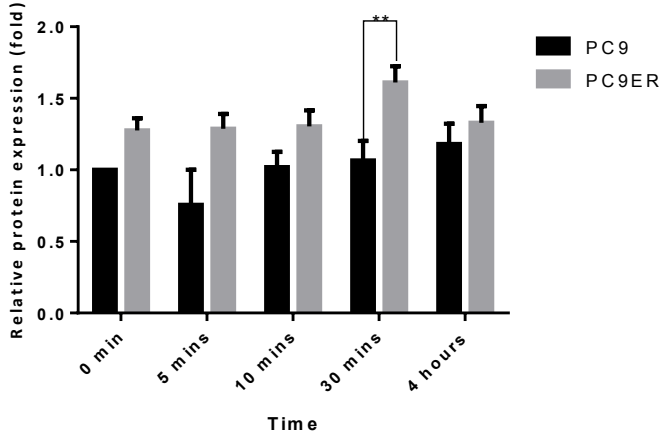
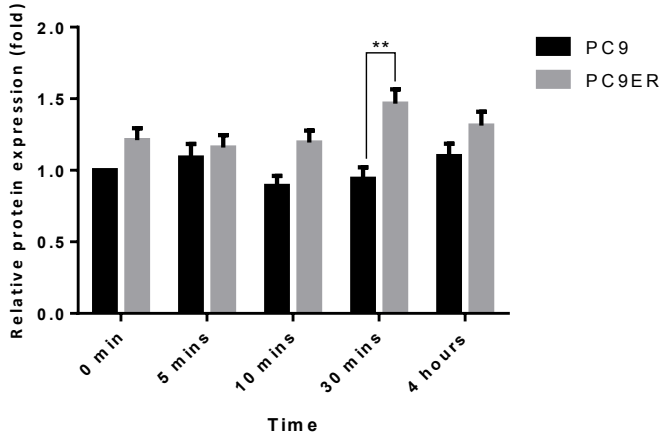
Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
cdc2	Cdc2 regulates eukaryotic cells entry into mitosis. The inhibitory phosphorylation at threonine 14 and tyrosine 15 are catalysed by WEE1 and Myelin Transcription Factor 1 (Myt1) (Wells et al., 1999b).	Cell Signaling Technologies	9112	rabbit	CDK1 (cdc2) <table><caption>Relative protein expression (fold) for CDK1 (cdc2)</caption><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>~1.6</td></tr><tr><td>5 mins</td><td>~1.1</td><td>~1.6</td></tr><tr><td>10 mins</td><td>~1.1</td><td>~1.5</td></tr><tr><td>30 mins</td><td>~1.4</td><td>~2.0</td></tr><tr><td>4 hours</td><td>~1.4</td><td>~1.5</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	~1.6	5 mins	~1.1	~1.6	10 mins	~1.1	~1.5	30 mins	~1.4	~2.0	4 hours	~1.4	~1.5
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4 hours	~1.4	~1.5																					
cdc25c P Ser216	Cdc25 is responsible for regulating eukaryotic cells into mitosis through its action as a protein phosphatase, responsible for dephosphorylating and activating cdc2 (Jesus and Ozon, 1995). During interphase c-TAK1 constitutively phosphorylates cdc25C at Serine 216, although during G2/M checkpoint this phosphorylation is DNA damage-dependent (Peng et al., 1997). Following phosphorylation at serine 216, cdc25C binds to 14-3-3 family members sequestering it to the cytoplasm and preventing entry into mitosis (Kumagai and Dunphy, 1999).	Cell Signaling Technologies	4901	rabbit	cdc25c P Ser216 <table><caption>Relative protein expression (fold) for cdc25c P Ser216</caption><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>~0.9</td></tr><tr><td>5 mins</td><td>~0.8</td><td>~1.2</td></tr><tr><td>10 mins</td><td>~1.1</td><td>~1.3</td></tr><tr><td>30 mins</td><td>~1.0</td><td>~1.1</td></tr><tr><td>4 hours</td><td>~0.9</td><td>~1.1</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	~0.9	5 mins	~0.8	~1.2	10 mins	~1.1	~1.3	30 mins	~1.0	~1.1	4 hours	~0.9	~1.1
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Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
CDK1 (p34cdc2) P Tyr15	Cyclin-dependent kinase 1 (CDK1) regulates eukaryotic cells into mitosis. This is controlled by a number of processes including cyclin binding and phosphorylation of cdc2 at Thr161 (Norbury et al., 1991). Furthermore, dephosphorylation at threonine 14 and tyrosine 15 is required for its activation and therefore, progression into mitosis (Wells et al., 1999b).	Cell Signaling Technologies	9111	rabbit	pCDK1 Tyr15 <table><caption>Relative protein expression (fold) for pCDK1 Tyr15</caption><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>~1.6</td></tr><tr><td>5 mins</td><td>~0.8</td><td>~1.4</td></tr><tr><td>10 mins</td><td>~1.0</td><td>~1.5</td></tr><tr><td>30 mins</td><td>~1.3</td><td>~1.9</td></tr><tr><td>4 hours</td><td>~1.2</td><td>~1.4</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	~1.6	5 mins	~0.8	~1.4	10 mins	~1.0	~1.5	30 mins	~1.3	~1.9	4 hours	~1.2	~1.4
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CDK2	Cyclin-dependent kinase 2 (CDK2) is a protein involved in G1/S transition. Its activation requires dephosphorylation at threonine 14 and tyrosine 15 residues and phosphorylation at threonine 160 by the CAK complex (CDK7 and cyclin H) (Gu et al., 1992, Fesquet et al., 1993). G1 to S phase transition is regulated by CDK2/cyclin E kinase activity, which also has a role in phosphorylation of Rb protein (Bertoli et al., 2013). In S-phase the activated complex phosphorylate E2F (Morgan, 1997).	Epitomics	1134-1	rabbit	CDK2 <table><caption>Relative protein expression (fold) for CDK2</caption><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>~1.2</td></tr><tr><td>5 mins</td><td>~1.1</td><td>~1.1</td></tr><tr><td>10 mins</td><td>~0.9</td><td>~1.1</td></tr><tr><td>30 mins</td><td>~1.0</td><td>~1.5</td></tr><tr><td>4 hours</td><td>~1.1</td><td>~1.2</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	~1.2	5 mins	~1.1	~1.1	10 mins	~0.9	~1.1	30 mins	~1.0	~1.5	4 hours	~1.1	~1.2
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Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
Chk1 P Ser345	Chk1 kinase acts downstream of ATM/ATR (Liu et al., 2000). Genotoxic stress results in activation of Chk1 by phosphorylation of the Serine 317 residue resulting in blocked DNA replication (Zhao and Piwnica-Worms, 2001).	Cell Signaling Technologies	2348	rabbit	<table border="1"><caption>Chk1 P Ser345</caption><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>~1.65 (***)</td></tr><tr><td>5 mins</td><td>~1.05</td><td>~1.75 (**)</td></tr><tr><td>10 mins</td><td>~1.05</td><td>~1.70 (**)</td></tr><tr><td>30 mins</td><td>~1.05</td><td>~1.75 (**)</td></tr><tr><td>4 hours</td><td>~1.65</td><td>~1.65 (***)</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	~1.65 (***)	5 mins	~1.05	~1.75 (**)	10 mins	~1.05	~1.70 (**)	30 mins	~1.05	~1.75 (**)	4 hours	~1.65	~1.65 (***)
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c-Jun N-term	c-Jun interacts with DNA sequences and regulates gene expression of a number of genes involved in cell growth (Bohmann et al., 1987).	Epitomics	1254-1	rabbit	<table border="1"><caption>c-Jun N-term</caption><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>~1.1 (***)</td></tr><tr><td>5 mins</td><td>~0.65</td><td>~1.0</td></tr><tr><td>10 mins</td><td>~0.75</td><td>~0.95</td></tr><tr><td>30 mins</td><td>~0.85</td><td>~1.3</td></tr><tr><td>4 hours</td><td>~1.25</td><td>~1.2</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	~1.1 (***)	5 mins	~0.65	~1.0	10 mins	~0.75	~0.95	30 mins	~0.85	~1.3	4 hours	~1.25	~1.2
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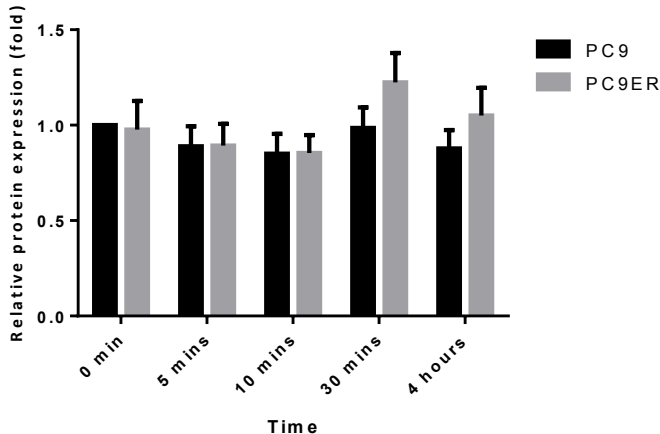
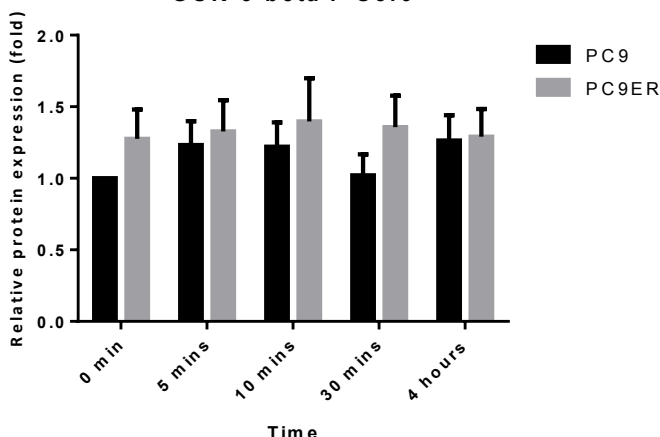
Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
c-Myc P Thr58,Ser62	c-Myc is a transcriptional regulator which has a function in a number of pathways including proliferation, differentiation and apoptosis (Baudino and Cleveland, 2001). Phosphorylation of c-Myc results in an increased protein stability (Wang et al., 2011).	Epitomics	1203-1	rabbit	c-Myc P Thr58,Ser62 <table><caption>Relative protein expression (fold) for c-Myc P Thr58,Ser62</caption><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>~1.4</td></tr><tr><td>5 mins</td><td>~1.15</td><td>~1.25</td></tr><tr><td>10 mins</td><td>~1.15</td><td>~1.3</td></tr><tr><td>30 mins</td><td>~1.15</td><td>~1.65</td></tr><tr><td>4 hours</td><td>~2.0</td><td>~1.8</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	~1.4	5 mins	~1.15	~1.25	10 mins	~1.15	~1.3	30 mins	~1.15	~1.65	4 hours	~2.0	~1.8
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CrkL	CrkL is involved in a number of signaling pathways include the modulation of cell adhesion, cell migration and immune cell responses initiated by different cytokines and growth factors (Feller, 2001). The Tyrosine 207 of CrkL is phosphorylated by BCR/Abl and potentially for other SH2-containing proteins (de Jong et al., 1997).	Cell Signaling Technologies	3182	mouse IgG1	CrkL <table><caption>Relative protein expression (fold) for CrkL</caption><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>~1.2</td></tr><tr><td>5 mins</td><td>~1.05</td><td>~1.05</td></tr><tr><td>10 mins</td><td>~1.1</td><td>~1.0</td></tr><tr><td>30 mins</td><td>~1.05</td><td>~1.35</td></tr><tr><td>4 hours</td><td>~1.1</td><td>~1.2</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	~1.2	5 mins	~1.05	~1.05	10 mins	~1.1	~1.0	30 mins	~1.05	~1.35	4 hours	~1.1	~1.2
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Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
CrkL P Tyr207		Cell Signaling Technologies	3181	rabbit	CrkL P Tyr207  Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (gray) <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.6*</td></tr><tr><td>5 mins</td><td>1.5</td><td>1.8</td></tr><tr><td>10 mins</td><td>1.8</td><td>2.1</td></tr><tr><td>30 mins</td><td>1.8</td><td>2.2</td></tr><tr><td>4 hours</td><td>2.1</td><td>2.3</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.6*	5 mins	1.5	1.8	10 mins	1.8	2.1	30 mins	1.8	2.2	4 hours	2.1	2.3
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Cyclin D1	Cyclin D1 is a regulator of cell cycle G1 to S phase progression (Sherr, 1996). Cyclin D1 is known to bind to cyclin dependent kinase 4 and 6, and these complexes promote progression through the phosphorylation of retinoblastoma protein (RB) (Kato et al., 1993, Lundberg and Weinberg, 1998, Weinberg, 1995). In addition it has a role in transcriptional modulation, through regulation of transcription factors as well as histone deacetylase (Coqueret, 2002).	Cell Signaling Technologies	2926	mouse IgG2a	Cyclin D1  Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (gray) <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.05</td></tr><tr><td>5 mins</td><td>0.9</td><td>1.0</td></tr><tr><td>10 mins</td><td>1.0</td><td>1.05</td></tr><tr><td>30 mins</td><td>0.95</td><td>1.2</td></tr><tr><td>4 hours</td><td>1.3</td><td>1.15</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.05	5 mins	0.9	1.0	10 mins	1.0	1.05	30 mins	0.95	1.2	4 hours	1.3	1.15
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E-Cadherin	E-Cadherin is a calcium-dependent cell-cell adhesion glycoprotein composed of five extracellular cadherin repeats (Wheelock and Johnson, 2003).	Cell Signaling Technologies	3195	Rabbit	E-Cadherin  Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (gray) <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.3</td></tr><tr><td>5 mins</td><td>0.8</td><td>1.3</td></tr><tr><td>10 mins</td><td>1.0</td><td>1.3</td></tr><tr><td>30 mins</td><td>1.1</td><td>1.6</td></tr><tr><td>4 hours</td><td>1.2</td><td>1.3</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.3	5 mins	0.8	1.3	10 mins	1.0	1.3	30 mins	1.1	1.6	4 hours	1.2	1.3
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FAK1	Focal adhesion kinase (FAK) is a widely expressed cytoplasmic protein tyrosine kinase involved in integrin-mediated signal transduction (Parsons et al., 2000). FAK is activated by integrin clustering, which leads to autophosphorylation, providing a binding site for both PI3K and PLCγ (Schaller et al., 1994, Cobb et al., 1994, Chen et al., 1996, Zhang et al., 1999b).	Cell Signaling Technologies	3285	Rabbit	FAK 1  Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (gray) <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.2</td></tr><tr><td>5 mins</td><td>1.1</td><td>1.2</td></tr><tr><td>10 mins</td><td>0.9</td><td>1.2</td></tr><tr><td>30 mins</td><td>1.0</td><td>1.5</td></tr><tr><td>4 hours</td><td>1.1</td><td>1.3</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.2	5 mins	1.1	1.2	10 mins	0.9	1.2	30 mins	1.0	1.5	4 hours	1.1	1.3
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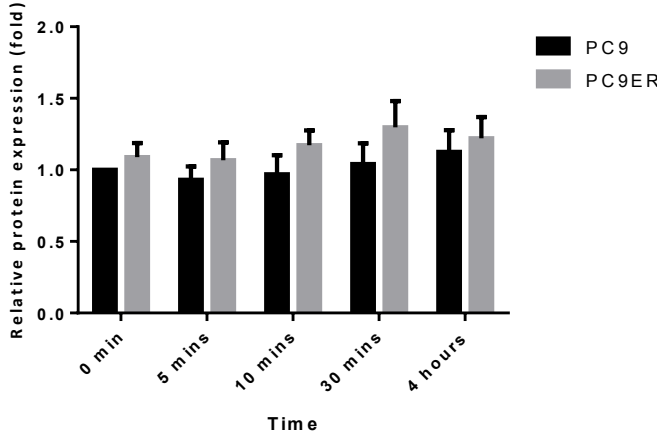
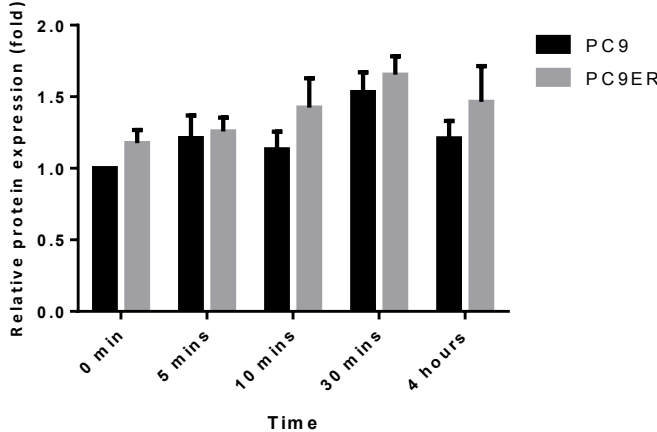
Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
FAK1 P TYR 397		Cell Signaling Technologies	3283	Rabbit	FAK1 P Y397 Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>~1.3</td></tr><tr><td>5 mins</td><td>~0.6</td><td>~1.5</td></tr><tr><td>10 mins</td><td>~1.2</td><td>~1.6</td></tr><tr><td>30 mins</td><td>~1.3</td><td>~1.8</td></tr><tr><td>4 hours</td><td>~1.0</td><td>~1.1</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	~1.3	5 mins	~0.6	~1.5	10 mins	~1.2	~1.6	30 mins	~1.3	~1.8	4 hours	~1.0	~1.1
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FLT3 P Tyr591 P Tyr591	FLT3, also known as Flk2, is a member of the type III receptor tyrosine kinase family, which includes c-Kit, PDGFR and M-CSF receptors. It has shown to have a role in growth and differentiation following activation by ligand (FL) binding, which results in activation of a number of downstream signalling pathways (Namikawa et al., 1996). Known downstream substrates of FLT3 include p85 subunit of PI3K, SHP2, GRB2 and Shc (Beslu et al., 1996, Zhang and Broxmeyer, 2000, Zhang et al., 1999a). Phosphorylation at Tyrosine 589 and 591 are required for its activation (Vempati et al., 2008)	Cell Signaling Technologies	3461	Rabbit	FLT3 P Tyr591 P Tyr591 Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>~1.3</td></tr><tr><td>5 mins</td><td>~1.0</td><td>~1.6</td></tr><tr><td>10 mins</td><td>~1.3</td><td>~1.6</td></tr><tr><td>30 mins</td><td>~1.4</td><td>~2.0</td></tr><tr><td>4 hours</td><td>~1.3</td><td>~1.5</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	~1.3	5 mins	~1.0	~1.6	10 mins	~1.3	~1.6	30 mins	~1.4	~2.0	4 hours	~1.3	~1.5
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FRA1 (R20)	Fos-related antigen 1 (FRA1) is part of the FOS family of proteins consisting of c-Fos, FosB, FRA1, and Fos-related antigen 2 (FRA2) (Tulchinsky, 2000). Both FRA1 and FRA2 act as transcriptional activators through their ability to form heterodimers with Jun proteins (Tulchinsky, 2000)	Santa Cruz	sc-605	Rabbit	FRA 1 (R20) Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.5</td></tr><tr><td>5 mins</td><td>1.1</td><td>1.5</td></tr><tr><td>10 mins</td><td>1.1</td><td>1.5</td></tr><tr><td>30 mins</td><td>1.0</td><td>2.0</td></tr><tr><td>4 hours</td><td>2.6</td><td>2.3</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.5	5 mins	1.1	1.5	10 mins	1.1	1.5	30 mins	1.0	2.0	4 hours	2.6	2.3
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GAPDH	GAPDH is an enzyme that catalyses the phosphorylation of glyceraldehyde-3-phosphate during glycolysis (Krasnov et al., 2013)	Abcam	ab9484	mouse IgG2b	GAPDH Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.2</td></tr><tr><td>5 mins</td><td>1.2</td><td>1.3</td></tr><tr><td>10 mins</td><td>1.3</td><td>1.2</td></tr><tr><td>30 mins</td><td>1.3</td><td>1.6*</td></tr><tr><td>4 hours</td><td>1.2</td><td>1.3</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.2	5 mins	1.2	1.3	10 mins	1.3	1.2	30 mins	1.3	1.6*	4 hours	1.2	1.3
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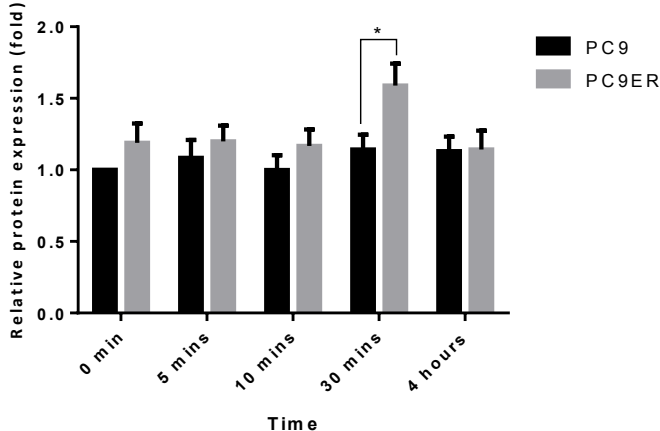
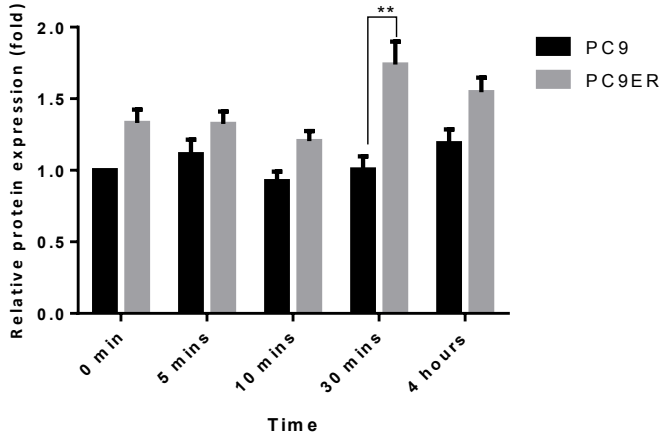
Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
GFAP	Glial Fibrillary Acidic Protein (GFAP), a class-III intermediate filament, is a cell-specific marker that, during the development of the central nervous system, distinguishes astrocytes from other glial cells (Eng, 1985, Eng et al., 2000)	Abcam	ab7260	Rabbit	GFAP  Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.0</td></tr><tr><td>5 mins</td><td>0.9</td><td>0.9</td></tr><tr><td>10 mins</td><td>0.85</td><td>0.85</td></tr><tr><td>30 mins</td><td>1.0</td><td>1.25</td></tr><tr><td>4 hours</td><td>0.9</td><td>1.05</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.0	5 mins	0.9	0.9	10 mins	0.85	0.85	30 mins	1.0	1.25	4 hours	0.9	1.05
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GSK-3-beta	Glycogen synthase kinase-3 (GSK-3) is an enzyme which regulates glycogen synthesis in response to insulin (Welsh et al., 1996). GSK-3 phosphorylates and inactivates glycogen synthase. GSK-3 can be inhibited by phosphorylated at Serine 9 by AKT (Srivastava and Pandey, 1998, Cross et al., 1995b).	Cell Signaling Technologies	9315	Rabbit	GSK-3-beta P Ser9  Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.0</td></tr><tr><td>5 mins</td><td>1.25</td><td>1.35</td></tr><tr><td>10 mins</td><td>1.25</td><td>1.4</td></tr><tr><td>30 mins</td><td>1.05</td><td>1.4</td></tr><tr><td>4 hours</td><td>1.3</td><td>1.3</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.0	5 mins	1.25	1.35	10 mins	1.25	1.4	30 mins	1.05	1.4	4 hours	1.3	1.3
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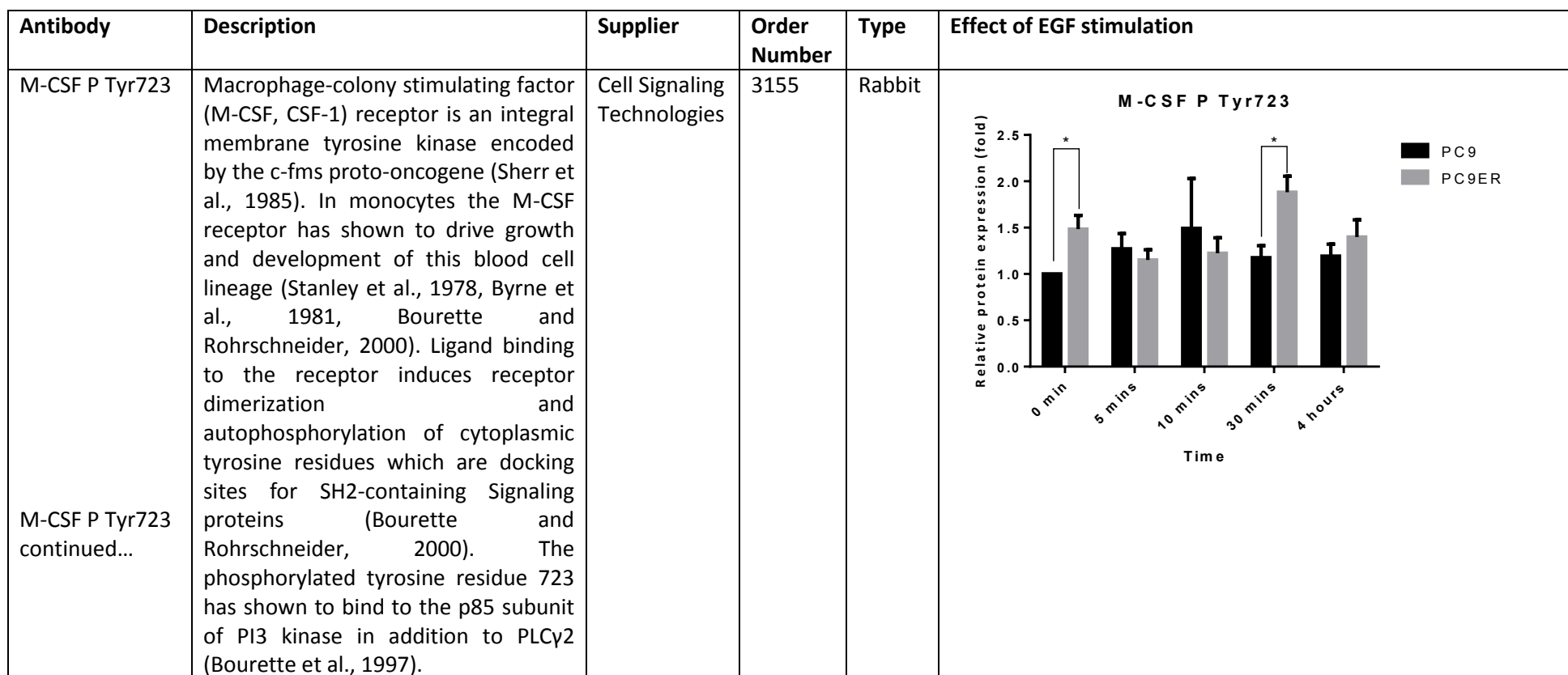
Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
GSK-3-beta P Ser9		Cell Signaling Technologies	9336	Rabbit	GSK-3-beta P Ser9 <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.3</td></tr><tr><td>5 mins</td><td>1.25</td><td>1.35</td></tr><tr><td>10 mins</td><td>1.25</td><td>1.4</td></tr><tr><td>30 mins</td><td>1.05</td><td>1.35</td></tr><tr><td>4 hours</td><td>1.3</td><td>1.3</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.3	5 mins	1.25	1.35	10 mins	1.25	1.4	30 mins	1.05	1.35	4 hours	1.3	1.3
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Hexokinase II	Hexokinases catalyse glucose conversion into glucose-6-phosphate. Hexokinase I, II and III are responsible for maintaining elevated rate of aerobic glycolysis in cancer cells (Warburg, 1956a).	Cell Signaling Technologies	2867	Rabbit	Hexokinase <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.1</td></tr><tr><td>5 mins</td><td>1.1</td><td>1.2</td></tr><tr><td>10 mins</td><td>0.95</td><td>1.25</td></tr><tr><td>30 mins</td><td>1.15</td><td>1.55</td></tr><tr><td>4 hours</td><td>1.45</td><td>1.5</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.1	5 mins	1.1	1.2	10 mins	0.95	1.25	30 mins	1.15	1.55	4 hours	1.45	1.5
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Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
HSP27 (HSPB1)	Hsp27 is a chaperone of the sHsp (small heat shock protein) family which facilitates refolding of damaged proteins in response to heat shock, oxidative and chemical stress (Jakob et al., 1993, Rogalla et al., 1999, Arrigo et al., 2005, Lanneau et al., 2008).	Cell Signaling Technologies	2402	mouse IgG1	HSP27 (HSPB1) <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.1</td></tr><tr><td>5 mins</td><td>1.05</td><td>1.05</td></tr><tr><td>10 mins</td><td>1.2</td><td>1.1</td></tr><tr><td>30 mins</td><td>1.05</td><td>1.15</td></tr><tr><td>4 hours</td><td>1.2</td><td>1.2</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.1	5 mins	1.05	1.05	10 mins	1.2	1.1	30 mins	1.05	1.15	4 hours	1.2	1.2
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IGF-1R beta P Tyr1162, Tyr1163	Type I insulin-like growth factor receptor (IGF-IR) is a transmembrane receptor tyrosine kinase. Phosphorylation of the receptor at tyrosine 1162 and 1163 results in receptor activation (Hubbard, 1997). The activated receptor signals to several pathways including MAPK, Raf/Ras and PI3K cascades (Denduluri et al., 2015).	Invitrogen (Biosource)	44-804G	Rabbit	IGF-1R beta P Tyr1162,Tyr1163 <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.35</td></tr><tr><td>5 mins</td><td>0.95</td><td>1.55</td></tr><tr><td>10 mins</td><td>1.3</td><td>1.55</td></tr><tr><td>30 mins</td><td>1.35</td><td>1.9</td></tr><tr><td>4 hours</td><td>1.35</td><td>1.6</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.35	5 mins	0.95	1.55	10 mins	1.3	1.55	30 mins	1.35	1.9	4 hours	1.35	1.6
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Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
IkB-alpha	IkB-alpha is an inhibitor of NF-κB/Rel transcription factors. Phosphorylation at serine 32 and 35 residues result in IkB-alpha proteasomal degradation and release of activated NF-κB (Finco et al., 1994, Brown et al., 1995).	Cell Signaling Technologies	4812	Rabbit	IkB -alpha <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.1</td></tr><tr><td>5 mins</td><td>0.95</td><td>1.08</td></tr><tr><td>10 mins</td><td>1.0</td><td>1.18</td></tr><tr><td>30 mins</td><td>1.05</td><td>1.32</td></tr><tr><td>4 hours</td><td>1.12</td><td>1.25</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.1	5 mins	0.95	1.08	10 mins	1.0	1.18	30 mins	1.05	1.32	4 hours	1.12	1.25
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IkB-alpha P Ser32	Cell Signaling Technologies	2859	Rabbit	IkB -alpha P Ser32 <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.2</td></tr><tr><td>5 mins</td><td>1.22</td><td>1.28</td></tr><tr><td>10 mins</td><td>1.15</td><td>1.45</td></tr><tr><td>30 mins</td><td>1.55</td><td>1.68</td></tr><tr><td>4 hours</td><td>1.22</td><td>1.48</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.2	5 mins	1.22	1.28	10 mins	1.15	1.45	30 mins	1.55	1.68	4 hours	1.22	1.48	
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Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
JAK1	Janus kinase 1 (JAK1) is a member of the Janus family of tyrosine kinases (JAK1, JAK2, JAK3, and Tyk2), which are activated by ligands binding to a number of associated cytokine receptors (Leonard and O'Shea, 1998). Activation of cytokine receptors result in autophosphorylation of JAK proteins, which then phosphorylate their associated receptor creating binding sites for signalling proteins including STATs, SHC, FAK etc. (Darnell, 1997, Zhu et al., 1998, VanderKuur et al., 1995).	Cell Signaling Technologies	3332	rabbit	JAK 1 Relative protein expression (fold) Time Legend: PC 9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC 9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.2</td></tr><tr><td>5 mins</td><td>1.3</td><td>1.4</td></tr><tr><td>10 mins</td><td>1.2</td><td>1.3</td></tr><tr><td>30 mins</td><td>1.1</td><td>1.8*</td></tr><tr><td>4 hours</td><td>1.2</td><td>1.4</td></tr></tbody></table>	Time	PC 9	PC9ER	0 min	1.0	1.2	5 mins	1.3	1.4	10 mins	1.2	1.3	30 mins	1.1	1.8*	4 hours	1.2	1.4
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JAK1 P Tyr1022,Thr1023	JAK phosphorylation at tyrosine 1022 and 1023 residues have been implicated in kinase activity due to the homologous tyrosine residues 1054/1055 in Tyk2 (Gauzzi et al., 1996)	Invitrogen (Biosource)	44-422G	rabbit	JAK1 P Tyr1022,Thr1023 Relative protein expression (fold) Time Legend: PC 9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC 9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.3</td></tr><tr><td>5 mins</td><td>0.8</td><td>1.3</td></tr><tr><td>10 mins</td><td>1.0</td><td>1.2</td></tr><tr><td>30 mins</td><td>1.1</td><td>1.5</td></tr><tr><td>4 hours</td><td>1.1</td><td>1.4</td></tr></tbody></table>	Time	PC 9	PC9ER	0 min	1.0	1.3	5 mins	0.8	1.3	10 mins	1.0	1.2	30 mins	1.1	1.5	4 hours	1.1	1.4
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Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
LKB1	LKB1 is a serine/threonine kinase, tumour suppressor that is involved in the control cell structure, apoptosis and energy homeostasis through regulation of numerous downstream kinases (Baas et al., 2004, Hardie, 2004). LKB1 is activated when in complex with, putative kinase STRAD, and the MO25 scaffold protein (Hawley et al., 2003). When activated it is able to phosphorylate and activate AMPK and several AMPK-related kinases (Baas et al., 2003).	Cell Signaling Technologies	3047	Rabbit	L K B 1  Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><caption>Approximate data for LKB1 chart</caption><thead><tr><th>Time</th><th>PC9 (fold)</th><th>PC9ER (fold)</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.2</td></tr><tr><td>5 mins</td><td>1.1</td><td>1.2</td></tr><tr><td>10 mins</td><td>1.0</td><td>1.2</td></tr><tr><td>30 mins</td><td>1.15</td><td>1.6*</td></tr><tr><td>4 hours</td><td>1.15</td><td>1.15</td></tr></tbody></table>	Time	PC9 (fold)	PC9ER (fold)	0 min	1.0	1.2	5 mins	1.1	1.2	10 mins	1.0	1.2	30 mins	1.15	1.6*	4 hours	1.15	1.15
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MAPKAPK-2	MAP kinase-activated protein kinase 2 (MAPKAPK-2) is a serine /threonine protein kinase regulated through direct phosphorylation by p38 MAP kinase (Rouse et al., 1994). Activated MAPKAPK-2 a wide range of proteins which leads to regulation of a number of signalling pathways.	Epitomics	1497-1	rabbit	MAP K A P K - 2  Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><caption>Approximate data for MAPKAPK-2 chart</caption><thead><tr><th>Time</th><th>PC9 (fold)</th><th>PC9ER (fold)</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.35</td></tr><tr><td>5 mins</td><td>1.15</td><td>1.35</td></tr><tr><td>10 mins</td><td>0.95</td><td>1.25</td></tr><tr><td>30 mins</td><td>1.05</td><td>1.75**</td></tr><tr><td>4 hours</td><td>1.25</td><td>1.6</td></tr></tbody></table>	Time	PC9 (fold)	PC9ER (fold)	0 min	1.0	1.35	5 mins	1.15	1.35	10 mins	0.95	1.25	30 mins	1.05	1.75**	4 hours	1.25	1.6
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MEK1/2	MEK1 and MEK2 are protein kinases which have a role in cell growth and proliferation through activation of protein kinase cascade (Crews et al., 1992, Cowley et al., 1994). It is activated through phosphorylation at serine 217 and 221 (Alessi et al., 1994)	Cell Signaling Technologies	9122	Rabbit	MEK 1/2 <table><thead><tr><th>Time</th><th>PC 9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.35</td></tr><tr><td>5 mins</td><td>1.0</td><td>1.1</td></tr><tr><td>10 mins</td><td>0.85</td><td>1.05</td></tr><tr><td>30 mins</td><td>0.95</td><td>1.5</td></tr><tr><td>4 hours</td><td>1.2</td><td>1.25</td></tr></tbody></table>	Time	PC 9	PC9ER	0 min	1.0	1.35	5 mins	1.0	1.1	10 mins	0.85	1.05	30 mins	0.95	1.5	4 hours	1.2	1.25
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MEK1/2 P Ser217/221	Cell Signaling Technologies	9154	Rabbit	MEK 1/2 P Ser217/221 <table><thead><tr><th>Time</th><th>PC 9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.0</td></tr><tr><td>5 mins</td><td>2.9</td><td>2.8</td></tr><tr><td>10 mins</td><td>3.6</td><td>3.5</td></tr><tr><td>30 mins</td><td>2.8</td><td>3.2</td></tr><tr><td>4 hours</td><td>2.2</td><td>1.8</td></tr></tbody></table>	Time	PC 9	PC9ER	0 min	1.0	1.0	5 mins	2.9	2.8	10 mins	3.6	3.5	30 mins	2.8	3.2	4 hours	2.2	1.8	
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Met	Met, a high affinity tyrosine kinase receptor for hepatocyte growth factor (HGF) (Cooper et al., 1984, Bottaro et al., 1991). Phosphorylation of tyrosine residues 1234/1235 are critical for MET activation (Longati et al., 1994). The phosphorylation at Tyrosine 1349 provides a docking site for Gab1 (Schaeper et al., 2000).	Cell Signaling Technologies	4560	rabbit	M E T <table><caption>Relative protein expression (fold) for MET</caption><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.2</td></tr><tr><td>5 mins</td><td>1.1</td><td>1.25</td></tr><tr><td>10 mins</td><td>1.15</td><td>1.25</td></tr><tr><td>30 mins</td><td>1.2</td><td>1.55</td></tr><tr><td>4 hours</td><td>1.35</td><td>1.35</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.2	5 mins	1.1	1.25	10 mins	1.15	1.25	30 mins	1.2	1.55	4 hours	1.35	1.35
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Met P Tyr1234	Signalway	11227-1	rabbit	Met P Tyr1234 <table><caption>Relative protein expression (fold) for Met P Tyr1234</caption><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.6</td></tr><tr><td>5 mins</td><td>1.1</td><td>1.45</td></tr><tr><td>10 mins</td><td>1.4</td><td>1.4</td></tr><tr><td>30 mins</td><td>1.25</td><td>1.85</td></tr><tr><td>4 hours</td><td>1.35</td><td>1.65</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.6	5 mins	1.1	1.45	10 mins	1.4	1.4	30 mins	1.25	1.85	4 hours	1.35	1.65	
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Met P Tyr1349		Signalway	11238	rabbit	Met P Tyr1349 <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.2</td></tr><tr><td>5 mins</td><td>0.8</td><td>1.6*</td></tr><tr><td>10 mins</td><td>1.2</td><td>1.5</td></tr><tr><td>30 mins</td><td>1.2</td><td>1.8</td></tr><tr><td>4 hours</td><td>1.1</td><td>1.3</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.2	5 mins	0.8	1.6*	10 mins	1.2	1.5	30 mins	1.2	1.8	4 hours	1.1	1.3
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4 hours	1.1	1.3																					
MNK1 (MKNK) P Thr197,Thr202	MNK1 a CAM kinase of the MAPKKAPK family that can regulate translation through phosphorylation of eIF4E at Ser209, increasing its affinity for the 7-methylguanosine-containing mRNA cap (Waskiewicz et al., 1999). MNK1 is activated by MAPK phosphorylation at threonine 197 and 202 residues (Waskiewicz et al., 1999).	Cell Signaling Technologies	2111	rabbit	MNK1 (MKNK) P Thr197,Thr202 <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.4</td></tr><tr><td>5 mins</td><td>1.0</td><td>1.5</td></tr><tr><td>10 mins</td><td>1.4</td><td>1.7</td></tr><tr><td>30 mins</td><td>1.7</td><td>1.9</td></tr><tr><td>4 hours</td><td>1.4</td><td>1.5</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.4	5 mins	1.0	1.5	10 mins	1.4	1.7	30 mins	1.7	1.9	4 hours	1.4	1.5
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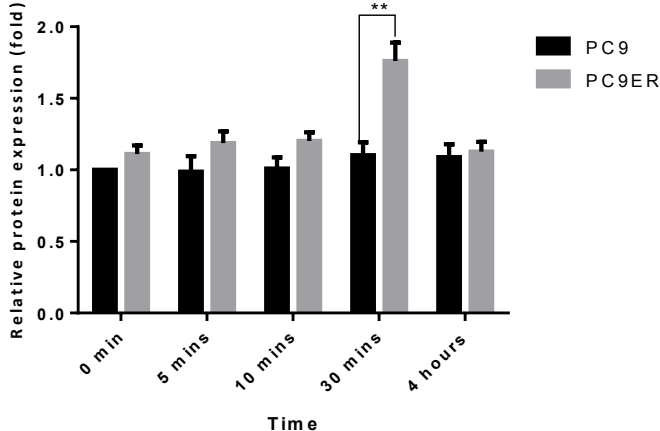
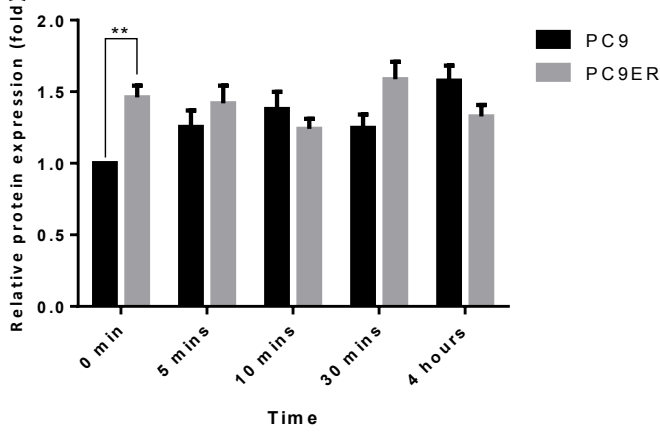
Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
MSK1 P Ser376	MSK1 is a mitogen and stress activated protein kinase (Deak et al., 1998). It is activated by ERK in response to growth factor stimulation and p38 MAPK in response cellular stress (Deak et al., 1998). Phosphorylation of MSK1 at Ser360 and Ser376 have been implicated in increasing MSK1 activity (McCoy et al., 2005).	Cell Signaling Technologies	9591	rabbit	MSK1 P Ser376 <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.4</td></tr><tr><td>5 mins</td><td>1.8</td><td>2.0</td></tr><tr><td>10 mins</td><td>2.6</td><td>2.4</td></tr><tr><td>30 mins</td><td>2.3</td><td>3.2</td></tr><tr><td>4 hours</td><td>1.4</td><td>2.1</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.4	5 mins	1.8	2.0	10 mins	2.6	2.4	30 mins	2.3	3.2	4 hours	1.4	2.1
Time	PC9	PC9ER																					
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4 hours	1.4	2.1																					
NFkB p105/p50	NFkB p105/p50 is part of the nuclear factor κ B (NF- κ B)/Rel family it is proteolytically processed to produce p50 and p52. p50 and p52 form dimers with Rel proteins, which regulate transcription by DNA binding (Baeuerle and Baltimore, 1996).	GeneTex	GTX110585	Rabbit	NFkB p105/p50 <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.2</td></tr><tr><td>5 mins</td><td>1.2</td><td>1.4</td></tr><tr><td>10 mins</td><td>1.3</td><td>1.3</td></tr><tr><td>30 mins</td><td>1.4</td><td>1.6</td></tr><tr><td>4 hours</td><td>1.3</td><td>1.3</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.2	5 mins	1.2	1.4	10 mins	1.3	1.3	30 mins	1.4	1.6	4 hours	1.3	1.3
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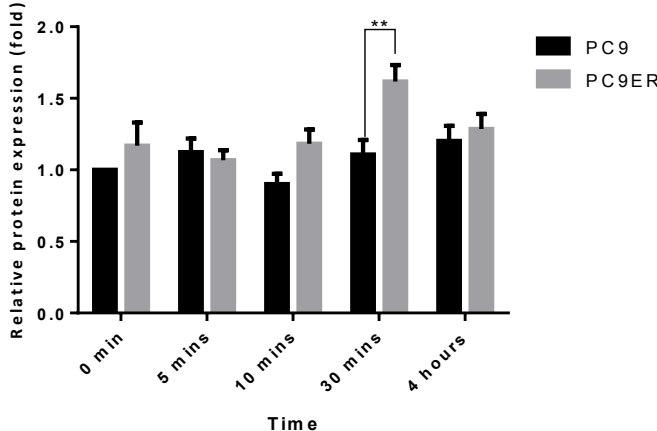
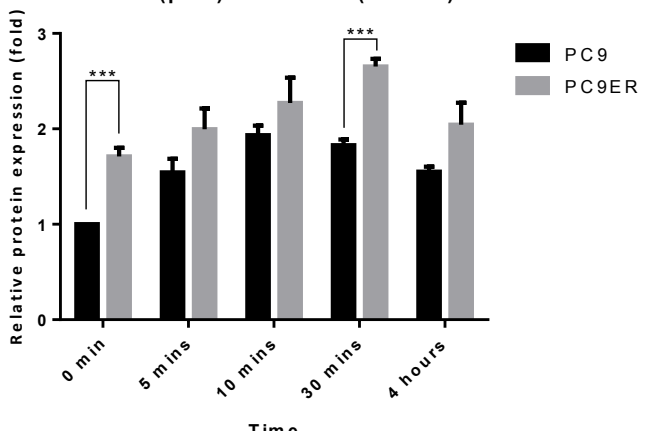
Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
NFkB p65 Ser536	NFkB-p65 is a subunit of NF-kappa-B transcription complex the inhibitory action of I-kappa-B is predominantly through interaction with the p65 subunit (Mattioli et al., 2004). Additionally the p65 subunit has shown to contribute to the DNA binding of the NF-kappa-B complex (Hansen et al., 1992).	Cell Signaling Technologies	3033	rabbit	NFkB p65 Ser536 <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>0.95</td></tr><tr><td>5 mins</td><td>0.55</td><td>0.8</td></tr><tr><td>10 mins</td><td>1.05</td><td>0.95</td></tr><tr><td>30 mins</td><td>1.15</td><td>1.0</td></tr><tr><td>4 hours</td><td>0.85</td><td>1.15</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	0.95	5 mins	0.55	0.8	10 mins	1.05	0.95	30 mins	1.15	1.0	4 hours	0.85	1.15
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p21 CIP/WAF1	The tumour suppressor p21Cip/WAF1, Is an important regulator of cell cycle progression through its ability to inhibit CDK2 complexes blocking progression through G1/S (Pestell et al., 1999). Phosphorylation at threonine 145 and serine 146 by AKT has been implicated to increase stability of p21Cip/WAF1 as well as promotes cell survival (Li et al., 2002).	Cell Signaling Technologies	2946	mouse IgG2a	p21 CIP/WAF1 <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.05</td></tr><tr><td>5 mins</td><td>1.0</td><td>1.1</td></tr><tr><td>10 mins</td><td>1.1</td><td>1.05</td></tr><tr><td>30 mins</td><td>1.1</td><td>1.25</td></tr><tr><td>4 hours</td><td>1.35</td><td>1.2</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.05	5 mins	1.0	1.1	10 mins	1.1	1.05	30 mins	1.1	1.25	4 hours	1.35	1.2
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Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
p21 CIP/WAF1 p Thr145		Santa Cruz	20220-R	rabbit	p21 CIP/WAF1 p Thr145 <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.15</td></tr><tr><td>5 mins</td><td>1.0</td><td>1.05</td></tr><tr><td>10 mins</td><td>1.05</td><td>1.0</td></tr><tr><td>30 mins</td><td>1.05</td><td>1.05</td></tr><tr><td>4 hours</td><td>1.05</td><td>1.1</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.15	5 mins	1.0	1.05	10 mins	1.05	1.0	30 mins	1.05	1.05	4 hours	1.05	1.1
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4 hours	1.05	1.1																					
p38 MAPK	p38 MAP kinase (MAPK) is involved in a number of signalling cascades including cellular response to cytokines and stress (Rouse et al., 1994, Han et al., 1994, Lee et al., 1994, Freshney et al., 1994). Indeed, it is activated by a number of cellular stresses including osmotic shock, inflammatory cytokines, lipopolysaccharide (LPS), UV light, and growth factors (Rouse et al., 1994, Han et al., 1994, Lee et al., 1994, Freshney et al., 1994, Raingeaud et al., 1995). Phosphorylation by MKK3,	Cell Signaling Technologies	9212	Rabbit	p38 MAPK <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>0.9</td></tr><tr><td>5 mins</td><td>1.1</td><td>0.9</td></tr><tr><td>10 mins</td><td>1.2</td><td>1.0</td></tr><tr><td>30 mins</td><td>1.45</td><td>1.25</td></tr><tr><td>4 hours</td><td>1.45</td><td>1.05</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	0.9	5 mins	1.1	0.9	10 mins	1.2	1.0	30 mins	1.45	1.25	4 hours	1.45	1.05
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Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
p38 MAPK PThr180,Tyr182	MKK6, and SEK at threonine 180 and tyrosine 182 result in its activation and ability to phosphorylate downstream signalling proteins MAPKAP kinase 2 (Lee et al., 1994), the transcription factors ATF-2 (Raingeaud et al., 1995), Max (Zervos et al., 1995) and MEF2 (Raingeaud et al., 1995).	Cell Signaling Technologies	9211	Rabbit	p38 MAPK PThr180,Tyr182 <table><caption>Relative protein expression (fold) for p38 MAPK PThr180,Tyr182</caption><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>~1.4</td></tr><tr><td>5 mins</td><td>~0.6</td><td>~0.9</td></tr><tr><td>10 mins</td><td>~1.8</td><td>~1.5</td></tr><tr><td>30 mins</td><td>~1.0</td><td>~1.2</td></tr><tr><td>4 hours</td><td>~0.6</td><td>~0.9</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	~1.4	5 mins	~0.6	~0.9	10 mins	~1.8	~1.5	30 mins	~1.0	~1.2	4 hours	~0.6	~0.9
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p53	P53 is important in the cells response to DNA damage. The activation of p53 results in either cell cycle arrest and DNA repair or apoptosis (Chehab et al., 1999). DNA damage results in the phosphorylation of p53 at Ser15 and Ser20. Phosphorylation at these sites leads to a reduced in its interaction with its negative regulator, MDM2 (Milczarek et al., 1997).	Cell Signaling Technologies	9282	Rabbit	p53 <table><caption>Relative protein expression (fold) for p53</caption><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>~1.5</td></tr><tr><td>5 min</td><td>~1.0</td><td>~1.4</td></tr><tr><td>10 min</td><td>~0.9</td><td>~1.5</td></tr><tr><td>30 min</td><td>~0.9</td><td>~1.6</td></tr><tr><td>4 hrs</td><td>~0.9</td><td>~1.3</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	~1.5	5 min	~1.0	~1.4	10 min	~0.9	~1.5	30 min	~0.9	~1.6	4 hrs	~0.9	~1.3
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Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
p53 P Ser15		Cell Signaling Technologies	9284	Rabbit	p 53 P Ser15 Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (gray) <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.4</td></tr><tr><td>5 min</td><td>1.1</td><td>1.35</td></tr><tr><td>10 min</td><td>1.05</td><td>1.45</td></tr><tr><td>30 min</td><td>1.02</td><td>1.6</td></tr><tr><td>4 hrs</td><td>1.03</td><td>1.42</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.4	5 min	1.1	1.35	10 min	1.05	1.45	30 min	1.02	1.6	4 hrs	1.03	1.42
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30 min	1.02	1.6																					
4 hrs	1.03	1.42																					
PARP	PARP is polymerase involved in DNA repair in response to environmental stress (Sato and Lindahl, 1992)	Cell Signaling Technologies	9542	Rabbit	PARP Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (gray) <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.3</td></tr><tr><td>5 mins</td><td>1.25</td><td>1.25</td></tr><tr><td>10 mins</td><td>1.08</td><td>1.35</td></tr><tr><td>30 mins</td><td>1.18</td><td>1.85</td></tr><tr><td>4 hours</td><td>1.35</td><td>1.4</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.3	5 mins	1.25	1.25	10 mins	1.08	1.35	30 mins	1.18	1.85	4 hours	1.35	1.4
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Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
PDGFR P Tyr1021	Platelet derived growth factor receptor (PDGFR) binds to ligand resulting in dimerization of receptor followed by autophosphorylation at Tyrosine 1009 and 1021 residues followed by activation of signal transduction molecules containing SH2 (Rönnstrand et al., 1992).	Cell Signaling Technologies	2227	Rabbit	PDGFR P Tyr1021  Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.1</td></tr><tr><td>5 mins</td><td>1.0</td><td>1.2</td></tr><tr><td>10 mins</td><td>1.0</td><td>1.2</td></tr><tr><td>30 mins</td><td>1.1</td><td>1.8**</td></tr><tr><td>4 hours</td><td>1.1</td><td>1.1</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.1	5 mins	1.0	1.2	10 mins	1.0	1.2	30 mins	1.1	1.8**	4 hours	1.1	1.1
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30 mins	1.1	1.8**																					
4 hours	1.1	1.1																					
PDK-1 P Ser241	Phosphoinositide-dependent protein kinase 1 (PDK1) has a role in a number of signalling pathways through the activation of a number of substrates including PKC, PKA and p70 S6 kinase (Belham et al., 1999). Autophosphorylation of the the receptor at serine 241 is required for PDK1 activity (Casamayor et al., 1999).	Cell Signaling Technologies	3061	rabbit	PDK-1 P Ser241  Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.5**</td></tr><tr><td>5 mins</td><td>1.3</td><td>1.4</td></tr><tr><td>10 mins</td><td>1.4</td><td>1.3</td></tr><tr><td>30 mins</td><td>1.3</td><td>1.6</td></tr><tr><td>4 hours</td><td>1.6</td><td>1.4</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.5**	5 mins	1.3	1.4	10 mins	1.4	1.3	30 mins	1.3	1.6	4 hours	1.6	1.4
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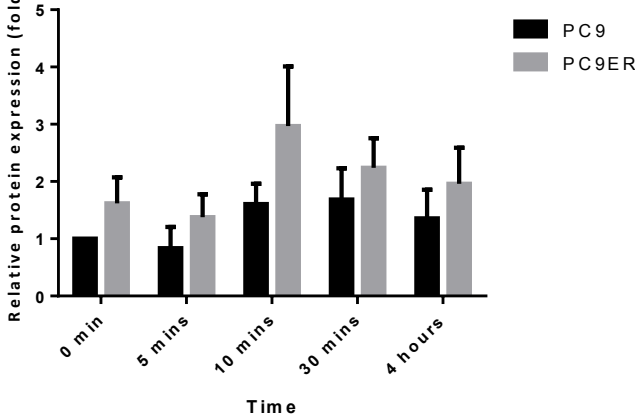
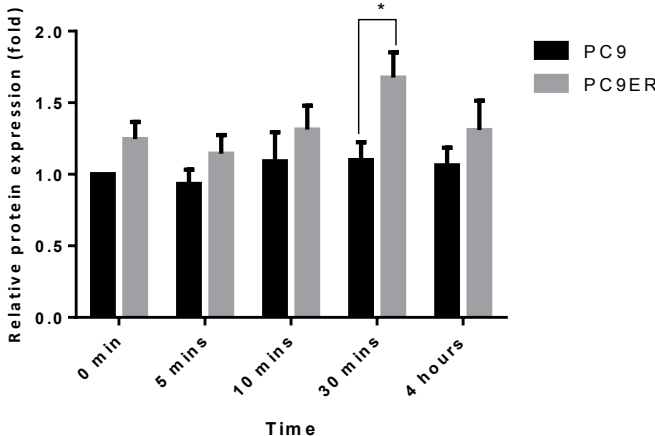
Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
PKA	Protein kinase A (PKA) activity is dependent on cellular levels of cyclic AMP (cAMP) (Hubbard and Till, 2000). PKA's catalytic subunit phosphorylate proteins on a target sequence predominantly [Arg-Arg-X-Ser/Thr-X] (X represents hydrophobic amino acid) on threonine and serine residues (Pearce et al., 2010). It is able to phosphorylate a number of molecules including enzymes, ion channels, chromosomal proteins e.g. Histone H1 as well as transcription factors e.g. cyclic AMP response element binding proteins (CREB) (Pearce et al., 2010).	Abcam	ab26322	rabbit	PKA  Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.2</td></tr><tr><td>5 mins</td><td>1.15</td><td>1.1</td></tr><tr><td>10 mins</td><td>0.95</td><td>1.2</td></tr><tr><td>30 mins</td><td>1.15</td><td>1.65</td></tr><tr><td>4 hours</td><td>1.25</td><td>1.35</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.2	5 mins	1.15	1.1	10 mins	0.95	1.2	30 mins	1.15	1.65	4 hours	1.25	1.35
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PKC (pan) P Ser660 (beta-2)	PKC is a family of 12 serine/threonine kinases including conventional (α , β , and γ), novel (δ , ϵ , η and θ), atypical (ζ and ι/λ) and PKN and PKC-related (PKN1, PKN2 and PKN3) (Pearce et al., 2010). Each of which differ in cofactor requirement, tissue distribution and substrate specificity. PKC activity is controlled by distinct phosphorylation events, initial phosphorylation of the activation loop at threonine 500, autophosphorylation at 641 and phosphorylation at serine 660 the	Cell Signaling Technologies	9371	rabbit	PKC (pan) P Ser660 (beta-2)  Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.75</td></tr><tr><td>5 mins</td><td>1.6</td><td>2.0</td></tr><tr><td>10 mins</td><td>1.95</td><td>2.3</td></tr><tr><td>30 mins</td><td>1.85</td><td>2.7</td></tr><tr><td>4 hours</td><td>1.6</td><td>2.05</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.75	5 mins	1.6	2.0	10 mins	1.95	2.3	30 mins	1.85	2.7	4 hours	1.6	2.05
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Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
PKC-alpha	carboxy-terminal hydrophobic site (Keranen et al., 1995). Phosphorylation at threonine 638 of PKC-alpha although not required for activation its absence results in increased sensitivity to phosphatase action (Bornancin and Parker, 1996). PDK-1 has been shown phosphorylate PKCγ at threonine 514 resulting in a conformational change enabling phosphorylation at two carboxyl-terminal sites which results in its release into the cytoplasm (Seki et al., 2006).	Beckton Dickinson	610108	mouse IgG2b	PKC -alpha Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC9 (fold)</th><th>PC9ER (fold)</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.0</td></tr><tr><td>5 mins</td><td>0.9</td><td>1.0</td></tr><tr><td>10 mins</td><td>1.0</td><td>1.2</td></tr><tr><td>30 mins</td><td>1.0</td><td>1.1</td></tr><tr><td>4 hours</td><td>1.0</td><td>1.0</td></tr></tbody></table>	Time	PC9 (fold)	PC9ER (fold)	0 min	1.0	1.0	5 mins	0.9	1.0	10 mins	1.0	1.2	30 mins	1.0	1.1	4 hours	1.0	1.0
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PKC-alpha P Thr638	Abcam	ab32502	rabbit	PKC-alpha P Thr638 Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC9 (fold)</th><th>PC9ER (fold)</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.5</td></tr><tr><td>5 mins</td><td>1.1</td><td>1.4</td></tr><tr><td>10 mins</td><td>1.0</td><td>1.5</td></tr><tr><td>30 mins</td><td>1.0</td><td>2.0</td></tr><tr><td>4 hours</td><td>1.1</td><td>1.6</td></tr></tbody></table>	Time	PC9 (fold)	PC9ER (fold)	0 min	1.0	1.5	5 mins	1.1	1.4	10 mins	1.0	1.5	30 mins	1.0	2.0	4 hours	1.1	1.6	
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Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
PKC-gamma P Thr514		GeneTex	GTX25778	rabbit	PKC-gamma P Thr514 <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.5</td></tr><tr><td>5 mins</td><td>1.1</td><td>1.2</td></tr><tr><td>10 mins</td><td>1.0</td><td>1.2</td></tr><tr><td>30 mins</td><td>1.1</td><td>1.6</td></tr><tr><td>4 hours</td><td>1.3</td><td>1.6</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.5	5 mins	1.1	1.2	10 mins	1.0	1.2	30 mins	1.1	1.6	4 hours	1.3	1.6
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PKC-zeta		Cell Signaling Technologies	9372	rabbit	PKC-zeta <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.4</td></tr><tr><td>5 mins</td><td>1.4</td><td>1.6</td></tr><tr><td>10 mins</td><td>1.0</td><td>1.3</td></tr><tr><td>30 mins</td><td>1.8</td><td>1.6</td></tr><tr><td>4 hours</td><td>1.1</td><td>1.6</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.4	5 mins	1.4	1.6	10 mins	1.0	1.3	30 mins	1.8	1.6	4 hours	1.1	1.6
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Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
PKC substrate P (R/K)X(S*)(Hyd)(R/k)	Activated PKC phosphorylate a number of substrates containing serine or threonine, with arginine or Lysine residues at the -3, -2, and +2 positions, and a hydrophobic amino acid at position +1 (Pearson and Kemp, 1991, Obata et al., 2000, Nishikawa et al., 1997). CST has developed an antibody which recognizes this motif.	Cell Signaling Technologies	2261	Rabbit	PKC substrate P (R/K)X(S*)(Hyd)(R/k) <table><caption>Relative protein expression (fold) for PKC substrate P (R/K)X(S*)(Hyd)(R/k)</caption><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.25</td></tr><tr><td>5 mins</td><td>1.15</td><td>1.3</td></tr><tr><td>10 mins</td><td>1.1</td><td>1.2</td></tr><tr><td>30 mins</td><td>1.25</td><td>1.3</td></tr><tr><td>4 hours</td><td>1.3</td><td>1.35</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.25	5 mins	1.15	1.3	10 mins	1.1	1.2	30 mins	1.25	1.3	4 hours	1.3	1.35
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PKM2 XP(R)	Pyruvate kinase is a glycolytic enzyme that catalyses the conversion of phosphoenolpyruvate to pyruvate (Christofk et al., 2008a). The M2 isoform (PKM2) is expressed during embryonic development and in cancer cells (Christofk et al., 2008a). PKM2 is shown to be essential for aerobic glycolysis in tumour cells (Christofk et al., 2008a).	Cell Signaling Technologies	4053	Rabbit	PKM2 XP(R) <table><caption>Relative protein expression (fold) for PKM2 XP(R)</caption><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.5</td></tr><tr><td>5 mins</td><td>1.45</td><td>1.55</td></tr><tr><td>10 mins</td><td>1.1</td><td>1.45</td></tr><tr><td>30 mins</td><td>1.4</td><td>2.0</td></tr><tr><td>4 hours</td><td>1.55</td><td>1.5</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.5	5 mins	1.45	1.55	10 mins	1.1	1.45	30 mins	1.4	2.0	4 hours	1.55	1.5
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Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
Prohibitin	Prohibitin is a tumour suppressor protein involved in the control of cell cycle (McClung et al., 1995). In mitochondria prohibitin function as chaperone, maintaining mitochondrial protein stability during protein synthesis and transportation (Nijtmans et al., 2000). In the nucleus prohibitin interact with transcription factors such as Rb and p53 to regulate target gene transcription (Mishra et al., 2006, Fusaro et al., 2003). Extracellular prohibitin can bind and activate C3 to enhance complement activation (Mishra et al., 2007).	Santa Cruz	sc-28259	rabbit	Prohibitin <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.0</td></tr><tr><td>5 mins</td><td>0.6</td><td>0.9</td></tr><tr><td>10 mins</td><td>0.9</td><td>1.0</td></tr><tr><td>30 mins</td><td>1.0</td><td>1.3</td></tr><tr><td>4 hours</td><td>0.9</td><td>1.0</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.0	5 mins	0.6	0.9	10 mins	0.9	1.0	30 mins	1.0	1.3	4 hours	0.9	1.0
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Raf1 (C-12)	Serine/threonine-protein kinase that is involved in the recruitment of GTP-bound Ras, resulting in the activation of the MEK-MAP kinase pathway (Avruch et al., 1994).	Santa Cruz	sc-133	rabbit	Raf1 (C-12) <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.2</td></tr><tr><td>5 mins</td><td>1.2</td><td>1.1</td></tr><tr><td>10 mins</td><td>1.2</td><td>1.2</td></tr><tr><td>30 mins</td><td>1.3</td><td>1.6</td></tr><tr><td>4 hours</td><td>1.5</td><td>1.3</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.2	5 mins	1.2	1.1	10 mins	1.2	1.2	30 mins	1.3	1.6	4 hours	1.5	1.3
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Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
Rap1	Acts both as a regulator of telomere function and as a transcription regulator (Hardy et al., 1992).	Cell Signaling Technologies	4938	rabbit	RAP1  Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC9 (fold)</th><th>PC9ER (fold)</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.6</td></tr><tr><td>5 mins</td><td>0.8</td><td>1.4</td></tr><tr><td>10 mins</td><td>1.6</td><td>3.0</td></tr><tr><td>30 mins</td><td>1.7</td><td>2.3</td></tr><tr><td>4 hours</td><td>1.4</td><td>2.0</td></tr></tbody></table>	Time	PC9 (fold)	PC9ER (fold)	0 min	1.0	1.6	5 mins	0.8	1.4	10 mins	1.6	3.0	30 mins	1.7	2.3	4 hours	1.4	2.0
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Ras	Ras is a guanine-nucleotide binding proteins which cycles between active (GTP-bound) and inactive (GDP-bound) forms (Boguski and McCormick, 1993). Receptor tyrosine kinases and G protein-coupled receptors are able to activate Ras leading to the activation of Raf-MEK-MAPK pathway (Avruch et al., 1994).	Beckton Dickinson	610001	mouse IgG1	Ras  Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC9 (fold)</th><th>PC9ER (fold)</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.25</td></tr><tr><td>5 mins</td><td>0.95</td><td>1.15</td></tr><tr><td>10 mins</td><td>1.1</td><td>1.35</td></tr><tr><td>30 mins</td><td>1.1</td><td>1.7</td></tr><tr><td>4 hours</td><td>1.05</td><td>1.35</td></tr></tbody></table>	Time	PC9 (fold)	PC9ER (fold)	0 min	1.0	1.25	5 mins	0.95	1.15	10 mins	1.1	1.35	30 mins	1.1	1.7	4 hours	1.05	1.35
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Rb P Ser807,Ser811	The tumour suppressor protein Rb regulates cell proliferation via its control of progression through the G1-phase of the cell cycle (Sherr, 1996). Cell cycle-dependent phosphorylation of Rb by CDK inhibits Rb and thereby allows cell cycle progression (Knudsen and Wang, 1997).	Cell Signaling Technologies	9308	Rabbit	Rb P Ser807,Ser811 <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.1</td></tr><tr><td>5 mins</td><td>0.9</td><td>1.3</td></tr><tr><td>10 mins</td><td>1.3</td><td>1.5</td></tr><tr><td>30 mins</td><td>1.7</td><td>1.6</td></tr><tr><td>4 hours</td><td>1.5</td><td>1.3</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.1	5 mins	0.9	1.3	10 mins	1.3	1.5	30 mins	1.7	1.6	4 hours	1.5	1.3
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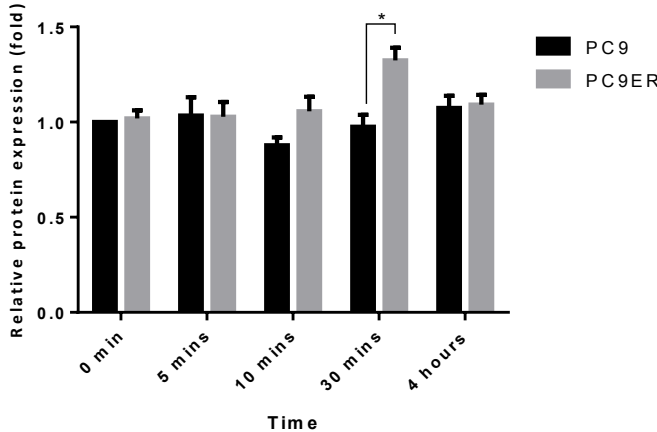
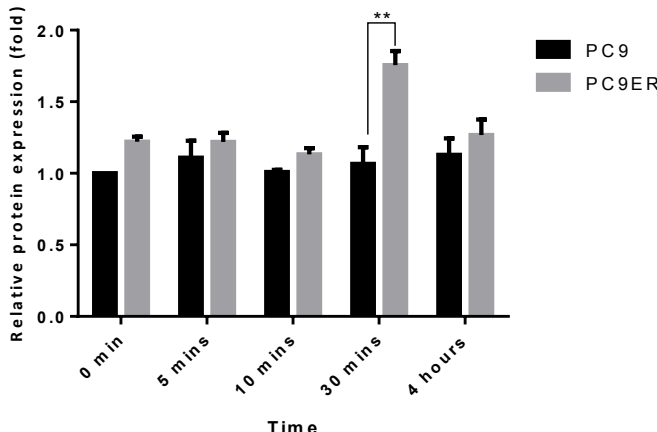
Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
Rsk2 Pser 227	The 90 kDa ribosomal S6 kinases (RSK1-4) are a family of widely expressed Ser/Thr kinases (Frodin and Gammeltoft, 1999). Phosphoinositide-dependent kinase-1 (PDK1) phosphorylates Serine 227 leading to substantial activation (Frodin et al., 2000)	Cell Signaling Technologies	3556	rabbit	Rsk2 Pser 227 Relative protein expression (fold) Time Legend: PC9 (black bar), PC9ER (gray bar) <table><caption>Approximate data from Rsk2 Pser 227 bar graph</caption><thead><tr><th>Time</th><th>PC9 (fold)</th><th>PC9ER (fold)</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.4</td></tr><tr><td>5 mins</td><td>1.6</td><td>1.7</td></tr><tr><td>10 mins</td><td>1.5</td><td>1.9</td></tr><tr><td>30 mins</td><td>1.5</td><td>2.1</td></tr><tr><td>4 hours</td><td>1.4</td><td>1.6</td></tr></tbody></table>	Time	PC9 (fold)	PC9ER (fold)	0 min	1.0	1.4	5 mins	1.6	1.7	10 mins	1.5	1.9	30 mins	1.5	2.1	4 hours	1.4	1.6
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SAPK/JNK P Thr183,Tyr185	Stress-activated protein kinase/Jun-amino-terminal kinase (SAPK/JNK), is activated by a number of environmental stresses including UV and gamma radiation, ceramides, inflammatory cytokines, as well as growth factors and GPCR agonists (Davis, 1999, Leppa and Bohmann, 1999, Whitmarsh and Davis, 1998, Kyriakis, 1999, Kyriakis and Avruch, 2001, Ichijo, 1999). Phosphorylation at threonine 183 and tyrosine 185 have shown to activate SAPK/JNK (Cobb and Goldsmith, 1995). It has shown to have a number of cellular function including regulating cell survival, apoptosis, and proliferation (Nishina et al., 2004).	Cell Signaling Technologies	4668	rabbit	SAPK/JNK P Thr183,Tyr185 Relative protein expression (fold) Time Legend: PC9 (black bar), PC9ER (grey bar) <table><thead><tr><th>Time</th><th>PC9 (fold)</th><th>PC9ER (fold)</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.1</td></tr><tr><td>5 mins</td><td>1.1</td><td>1.2</td></tr><tr><td>10 mins</td><td>3.3</td><td>2.1</td></tr><tr><td>30 mins</td><td>3.6</td><td>2.5</td></tr><tr><td>4 hours</td><td>1.3</td><td>1.0</td></tr></tbody></table>	Time	PC9 (fold)	PC9ER (fold)	0 min	1.0	1.1	5 mins	1.1	1.2	10 mins	3.3	2.1	30 mins	3.6	2.5	4 hours	1.3	1.0
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SHP2 P Tyr542	SHP-2 is a nonreceptor protein tyrosine phosphatase (PTP) which is involved in transducing mitogenic, pro-survival, cell-fate and/or pro-migratory signals from a number of growth-factor, cytokine and transduce mitogenic extracellular-matrix receptors (QU, 2000). Activation of growth receptors results in phosphorylation of SHP-2 in the carboxy-terminus at tyrosine 542 and 580 which relieve its inhibition and stimulate its tyrosine phosphatase activity (Bennett et al., 1994, Edouard et al., 2007).	Cell Signaling Technologies	3751	Rabbit	SHP2 P Tyr542 Relative protein expression (fold) Time PC9 PC9ER <table border="1"><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.3</td></tr><tr><td>5 mins</td><td>1.0</td><td>1.7</td></tr><tr><td>10 mins</td><td>1.4</td><td>1.9</td></tr><tr><td>30 mins</td><td>1.5</td><td>2.2</td></tr><tr><td>4 hours</td><td>1.4</td><td>1.6</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.3	5 mins	1.0	1.7	10 mins	1.4	1.9	30 mins	1.5	2.2	4 hours	1.4	1.6
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STAT3	STAT3 is a transcription factor and has shown to have a role as a signalling molecule for cytokines and growth factor receptors (Darnell et al., 1994).	Cell Signaling Technologies	9132	Rabbit	Stat3 Relative protein expression (fold) Time PC9 PC9ER <table border="1"><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.4</td></tr><tr><td>5 mins</td><td>1.5</td><td>1.4</td></tr><tr><td>10 mins</td><td>1.3</td><td>1.3</td></tr><tr><td>30 mins</td><td>1.3</td><td>1.7</td></tr><tr><td>4 hours</td><td>1.7</td><td>1.6</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.4	5 mins	1.5	1.4	10 mins	1.3	1.3	30 mins	1.3	1.7	4 hours	1.7	1.6
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Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
STAT5 P Tyr694	STAT5 is a transcription factor which is activated in response to a wide variety of stimuli including IL-2, GM-CSF, growth hormone and prolactin. Phosphorylation at Tyrosine 694 is required for STAT5 activation (Okutani et al., 2001).	Cell Signaling Technologies	9351	rabbit	Stat5 P Tyr694 Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC9 (fold)</th><th>PC9ER (fold)</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.3</td></tr><tr><td>5 mins</td><td>0.85</td><td>1.1</td></tr><tr><td>10 mins</td><td>1.1</td><td>0.95</td></tr><tr><td>30 mins</td><td>0.9</td><td>1.1</td></tr><tr><td>4 hours</td><td>0.85</td><td>1.3</td></tr></tbody></table>	Time	PC9 (fold)	PC9ER (fold)	0 min	1.0	1.3	5 mins	0.85	1.1	10 mins	1.1	0.95	30 mins	0.9	1.1	4 hours	0.85	1.3
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Survivin	Survivin is highly expressed in fetal development and in cancer (Reed and Reed, 1999). It binds and inhibits caspase-3s and controls G2/M-phase of cell cycle (Li et al., 1999). It is thereby able to inhibit apoptosis and promote cell division.	Cell Signaling Technologies	2808	Rabbit	Survivin Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC9 (fold)</th><th>PC9ER (fold)</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.4</td></tr><tr><td>5 mins</td><td>1.25</td><td>1.4</td></tr><tr><td>10 mins</td><td>1.15</td><td>1.45</td></tr><tr><td>30 mins</td><td>1.2</td><td>1.9</td></tr><tr><td>4 hours</td><td>1.35</td><td>1.6</td></tr></tbody></table>	Time	PC9 (fold)	PC9ER (fold)	0 min	1.0	1.4	5 mins	1.25	1.4	10 mins	1.15	1.45	30 mins	1.2	1.9	4 hours	1.35	1.6
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Tau	Tau is a microtubule-associated protein which promotes and stabilizes microtubule assembly (Kadavath et al., 2015). Phosphorylation of Tau at Serine 305 disrupts Tau-microtubule binding (Hanger et al., 2007).	Epitomics	1178-1	Rabbit	Tau <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.35</td></tr><tr><td>5 mins</td><td>1.2</td><td>1.3</td></tr><tr><td>10 mins</td><td>1.1</td><td>1.25</td></tr><tr><td>30 mins</td><td>1.15</td><td>1.75</td></tr><tr><td>4 hours</td><td>1.3</td><td>1.55</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.35	5 mins	1.2	1.3	10 mins	1.1	1.25	30 mins	1.15	1.75	4 hours	1.3	1.55
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TBK1/NAK	TBK1 (TANK-binding kinase 1)/NAK (NF-κB activating kinase) is a kinase that is able to activate IKK through direct phosphorylation (Tojima et al., 2000).	Cell Signaling Technologies	3504	Rabbit	T B K 1 / N A K  Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.05</td></tr><tr><td>5 mins</td><td>1.05</td><td>1.05</td></tr><tr><td>10 mins</td><td>0.9</td><td>1.1</td></tr><tr><td>30 mins</td><td>1.0</td><td>1.35*</td></tr><tr><td>4 hours</td><td>1.1</td><td>1.1</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.05	5 mins	1.05	1.05	10 mins	0.9	1.1	30 mins	1.0	1.35*	4 hours	1.1	1.1
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30 mins	1.0	1.35*																					
4 hours	1.1	1.1																					
Tsc-2	Tuberin (TSC-2) is a tumour suppressor protein that is involved in the regulation of cell proliferation and tumour development (Soucek et al., 1998). Phosphorylation at threonine 1462 regulates tuberin-hamartin complexes and tuberin activity (Manning et al., 2002). TSC-2 is able to inhibit mTOR resulting in inhibition of translation through a 4EBP1 mediated pathway (Goncharova et al., 2002, Manning et al., 2002, Inoki et al., 2002). In addition, AMPK phosphorylates TSC2 at serine 1387,	Cell Signaling Technologies	3612	rabbit	T s c - 2  Relative protein expression (fold) Time Legend: PC9 (black), PC9ER (grey) <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.25</td></tr><tr><td>5 mins</td><td>1.1</td><td>1.25</td></tr><tr><td>10 mins</td><td>1.0</td><td>1.15</td></tr><tr><td>30 mins</td><td>1.1</td><td>1.8**</td></tr><tr><td>4 hours</td><td>1.15</td><td>1.3</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.25	5 mins	1.1	1.25	10 mins	1.0	1.15	30 mins	1.1	1.8**	4 hours	1.15	1.3
Time	PC9	PC9ER																					
0 min	1.0	1.25																					
5 mins	1.1	1.25																					
10 mins	1.0	1.15																					
30 mins	1.1	1.8**																					
4 hours	1.15	1.3																					

Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
Tsc-2 P Threonine 1462	which controls the response to energy deprivation (Inoki et al., 2003).	Cell Signaling Technologies	3617	Rabbit	Tsc-2 P Thr 1462 <table><caption>Relative protein expression (fold) for Tsc-2 P Thr 1462</caption><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.1</td></tr><tr><td>5 mins</td><td>0.9</td><td>1.2</td></tr><tr><td>10 mins</td><td>0.75</td><td>1.25</td></tr><tr><td>30 mins</td><td>1.0</td><td>1.4</td></tr><tr><td>4 hours</td><td>1.0</td><td>1.15</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.1	5 mins	0.9	1.2	10 mins	0.75	1.25	30 mins	1.0	1.4	4 hours	1.0	1.15
Time		PC9	PC9ER																				
0 min	1.0	1.1																					
5 mins	0.9	1.2																					
10 mins	0.75	1.25																					
30 mins	1.0	1.4																					
4 hours	1.0	1.15																					
Tsc-2 P Serine 1387	Cell Signaling Technologies	5584	Rabbit	Tuberin P S1387 <table><caption>Relative protein expression (fold) for Tuberin P S1387</caption><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.3</td></tr><tr><td>5 mins</td><td>3.4</td><td>1.2</td></tr><tr><td>10 mins</td><td>1.0</td><td>1.2</td></tr><tr><td>30 mins</td><td>1.1</td><td>1.5</td></tr><tr><td>4 hours</td><td>1.0</td><td>1.2</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.3	5 mins	3.4	1.2	10 mins	1.0	1.2	30 mins	1.1	1.5	4 hours	1.0	1.2	
Time	PC9	PC9ER																					
0 min	1.0	1.3																					
5 mins	3.4	1.2																					
10 mins	1.0	1.2																					
30 mins	1.1	1.5																					
4 hours	1.0	1.2																					

Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
TYK2 P Tyr1054, Tyr1055	Tyrosine kinase 2 (TYK2), a member of the JAK family is activated by receptors for many cytokines e.g. IL-13, the IL-6 family, IL-10, and IFN- α and β (Velazquez et al., 1995, Leonard and O'Shea, 1998, Stahl et al., 1994). When bound to ligand TYK2 is phosphorylated at tyrosine 1054 and/or tyrosine 1055 resulting in its activation (Gauzzi et al., 1996). Tyk2 is needed for the tyrosine phosphorylation of STAT3 (Rani et al., 1999).	Cell Signaling Technologies	9321	Rabbit	Tyk2 P Tyr1054,Tyr1055 <table><caption>Relative protein expression (fold) for Tyk2 P Tyr1054,Tyr1055</caption><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.4</td></tr><tr><td>5 mins</td><td>1.1</td><td>1.8*</td></tr><tr><td>10 mins</td><td>1.6</td><td>1.9</td></tr><tr><td>30 mins</td><td>1.5</td><td>2.3*</td></tr><tr><td>4 hours</td><td>1.6</td><td>1.8</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.4	5 mins	1.1	1.8*	10 mins	1.6	1.9	30 mins	1.5	2.3*	4 hours	1.6	1.8
Time	PC9	PC9ER																					
0 min	1.0	1.4																					
5 mins	1.1	1.8*																					
10 mins	1.6	1.9																					
30 mins	1.5	2.3*																					
4 hours	1.6	1.8																					
Ubiquitin (P4D1)	Ubiquitin is involved in the proteasome degradation pathway (Lecker et al., 2006). Ubiquitin targets proteins for degradation via the 26S proteasome (Lecker et al., 2006).	Cell Signaling Technologies	3936	mouse IgG1	Ubiquitin (P4D1) <table><caption>Relative protein expression (fold) for Ubiquitin (P4D1)</caption><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.1</td></tr><tr><td>5 mins</td><td>0.9</td><td>1.1</td></tr><tr><td>10 mins</td><td>0.9</td><td>1.3</td></tr><tr><td>30 mins</td><td>0.9</td><td>1.5</td></tr><tr><td>4 hours</td><td>1.2</td><td>1.3</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.1	5 mins	0.9	1.1	10 mins	0.9	1.3	30 mins	0.9	1.5	4 hours	1.2	1.3
Time	PC9	PC9ER																					
0 min	1.0	1.1																					
5 mins	0.9	1.1																					
10 mins	0.9	1.3																					
30 mins	0.9	1.5																					
4 hours	1.2	1.3																					

Antibody	Description	Supplier	Order Number	Type	Effect of EGF stimulation																		
VEGFR2 P Tyr1175	Vascular endothelial growth factor receptor 2 (VEGFR2) is a major receptor for VEGF-induced signalling in endothelial cells. Upon ligand binding, VEGFR2 undergoes autophosphorylation and becomes activated (Meyer et al., 1999). Phosphorylation at Tyr1175 provides a docking site for p85 subunit of PI3 kinase, Phosphoinositide phospholipase C γ (PLC γ) and SH2 Domain Containing Adaptor Protein B (Shb) (Meyer et al., 1999, Kroll and Waltenberger, 1997, Rahimi et al., 2000).	Cell Signaling Technologies	2478	Rabbit	VEGFR P Tyr1175 <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.25</td></tr><tr><td>5 mins</td><td>1.1</td><td>1.15</td></tr><tr><td>10 mins</td><td>1.0</td><td>1.15</td></tr><tr><td>30 mins</td><td>1.15</td><td>1.45</td></tr><tr><td>4 hours</td><td>1.15</td><td>1.35</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.25	5 mins	1.1	1.15	10 mins	1.0	1.15	30 mins	1.15	1.45	4 hours	1.15	1.35
Time	PC9	PC9ER																					
0 min	1.0	1.25																					
5 mins	1.1	1.15																					
10 mins	1.0	1.15																					
30 mins	1.15	1.45																					
4 hours	1.15	1.35																					
XIAP	XIAP is an inhibitor of apoptotic cell death via its ability to bind to and inhibition of caspases 3, 7 and 9 (Deveraux et al., 1997).	Cell Signaling Technologies	2045	rabbit	XIAP <table><thead><tr><th>Time</th><th>PC9</th><th>PC9ER</th></tr></thead><tbody><tr><td>0 min</td><td>1.0</td><td>1.1</td></tr><tr><td>5 mins</td><td>1.05</td><td>1.05</td></tr><tr><td>10 mins</td><td>0.95</td><td>1.05</td></tr><tr><td>30 mins</td><td>1.15</td><td>1.4</td></tr><tr><td>4 hours</td><td>1.15</td><td>1.25</td></tr></tbody></table>	Time	PC9	PC9ER	0 min	1.0	1.1	5 mins	1.05	1.05	10 mins	0.95	1.05	30 mins	1.15	1.4	4 hours	1.15	1.25
Time	PC9	PC9ER																					
0 min	1.0	1.1																					
5 mins	1.05	1.05																					
10 mins	0.95	1.05																					
30 mins	1.15	1.4																					
4 hours	1.15	1.25																					

Appendix table 8.2: Full list of the assigned metabolites identified through NMR-based metabolic profiling. Keys refer to the peaks labelled on **Figure 3.1** and **table 3.1**. The chemical shifts for proton ($\delta^1\text{H}$) and carbon ($\delta^{13}\text{C}$) NMR are shown. a Multiplicity: singlet(s), doublet(d), triplet(t), quartet(q), doublet of doublets(dd), double of triplets (dt), multiplet(m). b The signals or the multiplicities were not determined.

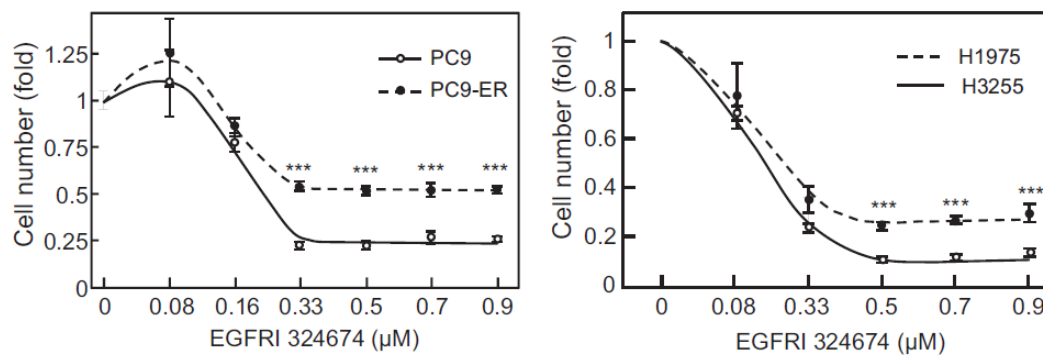
Key	Metabolite	Assignment	$\delta^1\text{H}$ (multiplicity ^a)	$\delta^{13}\text{C}$
1	Acetate	CH ₃	1.92(s)	26.1
		COO-		184.2
2	Adenosine	CH-ring	8.34(s)	8.34(s)
		CH-ring	8.178(s)	8.178(s)
		C1H-ribose	6.08(d)	6.08(d)
		C2H-ribose	4.77	4.77
		C4H-ribose	4.28	4.28
		C3H-ribose	4.42	4.42
		C5H-ribose	3.85/3.9	3.85/3.9
3	Alanine (Ala)	α -CH	3.78(q)	53.1
		β -CH ₃	1.48(d)	18.9
		COO(H)		178.5
4	Aspartate (Asp)	α -CH	3.90(m)	54.8
		β -CH	2.69(dd)	39.2
		β' -CH	2.81(dd)	
		γ COO(H)		
		COO(H)		
5	Choline	α -CH ₂	3.99	70
		β -CH ₂	3.56	58.6
		N-CH ₃	3.20(s)	56.6
6	Creatine	CH ₃	3.04(s)	39.6
		CH ₂	3.93(s)	56.5
		N=C		159.6
		COO(H)		177.3
7	Formate	H COO-	8.46(s)	B
8	Fumarate	C2,3H	6.52(s)	138.2
		COO-		B
9	Glutamate (Glu)	α -CH	3.76(t)	57.3
		β -CH ₂	2.08(m)	29.8
		γ -CH ₂	2.35(m)	36.2
		δ CO		183.9
		COO(H)		177.4
10	Glycerophosphocholine(GPC)	1-CH ₂	3.60(dd)	B
		2-CH	3.89(m)	B
		3-CH ₂	3.72(dd)	B
		α -CH ₂	4.32(t)	B
		β -CH ₂	3.68(t)	B
		N-CH ₃	3.23(s)	56.7
11	Glycine (Gly)	α -CH ₂	3.56(s)	44.1

Key	Metabolite	Assignment	$\delta^1\text{H}$ (multiplicity ^a)	$\delta^{13}\text{C}$
		COO(H)		175.1
12	Glycogen		5.41(broad peak)	102.4
			3.97	B
			3.85	B
			3.7	B
13	Guanosine	CH-ring	8.0(s)	140.9
		C1H-ribose	5.92(d)	91.7
		C3H-ribose	4.41(dd)	73.4
		C4H-ribose	4.24(dt)	88.21
		C5H-ribose	3.84	64
14	Histidine (His)	C4H,ring	7.08(s)	119.8
		C2H,ring	7.85(s)	137
		COO(H)		176.8
15	Inosine	CH-ring	8.35(s)	143.2
		CH-ring	8.24(s)	155.6
		C1H-ribose	6.10(d)	91.2
		C2H-ribose	4.78	76.65
		C3H-ribose	4.44	B
		C4H-ribose	4.28	B
		C5H-ribose	3.87	B
16	Inosine-5'-monophosphate(IMP)	CH-ring	8.54(s)	142.5
		CH-ring	8.24(s)	149.1
		C1H-ribose	6.14(d)	89.9
		C2H-ribose	4.77	77.1
		C3H-ribose	4.51	73.2
		C4H-ribose	4.37	87.5
		C5H-ribose	4.03	66.2
17	Isoleucine (Ile)	α -CH	3.67(m)	62.3
		β -CH	1.98(m)	38.6
		γ -CH	1.27(m)	27.8
		γ' -CH	1.47(m)	27.8
		δ -CH ₃	0.94(t)	13.9
		3- CH ₃	1.01(d)	17.3
		COO(H)		176.4
18	Lactate	α -CH	4.11(q)	71.2
		β -CH ₃	1.33(d)	22.7
		COO-		185.2
19	Leucine(Leu)	α -CH	3.73(t)	56
		β -CH ₂	1.72(m)	42.6
		γ -CH	1.69(m)	26.8
		δ -CH ₃	0.97(d)	24.7
		δ' -CH ₃	0.96(d)	23.7
		COO(H)		177.3
20	Myo-inositol	C1,3H	3.54(dd)	3.54(dd)

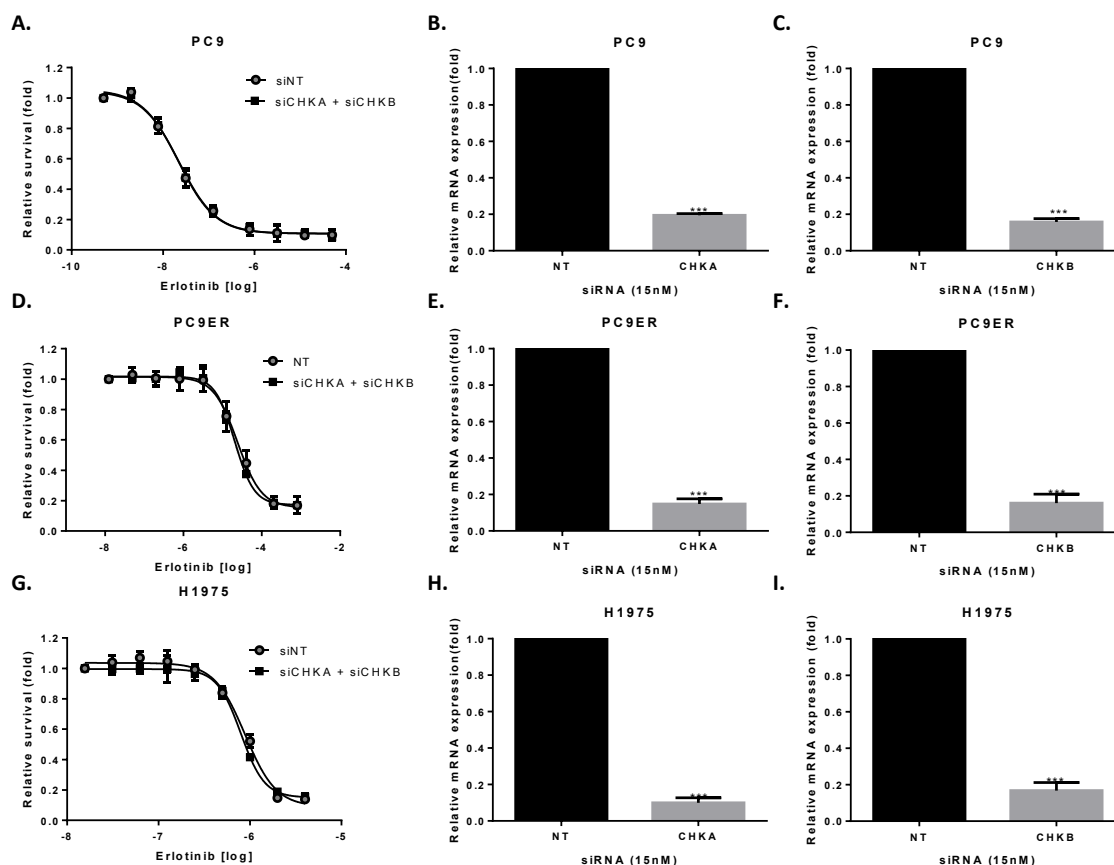
Key	Metabolite	Assignment	$\delta^1\text{H}$ (multiplicity ^a)	$\delta^{13}\text{C}$
		C2H	4.06(t)	4.06(t)
		C5H	3.26(t)	75.23
		C4,6H	3.62(t)	72.01
21	N-acetyl Aspartate	α -CH	4.39(dd)	54.01
		β -CH	2.68(dd)	40.54
		β' -CH	2.49(dd)	
		CH ₃	2.02(s)	22.85
22	NAD	N5ring	8.20(m)	131.2
		N4ring	8.83(d)	148.7
		N2ring	9.34(s)	142.9
		N6ring	9.14(d)	145.2
		A2Hring	8.0(s)	126
		A8H ring	8.43(s)	143.3
		A4ring		151.2
		A1'H	6.09(d)	102.6
		N1'H	5.99	B
23	Oxidative Glutathione (GSSG)	S-CH ₂	3.314(dd)/2.98(dd)	41.3
		CH	4.76	55.4
		CH ₂	3.76(m)	46
		CH ₂	2.16(m)	29.2
		CH ₂	2.51(m)	34.1
		C=O		177.8
		COOH		176.5
24	Phenylalanine (Phe)	α -CH	3.99(dd)	58.8
		β -CH	3.13(dd)	38.9
		β' -CH	3.27(dd)	38.9
		C1,ring		137.9
		C2,6,ring	7.33(m)	132
		C3,5,ring	7.42(m)	131.5
		C4,ring	7.38(m)	130.5
		COO(H)		176.7
25	Phosphocholine	α -CH ₂	3.60(m)	69.5
		β -CH ₂	4.17(m)	B
		N-CH ₃	3.22(s)	56.7
26	Reduced Glutathione	Glu α	3.78(t)	56.8
		Glu β	2.16(m)	29.2
		Glu γ	2.56(m)	34.1
		Cys α	4.57(dd)	58.6
		Cys β	2.94(m)	28.3
		Gly α	b	B
		C=O		174.5
				177.2
27	Succinate	CH ₂	2.40(s)	35.9
		COO-		183.6

Key	Metabolite	Assignment	$\delta^1\text{H}$ (multiplicity ^a)	$\delta^{13}\text{C}$
28	Taurine	N-CH ₂	3.27(t)	50.3
		S-CH ₂	3.42(t)	38.2
29	Tyrosine (Tyr)	C3,5H,ring	6.90(d)	118.6
		C2,6H,ring	7.19(d)	133.3
		C1,ring		129.5
		α -CH	3.94(dd)	58.9
		β -CH ₂	3.06	39
30	Uridine 5'-diphosphate (UDP)	CH-ring	7.99(d)	B
		CH-ring	5.97(d)	B
		C1H-ribose	5.96(broad,s)	B
		C3H-ribose	4.43(t)	B
		C2H-ribose	4.39(t)	B
		C4H-ribose	4.27(m)	B
		C5H-ribose	4.23(c)	B
31	UDP-glucose (UDPG)	G1-H	5.61	98.65
		C6,ring	7.96(d)	144.4
		C5,ring	5.98(d)	105.4
		C1'H,ribose	5.99(d)	91.3
		C2'3'H,ribose	4.38(m)	72.2/76.5
		C4',ribose	4.29(m)	85.9
		C5'H,ribose	4.26/4.21(m)	67.8
		G2-H	3.9	73.1
		G6-H	3.86/3.78	63.36
		G3-H	3.77	74.7
		G4-H	3.54	74
		G5-H	3.47	72
32	UDP-N-acetyl glucosamine	C6,ring	7.96(d)	144.1
		C1'H,ribose	5.98(d)	91.1
		C5,ring	5.97(d)	105.4
		C2'3'H,ribose	4.37(m)	72.2
		C5'H,ribose	4.23/4.17(m)	66.2
		C4'H,ribose	4.29(m)	86
		C2,ring		156.6
		G1-H	5.52(dd)	97.2
		G2-H	3.99	56.5
		G3-H	3.82	73.7
		G4-H	3.55	72.2
		G5-H	3.93/3.867	
		NA-H	2.08	24.9
		NA-C=O		177.4
33	UDP-N-Acetyl Galactosamine	G1-H	5.55(dd)	97.4
		G2-H	4.05(m)	71.2
		G3-H	3.97(dd)	70.3
		G4-H	3.76(m)	57.4

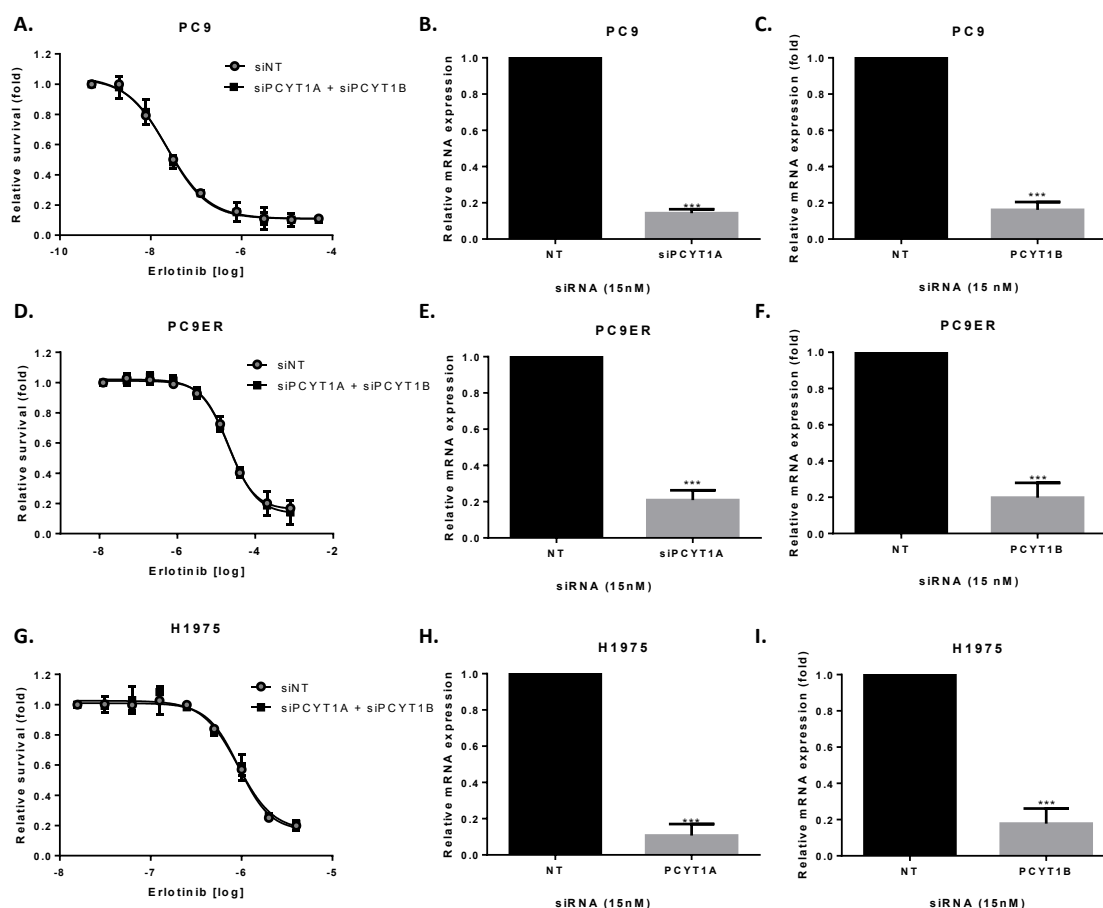
Key	Metabolite	Assignment	$\delta^1\text{H}$ (multiplicity ^a)	$\delta^{13}\text{C}$
		G5-H	3.79	63.6
		G6-H	3.78	63.6
		C2'3'H,ribose	4.37(m)	76.6
		C4',ribose	4.29(m)	85.9
		C5'H,ribose	4.25/4.19(m)	67.8
		NA-H	2.089(s)	24.9
		C1'H,ribose	5.99(d)	91.1
		C5,ring	5.97(d)	105.4
		C6,ring	7.96(d)	144.1
34	Uracil	C5H	5.80(d)	B
		C6H	7.54(d)	B
35	Uridine	C6,ring	7.87(d)	144.1
		C1'H,ribose	5.92(d)	92
		C5,ring	5.90(d)	105.1
		C3'H,ribose	4.34(t)	72.2
		C5'H,ribose	4.12(q)	66.2
		C4'H,ribose	4.23(t)	86
		C2,ring		156.6
36	Valine (Val)	α -CH	3.61(d)	63.1
		β -CH	2.28(m)	31.9
		γ -CH ₃	0.99(d)	19.5
		γ' -CH ₃	1.04(d)	20.7
		COO(H)		176.8



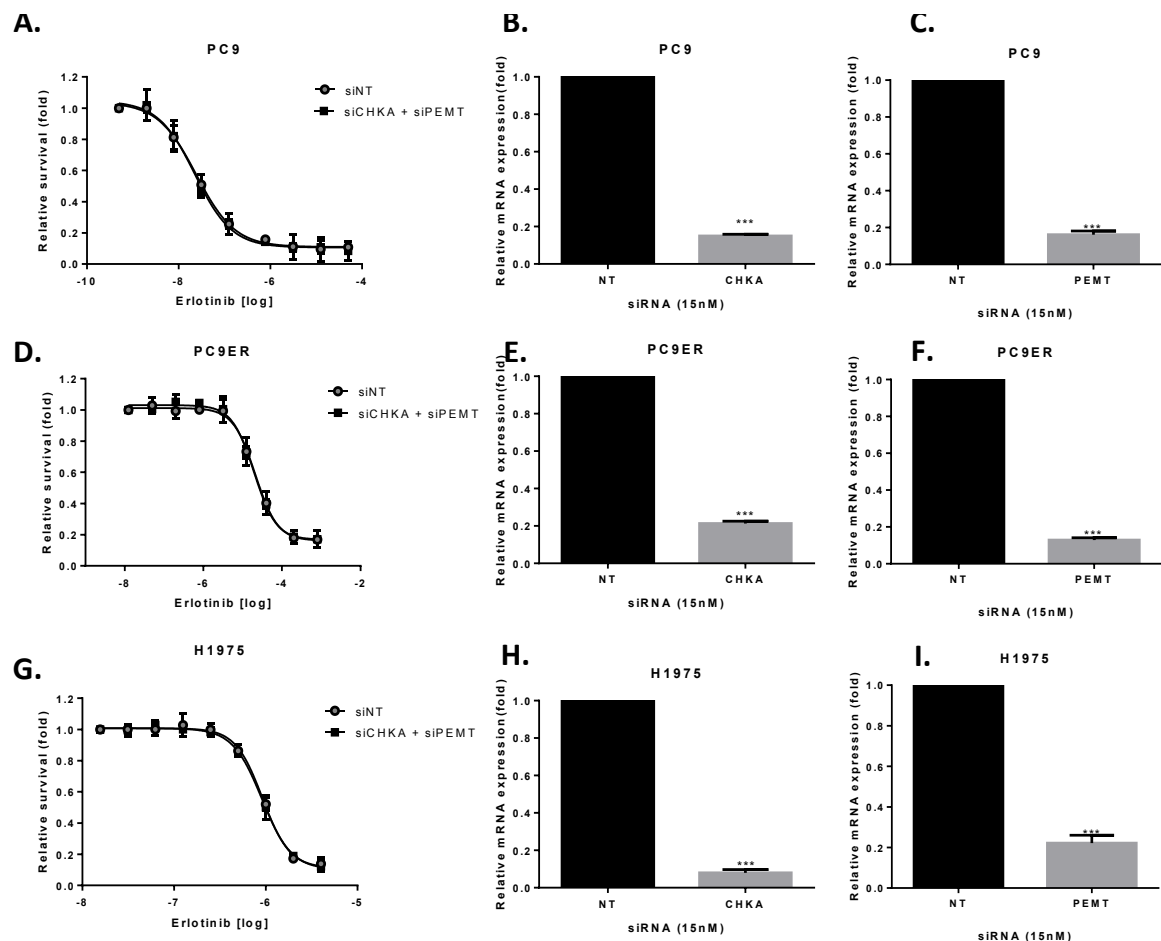
Appendix figure 8.1: EGFR 324674 survival assays. PC9, PC9ER, H3255 and H1975 cells were treated with increasing concentrations of EGFR 324674 (48 hours) and quantified by crystal violet staining. Figures represent the mean of 3 independent biological experiments $\pm$ SEM. Statistical analysis; ANOVA, Student t-test. ***, $p \leq 0.001$, *, $p \leq 0.05$.



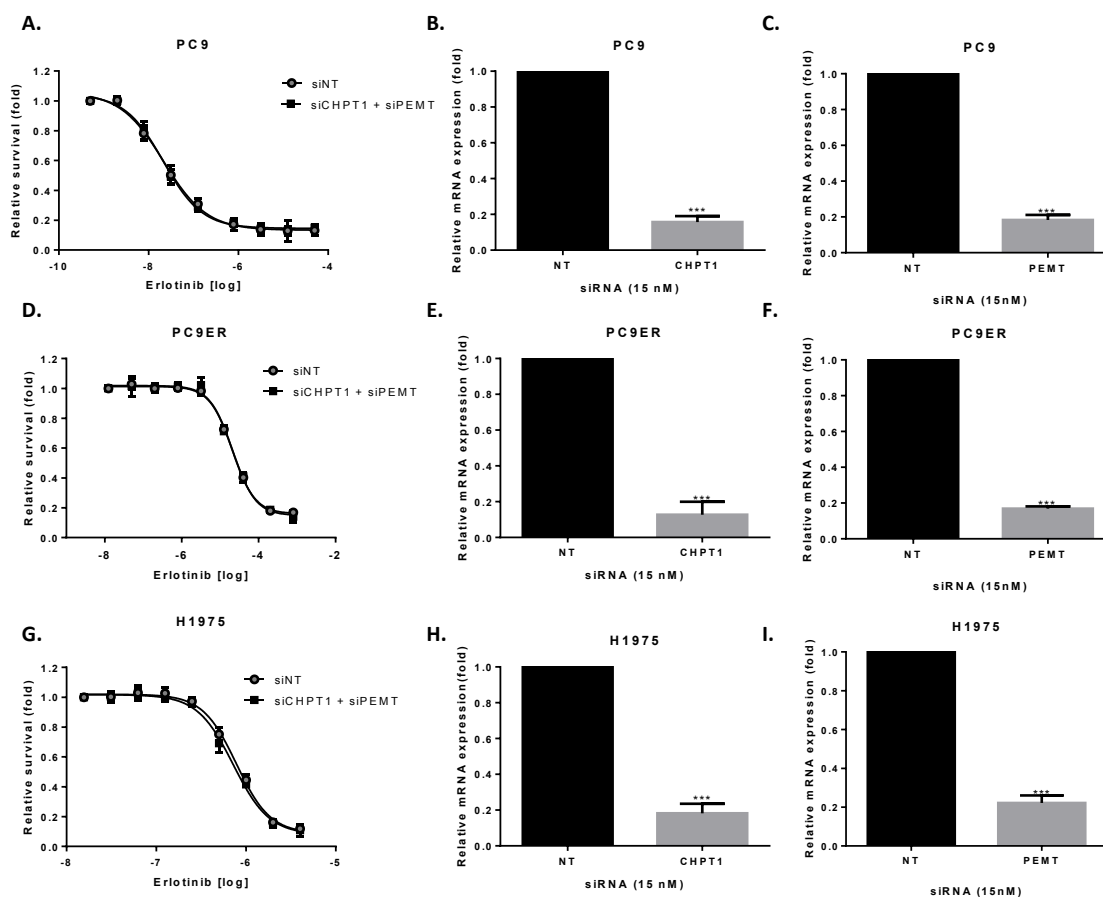
Appendix figure 8.2: Double knockdown of CHKA and CHKB does not affect survival to erlotinib treatment. PC9 (A, B and C), PC9ER (D, E and F) and H1975 (G, H and I) cells were transfected with siRNA against both CHKA and CHKB or NT control. Twenty-four hours post transfection cells were treated with increasing concentrations of erlotinib (48h) prior to fixation and crystal violet staining. The obtained values were normalised against the DMSO control. siRNA efficiency was confirmed by qPCR for CHKA (B, E and H) and CHKB (C, F and I). Figures represent the mean of three biological repeats with each replicate done in technical triplicate ($\pm$ SEM). Statistical analysis was done using Student's *t*-test. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.



Appendix figure 8.3: Double knockdown of PCYT1A and PCYT1B does not affect survival to erlotinib treatment. PC9 (A, B and C), PC9ER (D, E and F) and H1975 (G, H and I) cells were transfected with siRNA against both PCYT1A and PCYT1B or NT control. Twenty-four hours post transfection cells were treated with increasing concentrations of erlotinib (48h) prior to fixation and crystal violet staining. The obtained values were normalised against the DMSO control. siRNA efficiency was confirmed by qPCR for PCYT1A (B, E and H) and PCYT1B (C, F and I). Figures represent the mean of three biological repeats with each replicate done in technical triplicate ($\pm$ SEM). Statistical analysis was done using Student's *t*-test. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

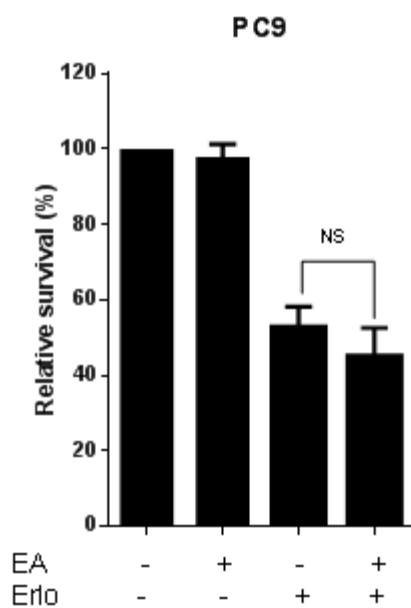


Appendix figure 8.4: Double knockdown of CHKA and PEMT does not affect survival to erlotinib treatment. PC9 (A, B and C), PC9ER (D, E and F) and H1975 (G, H and I) cells were transfected with siRNA against both CHKA and PEMT or NT control. Twenty-four hours post transfection cells were treated with increasing concentrations of erlotinib (48h) prior to fixation and crystal violet staining. The obtained values were normalised against the DMSO control. siRNA efficiency was confirmed by qPCR for CHKA (B, E and H) and PEMT (C, F and I). Figures represent the mean of three biological repeats with each replicate done in technical triplicate ($\pm$ SEM). Statistical analysis was done using Student's *t*-test. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

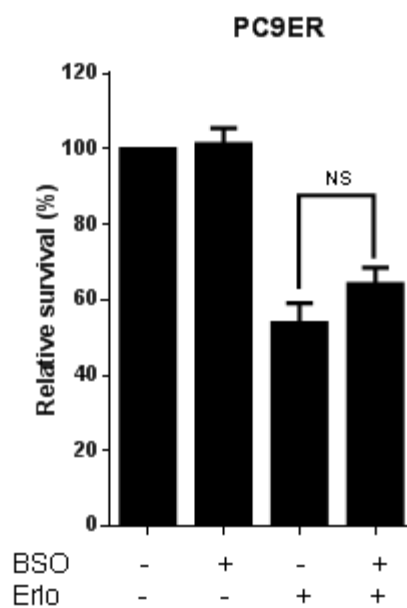


Appendix figure 8.5: Double knockdown of CHPT1 and PEMT does not affect survival to erlotinib treatment. PC9 (A, B and C), PC9ER (D, E and F) and H1975 (G, H and I) cells were transfected with siRNA against both CHPT1 and PEMT or NT control. Twenty-four hours post transfection cells were treated with increasing concentrations of erlotinib (48h) prior to fixation and crystal violet staining. The obtained values were normalised against the DMSO control. siRNA efficiency was confirmed by qPCR for CHPT1 (B, E and H) and PEMT (C, F and I). Figures represent the mean of three biological repeats with each replicate done in technical triplicate ($\pm$ SEM). Statistical analysis was done using Student's *t*-test. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

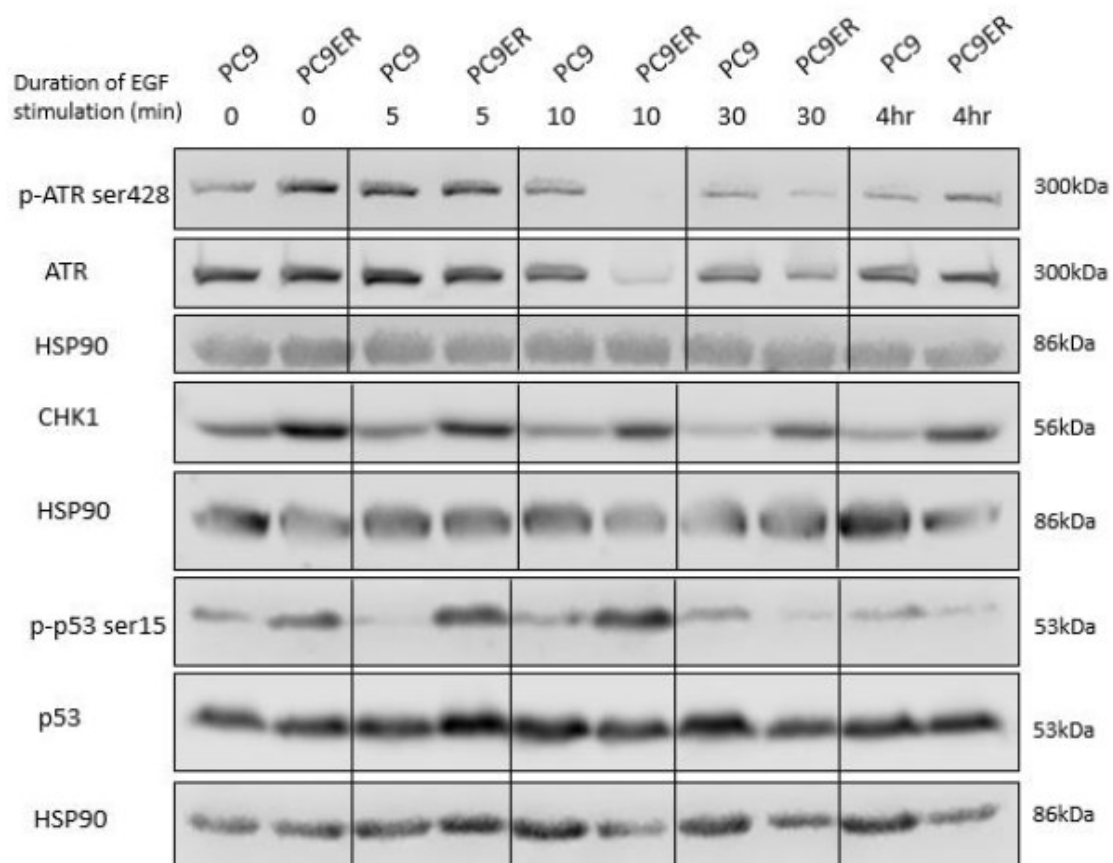
A.



B.



Appendix figure 8.6: Treatment of PC9 with EA and PC9ER with BSO did not significantly affect survival to erlotinib treatment. (A) PC9 cells were treated with EA alone and in combination with Erlotinib for 48 hours and survival were monitored by crystal violet assay. **(B)** PC9ER cells were treated with BSO alone and in combination with erlotinib for 48 hours and survival were monitored by crystal violet assay. Data are the relative responsiveness to erlotinib normalised to vehicle (-; DMSO). Figures represent the mean of three biological repeats with each replicate done in technical triplicate ($\pm$ SEM).



Appendix figure 8.7: Expression of cell cycle checkpoint proteins in PC9 and PC9ER following EGF stimulation. PC9 and PC9ER cells were stimulated with 10 ng/ml of EGF for a time course of 0, 5, 10, 30 min and 4 hours followed by SDS-PAGE/Western blotting for relevant proteins. HSP90 was used as a loading control. Figure is a representative of three biological repeats. I am grateful to Chun Hang Lau for his contributions to SDS PAGE / Western blotting under my supervision.

Chapter 9.0 References

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Chapter10.0 Publications

Decreased glutathione biosynthesis contributes to EGFR T790M-driven erlotinib resistance in non-small cell lung cancer

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Epidermal growth factor receptor (EGFR) inhibitors such as erlotinib are novel effective agents in the treatment of EGFR-driven lung cancer, but their clinical impact is often impaired by acquired drug resistance through the secondary T790M EGFR mutation. To overcome this problem, we analysed the metabonomic differences between two independent pairs of erlotinib-sensitive/resistant cells and discovered that glutathione (GSH) levels were significantly reduced in T790M EGFR cells. We also found that increasing GSH levels in erlotinib-resistant cells re-sensitised them, whereas reducing GSH levels in erlotinib-sensitive cells made them resistant. Decreased transcription of the GSH-synthesising enzymes (GCLC and GSS) due to the inhibition of NRF2 was responsible for low GSH levels in resistant cells that was directly linked to the T790M mutation. T790M EGFR clinical samples also showed decreased expression of these key enzymes; increasing intra-tumoural GSH levels with a small-molecule GST inhibitor re-sensitised resistant tumours to erlotinib in mice. Thus, we identified a new resistance pathway controlled by EGFR T790M and a therapeutic strategy to tackle this problem in the clinic.

Keywords: metabolomics; glutathione; lung cancer; drug resistance; TKI

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Introduction

Lung cancer is the main cancer killer and non-small cell lung cancer (NSCLC) represents ~85% of such cases. About 10% and 30% of NSCLCs in Western and Asian populations, respectively, express an activated mutant epidermal growth factor receptor (EGFRm) and the vast majority (90%) of such patients respond to ATP-competitive EGFR tyrosine kinase inhibitors (TKIs) such as gefitinib or erlotinib [1–3]. Unfortunately, most patients can quickly acquire TKI

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resistance limiting the benefits of these drugs to patients' survival.

Resistance mechanisms include Met amplification (~5% of cases) [4] and more frequently (50% of cases) an additional T790M gatekeeper mutation within the EGFRm [5–8]. The latter enhances kinase activity by increasing the affinity of EGFR for ATP, competing out TKI binding [9]. This led to the development of compounds irreversibly interacting with EGFRm/T790M, such as afatinib, 324674 and more recently AZD9291 and CO1696 [10–12]. However, thus far clinical trials of afatinib failed to demonstrate improved response in EGFRm/T790M patients [13], and although initial trials with the irreversible inhibitor AZD9291 showed great promises, additional resistance mechanisms to these inhibitors have already surfaced [14].

Changes in cellular metabolism accompany tumourigenesis and classical chemoresistance [15–17]. Hence, changes in metabolite concentrations can specifically reflect the onset of therapy resistance, providing response/outcome biomarkers and novel therapeutic strategies to reverse resistance [18, 19]. Both ¹H-nuclear magnetic resonance (NMR) and mass spectrometry are efficient tools to investigate these metabolic changes [20–23].

Here we used ¹H-NMR to compare the metabolic signatures of paired NSCLC cell lines expressing EGFRm without (erlotinib sensitive) or with the additional T790M mutation (erlotinib resistant). We showed that glutathione (GSH) levels were reduced in erlotinib-resistant NSCLC cells in a T790M-dependent manner due to the decreased expression of GSH-synthesising enzymes. Correcting this defect re-sensitised resistant cells to erlotinib *in vitro* and *in vivo*. Moreover, ethacrynic acid (EA), a glutathione-*S*-transferase inhibitor, reversed erlotinib resistance in T790M NSCLC cells *in vitro* and *in vivo* by increasing GSH levels. As EA is a clinically used diuretic, it could be repurposed to reverse T790M-mediated erlotinib resistance in NSCLC patients. Overall, our work demonstrated the power of metabonomic screening to generate novel research hypotheses and discover unexplored strategies to tackle drug resistance in lung cancer treatments.

Results

¹H-NMR-based metabolic profiling reveals decreased GSH levels in erlotinib-resistant NSCLC cells

Two pairs of cell lines were employed to obtain generic metabonomic phenotypes for the erlotinib-sensitive and erlotinib-resistant NSCLC cells. The

first pair were the isogenically matched PC9 (erlotinib sensitive) and PC9ER (erlotinib resistant) cells both containing ΔE746-A750 EGFRm with an additional T790M (EGFRm/T790M) mutation in PC9ER cells. The second pair included the H3255 and genetically unrelated erlotinib-resistant H1975 cell lines sharing L858R EGFRm, but with an additional T790M mutation in the H1975. PC9ER and H1975 cells displayed significant resistance to erlotinib as compared with their sensitive counterparts (Supplementary Figure S1A). This resistance was limited to EGFR TKIs as PC9ER and PC9 cells were equally sensitive to conventional chemotherapeutic agents (Supplementary Figure S1B). It has been suggested that the EGFR T790M-mediated TKI resistance is due to increased affinity of the receptor for ATP, which displaces competitive inhibitors such as erlotinib [24]. However, both PC9ER and H1975 showed significant resistance even to the irreversible EGFR inhibitor 324674 compared with PC9 and H3255 cells, respectively (Supplementary Figure S1C). This clearly suggests that other unidentified molecular mechanisms also contribute to T790M-mediated TKI resistance.

To identify these, we comprehensively analysed the ¹H-NMR metabonomic profiles of our erlotinib-sensitive and -resistant cells. ¹H-NMR analysis of cell extracts from our cell lines identified 36 metabolites (Figure 1a) for which unambiguous assignments were obtained using various two-dimensional NMR methods (Supplementary Table S1). Statistical analysis of the spectral data by orthogonal projections to latent structures discriminant analysis (OPLS-DA) showed significant metabonomic differences between the erlotinib-resistant and -sensitive cells (Figure 1b and c). Changes in 14 metabolites mainly involved in GSH, amino acids, nucleotides and choline metabolism (Supplementary Figure S2A–C) correlated with resistance in both cell line pairs (Figure 1d; Supplementary Table S2). Noticeably, a significant drop in the intracellular levels of GSH accompanied erlotinib resistance (Figure 1d; Supplementary Table S2). Such GSH decrease observed by NMR was independently confirmed using a colorimetric assay (Figure 1e and f). This was intriguing, as drug resistance was traditionally associated with increased GSH levels [25, 26]. Nevertheless, GSH covalently binds some chemotherapeutic drugs leading to their glutathione-*S*-transferase-mediated extracellular export and resistance of cancer cells to these compounds [27, 28]. Hence, the increased export of this metabolite in complex with erlotinib could account for the lower GSH levels in these resistant cell lines. ¹H-NMR

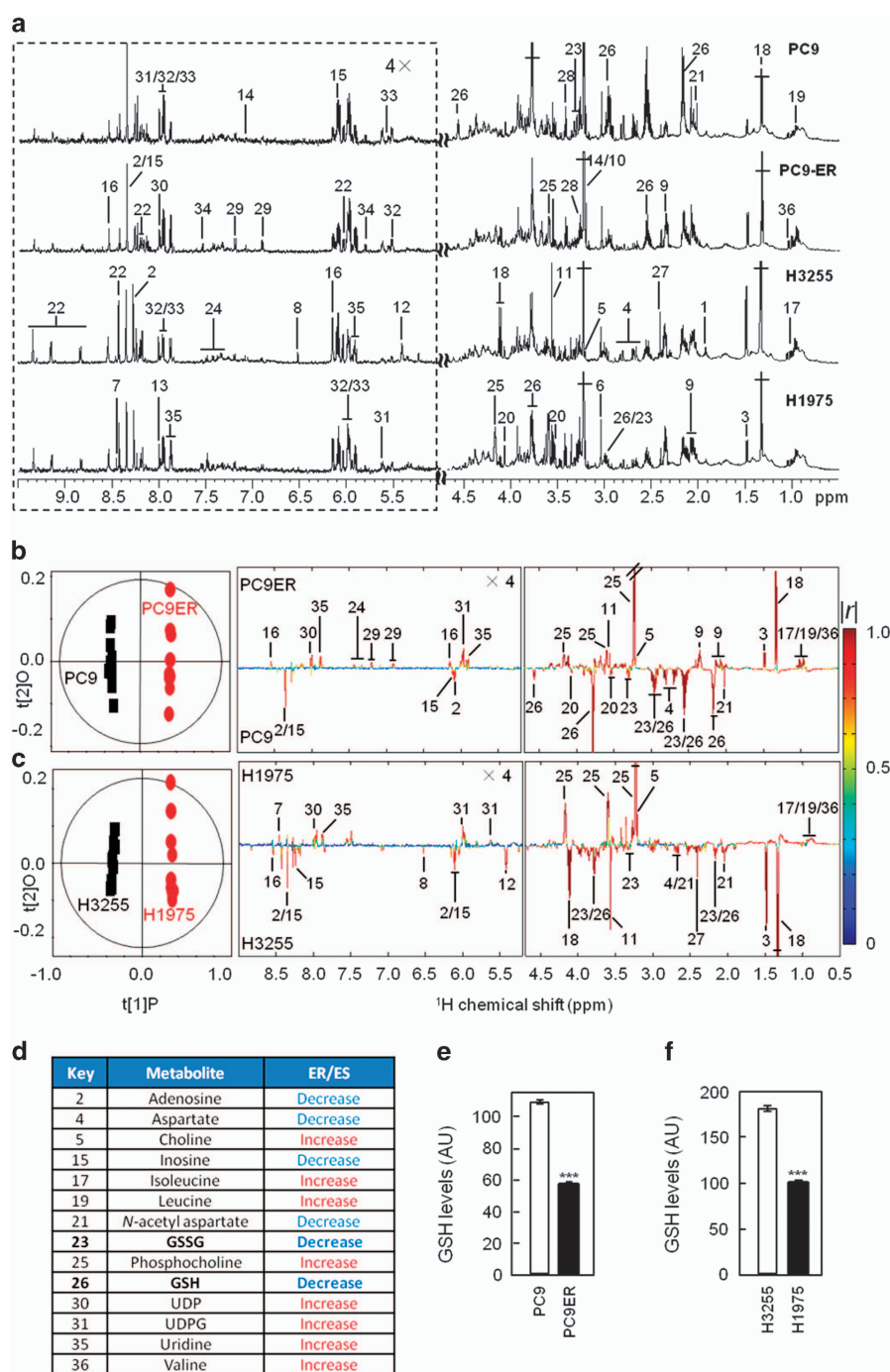
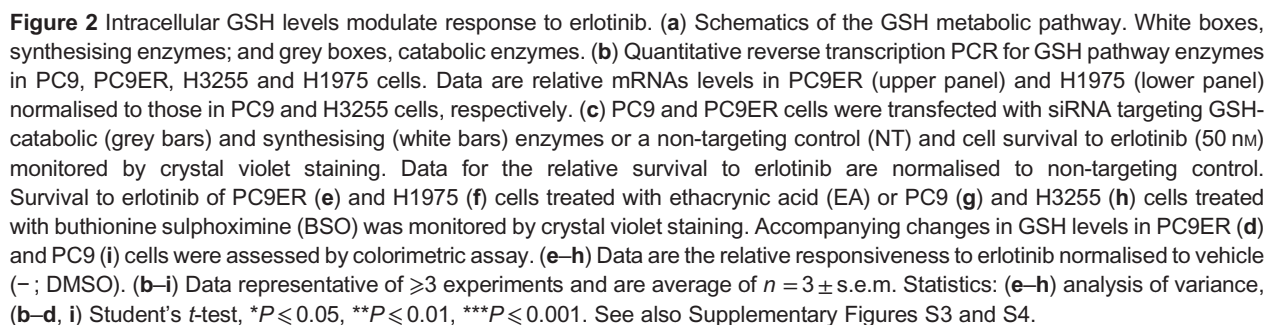


Figure 1 Metabolic characteristics for the erlotinib-resistant and -sensitive cells. **(a)** Typical 600 MHz ^1H -NMR spectra of aqueous extracts from PC9, PC9ER, H3255 and H1975 cells. The region (δ 5.0–9.5) is vertically expanded four times ($4\times$). Data representative of $n = 10$. Orthogonal projections to latent structures discriminant analysis (OPLS-DA) score plots (left) and coefficient plots (right) for ^1H -NMR spectra of aqueous cellular extracts from PC9ER and PC9 showing significantly differentiated metabolites **(b)**, H1975 and H3255 **(c)**. Models validated by CV-ANOVA, $P = 2.36 \times 10^{-17}$ **(b)** and $P = 3.04 \times 10^{-19}$ **(c)**. The Q^2 is 0.99 for both models. The colour scale for coefficient plots reflects the differences in the contribution of metabolite variations between groups. $|r|$ cutoff value is 0.602 ($n = 10$, $P < 0.05$). For identification of peak numbers, see Supplementary Table S1 and **d**. **(d)** Metabolites showed statistically significant differences between resistant and sensitive cells in both cell line pairs with statistically significant ‘decreases’ or ‘increases’ detected in the erlotinib-resistant (ER) cells as compared with erlotinib-sensitive (ES) ones. **(e, f)** GSH levels in PC9 and PC9ER **(e)** or H3255 and H1975 **(f)** cells determined by colorimetric assay. Data are average $\pm$ s.e.m. of $n = 4$. Statistics: Student’s t -test. *** $P < 0.001$. See also Supplementary Figures S1 and S2.



Erlotinib-resistant cells have lower expression of GSH-synthesising enzymes

RNA (mRNA) levels for GSH-synthesising enzymes (GCLC, GSS and GSR) in erlotinib-resistant cells compared with sensitive ones (Figure 2a and b). In addition, mRNA levels for GCLM, the modulatory subunit of GCLC, were significantly lower in H1975 than in H3255 cells. In contrast, changes in the levels for GSH-catabolic enzymes (GPX1/2/3, GGT and GSTpi/ml/zi) varied greatly between cell line pairs and enzyme subtypes indicating no clear pattern (Figure 2b). Therefore, a reduction in GSH biosynthesis becomes a sound explanation for the decreased GSH levels in EGFRm/T790M erlotinib-resistant cells.

Targeting GSH metabolism modulates the cellular response to erlotinib

NMR results suggested that lower GSH levels associated with erlotinib resistance. To strengthen this link, we employed small interfering RNAs (siRNAs) for GSH-metabolic enzymes to modulate GSH levels in our cell lines. Silencing of GSH-catabolic enzymes (GGT1, GPX1 and GSTpi) increased the response

to erlotinib in both the EGFRm PC9 and EGFRm/T790M PC9ER and H1975 cells (Figure 2c; Supplementary Figure S3C). This correlated with efficient targets' downregulation and a corresponding increase in GSH levels (Supplementary Figure S3A and B). Conversely, silencing GSH-synthesising enzymes (GCLC, GSS and GSR) lowered cellular GSH levels (Supplementary Figure S3A and B) and rendered the sensitive PC9 cells erlotinib-resistant (Figure 2c).

To validate our siRNA data, we used small-molecule inhibitors targeting the activity of GSH pathway enzymes. Treatment with EA, a known GST inhibitor, increased GSH levels in erlotinib-resistant cells (Figure 2d) causing re-sensitisation of PC9ER and H1975 cells to erlotinib (Figure 2e and f). Similarly, GPXs inhibition using mercaptosuccinate increased intracellular GSH levels (Supplementary Figure S3D) and the response of H1975 cells to erlotinib (Supplementary Figure S3E). Conversely, GCLC inhibition using buthionine sulfoximine in sensitive cells made them erlotinib resistant (Figure 2g and h), an effect associated with decreased GSH levels (Figure 2i). Furthermore, EA was also able to sensitise PC9ER cells to gefitinib by increasing intracellular GSH (Supplementary Figure S4A–C). Taken together, these data suggest that manipulating GSH levels controls the responsiveness of our cell lines to erlotinib.

The NRF2 pathway controls GSH synthesis and responsiveness to erlotinib

GCLC, GSS and GSR are transcriptional targets of NFE2-related factor 2 (NRF2) [29–31], a downstream target of EGFR [32]. We therefore hypothesised that NRF2 activity might be impaired in EGFRm/T790M cells. NRF2's transcriptional activity requires its nuclear localisation and NRF2 is also degraded through binding to KEAP1, a process counteracted by

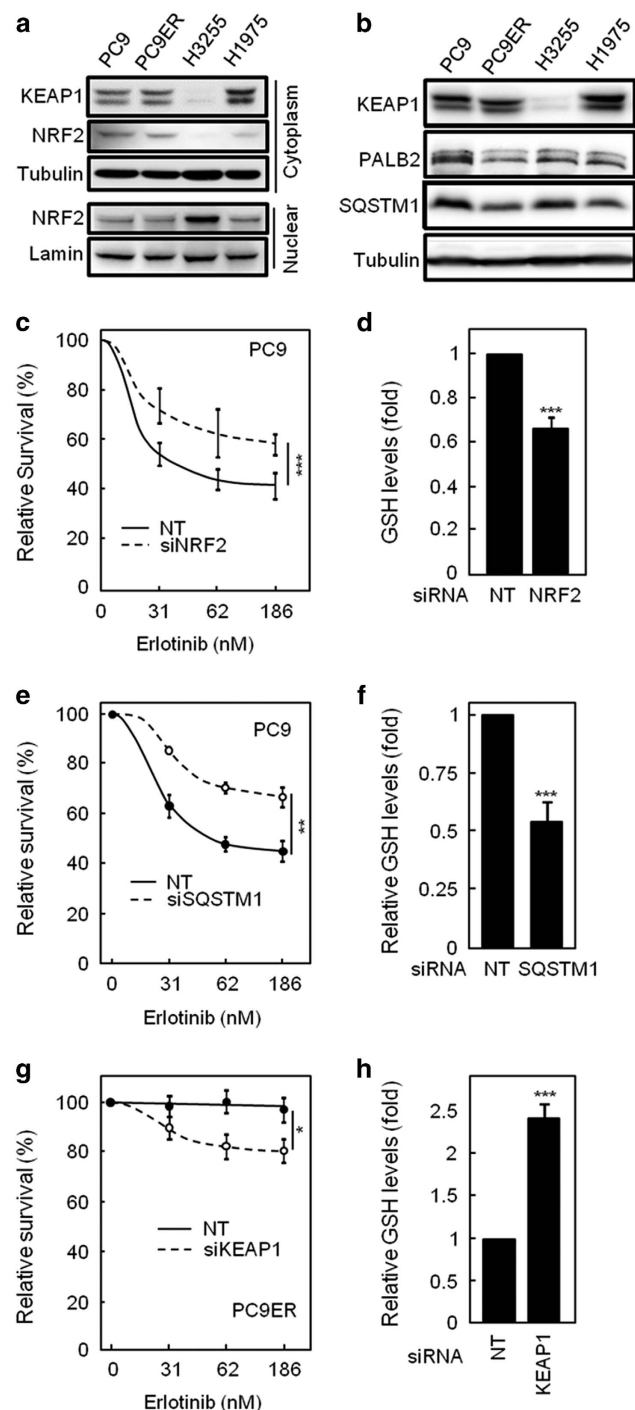


Figure 3 Erlotinib resistance correlates with decreased NRF2 activity. (a, b) Subcellular fractions (a) and total lysates (b) from PC9, PC9ER, H3255 and H1975 cells were analysed by SDS-PAGE/western blotting for the indicated proteins. Detection of lamin and tubulin was used as loading controls for nuclear fractions and total lysates or cytoplasmic fractions, respectively. (c–h) PC9 cells transfected with non-targeting (NT), NRF2 or SQSTM1 siRNAs (c–f) or PC9ER cells transfected with KEAP1 or NT siRNAs (g, h) were treated with erlotinib and survival assessed by crystal violet staining (c, e, g). GSH levels were measured by colorimetric assay (d, f, h). (c–h) Data are average of $n = 4 \pm$ s.e.m. Statistics: Student's *t*-test, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. See also Supplementary Figures S5 and S6.

competitive interaction of the latter protein with PALB2 and/or SQSTM1. Analysis of nucleocytoplasmic fractions and total lysates from our four cell lines revealed that NRF2 or KEAP1 localisation/expression had no differences between PC9 and PC9ER cells, whereas H3255 cells showed higher level of nuclear NRF2 than H1975 cells (Figure 3a; Supplementary Figure S5A). This correlated with increased KEAP1 expression in H1975 as compared with H3255 cells (Figure 3a; Supplementary Figure S5B). Although these results alone may explain the difference in GSH pathway enzymes expression between the latter two cell lines, they cannot account for that seen between PC9 and PC9ER cells. However, SQSTM1 was downregulated in both PC9ER and H1975 cells as compared with their erlotinib-sensitive counterparts (Figure 3b; Supplementary Figure S5C), whereas PALB2 levels were lower in PC9ER as compared with PC9 cells (Figure 3b; Supplementary Figure S5D). Furthermore, NRF2 has been shown to be a transcriptional regulator of SQSTM1 and, indeed, mRNA levels of SQSTM1 were found to be significantly lower in both the resistant cell line pair (Supplementary Figure S5E and F). Hence, inhibition of NRF2 activity through various mechanisms may be linked to erlotinib resistance in NSCLC cells.

To test this hypothesis, we silenced NRF2, SQSTM1, PALB2 and KEAP1 in our cells. siRNA-mediated silencing of NRF2 (Supplementary Figure S6A) rendered PC9 cells erlotinib resistant, a change associated with lower intracellular GSH (Figure 3c and d). Indeed, NRF2-silenced cells showed downregulation of the GSH-synthesising enzymes GCLC and GSR (Supplementary Figure S6B), demonstrating a direct link between NRF2 activity and GSH synthesis. Similarly, SQSTM1 silencing (Supplementary Figure S6C and D) decreased the sensitivity of PC9 cells to erlotinib (Figure 3e) in association with a drop in GSH levels (Figure 3f). Conversely, KEAP1 downregulation (Supplementary Figure S6E) sensitised EGFRm/T790M PC9ER cells to erlotinib (Figure 3g), accompanied by the increased GSH levels (Figure 3h) and increased transcription of GSH-synthesising enzymes GCLC, GSR and GSS (Supplementary Figure S6F). Finally, despite the changes in PALB2 between PC9ER and PC9 cells (Figure 3b), silencing this protein in PC9 cells failed to induce erlotinib resistance or alter GSH levels (Supplementary Figure S7A and B). Hence, the modulation of NRF2 activity through KEAP1 and SQSTM1 regulates the sensitivity of NSCLC cells to erlotinib.

Inhibition of NRF2 activity and decreased GSH levels are direct consequences of the T790M mutation

Although lower GSH levels and NRF2 activity were associated with T790M-driven erlotinib resistance in our cell lines, this may still be incidental unless the T790M mutation directly induces these changes. We further expressed the active (L858R) or active/resistant (L858R/T790M) EGFR mutants in HEK293 cells that contain low endogenous EGFR levels (Figure 4a). Unlike expression of the L858R-EGFR, expression of the L858R/T790M double-mutant receptor reduced intracellular GSH levels (Figure 4b). This was associated with reduced PALB2 and SQSTM1 expression (Figure 4c). Conversely, transfection with two independent siRNA sequences previously shown to selectively target T790M-mutant EGFR [33]-sensitised PC9ER cells to erlotinib (Figure 4d; Supplementary Figure S7C and D) and increased GSH levels (Figure 4e). The latter correlated with a reversal of changes in the expression pattern of GSH metabolic enzymes observed between PC9 and PC9ER cells (Figure 4f vs Figure 2b) and with increased PALB2, SQSTM1 and NRF2 levels in T790M-silenced cells (Figure 4g). Therefore, lower GSH levels in T790M NSCLC cells are a direct consequence of acquiring this mutation and the accompanying impairment of NRF2 activity.

Decrease in GSH correlates with increased nitric oxide levels

As GSH buffers reactive oxidative species, we investigated whether lower GSH levels in erlotinib-resistant cells associated with elevated reactive oxidative species. We performed flow cytometry analysis in the presence of dihydroethidine and DAF-FM (4-amino-5-methylamino-2',7'-difluorofluorescein diacetate) to detect superoxide and nitric oxide (NO) species, respectively. Erlotinib-resistant cells showed an increase in NO species (Figure 5a), although they did not show increased superoxide levels. To assess whether this could influence erlotinib resistance, we first silenced the expression of the three NO synthases, NOS1–3. Although siRNA-mediated downregulation of NOS2 and 3 did not impact on erlotinib resistance (not shown), NOS1 silencing sensitised PC9ER cells to erlotinib (Figure 5b). Next, we quenched cellular NO in erlotinib-resistant cells with the NO-trap carboxy-PTIO and revealed that this partially re-sensitised PC9ER cells to erlotinib (Figure 5c). Although these data suggest a role for NO in erlotinib resistance, the levels of changes observed as compared with those seen earlier (Figures 2 and 3) suggest that changes in NO are

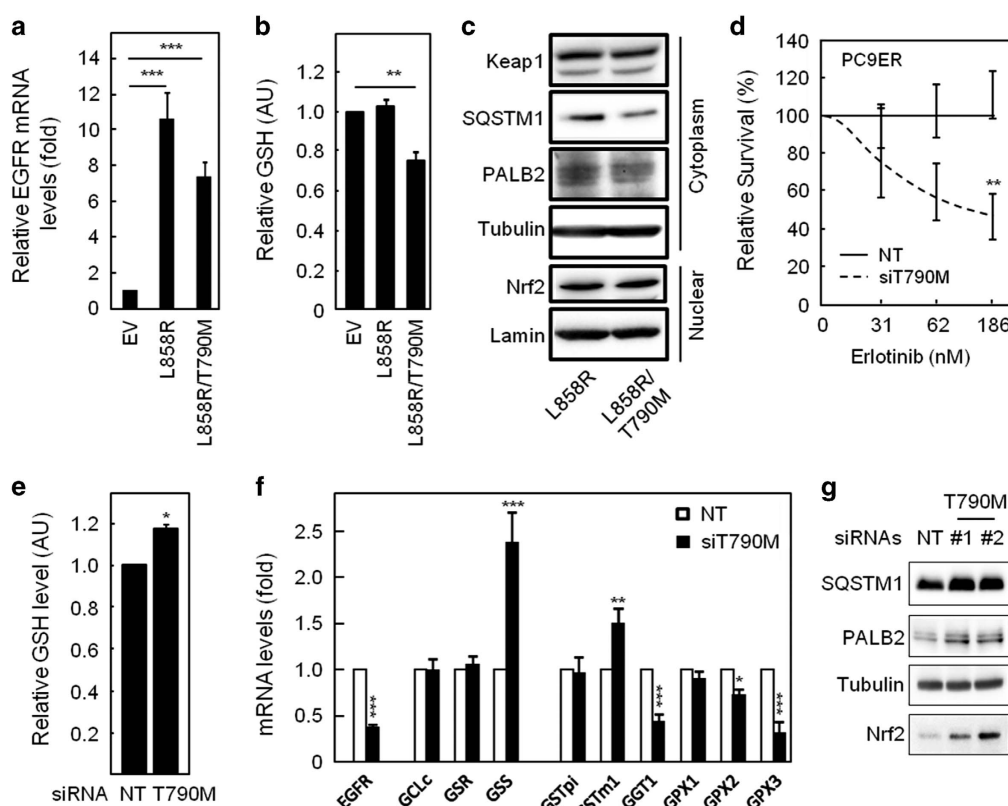


Figure 4 Expression of EGFRm/T790M decreases intracellular GSH levels and NRF2 activity. (a–d) HEK293 cells were transfected with empty vector control (EV), activated L858R-EGFR or activated/resistant L858R/T790M EGFR-mutant constructs. (a) Quantitative reverse transcription PCR for EGFR, (b) colorimetric assay for GSH levels and (c) cell fractionation followed by SDS-PAGE/western blotting for the indicated proteins were done on stable cell lines. Detection of lamin and tubulin was used as loading controls for nuclear and cytoplasmic fractions, respectively. (d–g) PC9ER cells transfected with an EGFR T790M-specific or NT siRNAs were subjected to (d) treatment with erlotinib before crystal violet staining, (e) colorimetric assay for intracellular GSH levels, (f) quantitative PCR for GSH metabolic enzymes or (g) SDS-PAGE/western blotting. All data representative of ≥ 3 experiments. (a,b,d–f) Values are average of $n = 4 \pm \text{s.e.m.}$ Statistics: (a,b) analysis of variance, (d,e,f) Student's *t*-test, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. See also Supplementary Figures S6 and S7.

not solely responsible for resistance downstream of decreased GSH levels.

EA administration re-sensitises EGFRm/T790M tumours to erlotinib in mouse xenografts

The GST inhibitor EA restored GSH levels and erlotinib sensitivity in EGFRm/T790M cells *in vitro* (Figure 2). EA is still used as a diuretic in humans for conditions including high blood pressure and heart failure [34]. Hence, we hypothesised that co-administration of physiologically relevant doses of EA might improve the responsiveness of EGFRm/T790M tumours to erlotinib *in vivo*. PC9 or PC9ER cells were injected subcutaneously in *nude* mice and tumours were left to grow to 100 mm^3 . The animals were then treated daily with erlotinib and EA alone or in combination. Co-administration of the drugs greatly inhibited tumour growth with 60% of the animals

showing tumour volumes $\leq 300 \text{ mm}^3$ at 25 days, whereas those treated with either drug alone showed more extensive disease (Figure 6a). This was associated with increased survival (Figure 6b) and intra-tumoural GSH levels in combination-treated animals (Figure 6c). EA did not have any effect on erlotinib sensitivity of PC9 xenografts in agreement with the lack of further added sensitisation to erlotinib obtained with this inhibitor *in vitro* (Supplementary Figure S7E). Thus, co-administration of EA is probably a viable strategy for the management of erlotinib-resistant cancers in humans.

Decreased GSH synthetic enzymes expression characterises erlotinib-resistant patients

Finally, we assessed whether the decrease in GSH-synthesising enzymes observed in EGFRm/T790M cell lines *in vitro* also occurred in patients.

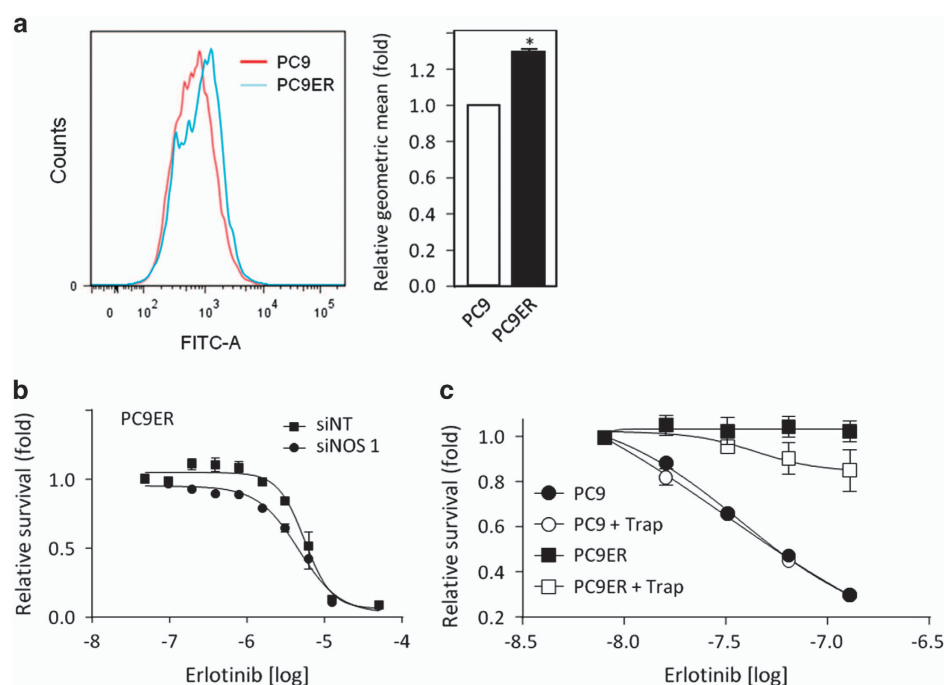


Figure 5 Changes in NO levels modulate erlotinib response. (a) NO levels in PC9 and PC9ER cells were compared by fluorescence-activated cell sorting (FACS) using DAF-FM. Left: FACS profile; right: fold changes in geometric mean. (b) PC9ER cells transfected with non-targeting (NT) or NOS1 siRNAs or (c) PC9 and PC9ER cells treated $\pm$ an NO-trap were exposed to a dose range of erlotinib. Cell survival was determined by crystal violet staining. Statistics: Student's *t*-test, * $P \leq 0.05$.

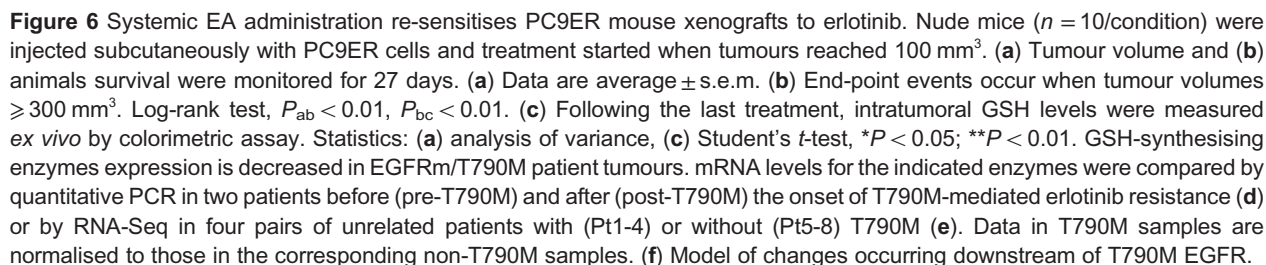
First, we performed quantitative PCR for GSS, GSR, GCLC and GCLM in paired biopsy samples from two patients before (EGFR^m alone) and after acquiring EGFR^m/T790M-mediated erlotinib resistance. In both cases, resistance was accompanied by a decrease in one or both of the rate-limiting enzymes for GSH biosynthesis, GCLC and GSS (Figure 6d). Moreover, this association was not limited to syngeneic samples, as RNA-Seq of four pairs of unrelated patients' biopsies revealed lower expression of at least one of these enzymes in T790M tumours as compared with non-T790M samples (Figure 6e). Therefore, decreased expression of GSH synthetic enzymes is probably associated with T790M-mediated erlotinib resistance in lung cancer patients.

Discussion

EGFR TKIs such as erlotinib offer therapeutic benefit to NSCLC patients harbouring EGFR^m [1–3]. However, the rapid development of resistance due in 50% of cases to acquisition of the secondary T790M EGFR mutation greatly limits the ability of these agents to prolong patient survival [5–8]. Although decreased affinity of the EGFR^m/T790M for erlotinib was thought responsible and new irreversible inhibitors

may be promising in circumventing this, additional mechanisms of resistance are likely to be present. Indeed, EGFR^m/T790M cells still demonstrate significant loss of sensitivity to an irreversible compound (Supplementary Figure S1C). This suggested that resistance to erlotinib in EGFR^m/T790M NSCLC cells is mediated through additional mechanisms.

Accumulating evidence suggests EGFR mutations to drive alteration in metabolic signatures, however, majority of them fail to demonstrate efficacy of targeting these molecules in clinical settings or *in vivo* models [35–37]. To identify novel resistance pathways, we performed ¹H-NMR metabonomic analysis of two independent NSCLC erlotinib-sensitive/resistant cell line pairs (PC9/PC9ER and H3255/H1975 cell lines). These were chosen according to the several criteria. First, both resistant cell lines shared the same T790M resistance mutation. Second, although PC9ER cells were obtained through selecting PC9 cells with erlotinib making these two lines relatively isogenic, H3255 and H1975 cells are genetically unrelated. Third, the primary EGFR-activating mutations in the two cell line pairs were different (Δ E746-A750 for PC9/PC9ER cells, L858R for H3255/H1975 cells). These criteria maximised the opportunity for metabolic changes shared by both cell line pairs to be solely



dependent on the T790M mutation. One of the most striking differences highlighted by our analysis was a decrease in GSH levels in erlotinib-resistant cells (Figure 1). The GSH pathway has long been involved in cancer drug resistance [27, 28]. However, this was traditionally associated with increased GSH levels [25, 26]. Indeed, GSH covalently binds to some drug molecules in a GST-dependent manner leading to their cellular export and quenches reactive oxidative species often requiring for these compounds to act [27, 28]. Therefore, an association between decreased GSH levels and EGFR TKI resistance was surprising and warranted further investigation of its relevance to erlotinib responses.

Our experiments demonstrated that inhibition of GSH biosynthesis by either RNAi or small molecules made erlotinib-sensitive cells resistant to the drug (Figure 2d–i). Conversely, inhibition of GSH-degradation re-sensitised resistant cells to erlotinib (Figure 2d–f). Hence, changes in GSH levels alone can modulate the response of NSCLC cells to this drug and decreased GSH levels accounts for erlotinib resistance in PC9ER and H1975 cells. Comparative analysis revealed a transcriptional downregulation of GSH-synthesising enzymes in T790M cells (Figure 2b) due to the impairment of NRF2, a downstream mediator of EGFR responsible for transcription of these enzymes (Figure 3a and b). This occurred via upregulation of the NRF2 inhibitor KEAP1 and/or downregulation of PALB2 and SQSTM1, two proteins involved NRF2 stabilisation. Indeed, siRNA-mediated silencing of KEAP1 in T790M cells sensitised them to erlotinib (Figure 3g and h), whereas that of SQSTM1 or NRF2 made sensitive cells resistant to this drug (Figure 3c–f). Importantly, decreased NRF2 activity and GSH levels in resistant cells were a direct consequence of acquiring the T790M mutation as introducing EGFRm/T790M in HEK293 cells, rather than EGFRm alone, reproduced the changes associated with erlotinib resistance (Figure 4a–c). Conversely, silencing EGFRm/T790M in PC9ER cells reverted the changes in GSH levels and metabolic enzymes seen upon acquisition of resistance by PC9 cells (Figure 4d–g).

It is unclear by what mechanism(s) the T790M mutation induces the observed transcriptional changes as the higher kinase activity of EGFRm/T790M [9] should further enhance NRF2 activity. However, mutant EGFRs differ from their wild-type counterparts in their subcellular localisation [38], which probably results in the EGFRm/T790M having different signalling partners as EGFRm or wild-type

EGFR. Further research will be required to investigate this possibility.

We next attempted to identify the mechanism by which decreased GSH levels cause erlotinib resistance. GSH is a major cellular antioxidant [39], and its reduced expression could result in increased reactive oxidative species. In addition, single-nucleotide polymorphisms in antioxidant genes have been demonstrated to be associated with survival outcome in patients receiving TKI therapy [40]. Although superoxide levels were unchanged, NO levels were raised in PC9ER as compared with PC9 cells (Figure 5a) and NOS1 silencing or NO quenching sensitised PC9ER cells to erlotinib (Figure 5b and c). GSH is known to neutralise NO and protect against protein nitrosylation [41, 42]. It is worth noting that EGFR is a target of S-nitrosylation [43], but the consequence of this on erlotinib response is currently unknown. However, although our data suggest that NO probably contributes to erlotinib resistance, this does not fully explain the effects of reduced GSH. Glutathionylation has a role in disease state by modifying the function of target proteins [44] and assessing changes to the glutathionylation profile may identify proteins involved in EGFRm/T790M-mediated erlotinib resistance.

Regardless of the mechanism underlying erlotinib resistance downstream of decreased GSH levels, we showed that the GSH pathway could be manipulated for therapeutic benefit. Indeed, systemic administration of clinically relevant doses of EA, a GST inhibitor [45], increased the intra-tumoural GSH levels (Figure 6c) and re-sensitised EGFRm/T790M tumours to erlotinib in a cancer cell xenograft mice model (Figure 6a–c). As EA is an orally available diuretic used in humans with limited toxicity [34], our findings could rapidly translate into clinical practice if this sensitisation also occurs in humans. Moreover, EA has already been used together with classical chemotherapeutics such as alkylating agents to prevent their GST-mediated cellular export [34], leading to improved clinical outcome. Therefore, EA may help manage erlotinib resistance in EGFRm-NSCLC patients and improve response to follow-on chemotherapeutic regimen. However, it is unclear whether decreased GSH levels only occurs downstream of the EGFRm/T790M or if this is a common feature of other erlotinib resistance pathways such as c-Met amplification. Answering this before clinical exploitation of our findings will help more accurate patient selection for EA/erlotinib combined trials.

Finally, we show our findings to be clinically relevant using EGFRm and EGFRm/T790M lung

cancer samples (Figure 6d and e). The reduced number of samples analysed reflects the fact that repeated biopsy in NSCLC following the onset of EGFR TKI resistance is rare, although this practice is now changing. Nevertheless, we demonstrate in both syngenic and unrelated patient samples that mRNA levels for GSH-synthesising enzymes are decreased in T790M tumours. Hence, probing for GSH-synthesising enzymes may help, in a recurrent setting, to predict the response to combinatorial therapies of erlotinib and GSH level increasing agents.

To sum up, we demonstrate that decreased intracellular level of GSH could mediate T790M-driven erlotinib resistance in NSCLC and highlight the molecular events involved (Figure 6f). Therapeutic strategies that increase intra-tumoural GSH levels may revert erlotinib resistance in the clinic.

Materials and Methods

Materials

Mercaptosuccinic acid (used at 50 μ M), buthionine sulfoximine (used at 40 μ M) and EA (used at 50 and 100 μ M in PC9 and H1975 cells, respectively) were purchased from Sigma (St Louis, MO, USA), whereas EGFR Inhibitor 324674 was from Santa Cruz (Dallas, TX, USA) and Merck (Kenilworth, NJ, USA), respectively. Antibodies against GSTpi, GPX1, GSS, GSR, GCLC, GSTpi, SQSTM1 and DPP3 were from Abcam (Cambridge, UK); antibodies targeting NRF2 and KEAP1 were from Santa Cruz and anti-PALB2 was from Novus (Abingdon, UK). The specificity of all antibodies employed here was assessed by disappearance of the respective signal following selective targeting of the expression of the corresponding protein by siRNA treatment. Quantitect primers targeting GSTpi, GPX1, GPX1, GSS, GSR and GCLC were from Qiagen (Valencia, CA, USA). All other primers were synthesised by Sigma. siRNAs were purchased from Dharmacon (Little Chalfont, UK). Dihydroethidine was from Invitrogen (Waltham, MA, USA) and DAF-FM from Sigma.

Cell culture

All cell lines were obtained from the CRUK cell line bank where they were authenticated and mycoplasma status assessed through regular testing in our lab. Cell lines were grown in Roswell Park Memorial Institute with 10% fetal bovine serum at 37 °C, 5% CO₂.

Extraction of the intracellular metabolites

Intracellular metabolites were extracted as reported previously [46, 47] with some modifications. In brief, 10⁷ cells per condition were trypsinised and washed thrice in ice-cold phosphate-buffered saline before metabolite extraction. Cell pellets were resuspended in 0.6 ml cold water/methanol (1:2) and subjected to three freeze–thaw cycles before sonication in a wet ice bath for 15 min (cycles: 1 min pulse followed by 1 min pause). Samples were then centrifuged (3200 g/4 °C, 10 min) and

supernatants transferred into cold Eppendorfs. The remaining pellets were extracted twice more by the same method. Supernatants from the three subsequent extractions were combined, centrifuged (12 000 g/4 °C, 10 min) and freeze-dried following vacuum-driven methanol evaporation. Lyophilised samples were stored at –80 °C. Ten biological replicates were used for each group of cells.

Cellular metabolomic analysis by ¹H-NMR

Freeze-dried intracellular metabolites extracts were dissolved in 600 μ l phosphate buffer 0.1 M (pH 7.4, 99.9% D₂O) containing 0.001% sodium 3-trimethylsilyl-1-[2,2,3,3-²H₄] propionate as previously described [48]. All samples were centrifuged (12 000 g/4 °C, 10 min) after short vortexing and supernatants transferred into the 5 mm NMR tubes for NMR detection. All one-dimensional ¹H-NMR spectra were acquired on a Bruker AVIII 600 MHz NMR spectrometer equipped with a cryogenic probe (BrukerBiospin, Rheinstetten, Germany) at 298 K. The first increment of NOESY pulse sequence was employed with continuous wave irradiation on the water peak during recycle delay and mixing time for water suppression. Recycle delay of 2 s and mixing time of 100 ms were set. The 90° pulse was adjusted to 10 μ s approximately and 64 scans were collected into 32 k data points with the spectral width of 20 p.p.m. For metabolite assignments, two-dimensional NMR spectra including ¹H–¹H COSY, ¹H–¹H TOCSY, ¹H *J*-resolved, ¹H–¹³C HSQC and ¹H–¹³C HMBC for typical samples were acquired and processed as described previously [49].

NMR data analysis

The spectral region at δ 0.5–9.5 was integrated into bins with the width of 0.002 p.p.m. (1.2 Hz) using AMIX package (v3.8, BrukerBiospin). The range (δ 4.7–5.2) was removed to eliminate the effects of water peak suppression. Each bin area was normalised to the total area of the respective spectrum. Multivariate data analysis was performed with the software SIMCA-P+ (v 12.0, Umetrics, Umea, Sweden). The model was built using the orthogonal projection to latent structure-discriminant analysis (OPLS-DA) [50] with Pareto variance (Par) scaling and sevenfold cross-validation. The parameter R^2X was the variation of *X* explained by the model and Q^2 represented the predictability of the model. The validation of all the models was further ensured by CV-analysis of variance ($P < 0.05$) [51]. To assist the biological interpretation of the loadings generated from the models, the loadings were firstly back-transformed [52] and then plotted with colour-coded OPLS-DA coefficients in MATLAB 7.1 using an in-house script [53]. The colour code corresponded to the absolute value of the OPLS-DA coefficients ($|r|$), indicating the contribution of each variable to explain the intergroup differentiation. The value of $|r|$, > 0.602 , was considered to be significant ($n = 10$, $P < 0.05$).

GSH colorimetric assay

A GSH colorimetric assay kit was purchased from BioAssay Systems (Hayward, CA, USA) and used according to the manufacturer's instructions.

siRNA transfection

A total of 1×10^4 PC9, PC9ER or H1975 cells per well in six-well plates were transfected with siRNAs at 25 nM (Dharmacon) for 24 h using RNAiMax (Invitrogen) following the manufacturer's protocol. Each protein was targeted with a mix of four sequences. A total of 4×10^3 cells were re-seeded and then incubated at 37 °C/5% CO₂ for 24 h for target silencing before further experiment steps.

Cell survival assay

For EA, buthionine sulfoximine and mercaptosuccinic acid, cells were pretreated for 4 h before erlotinib addition (100 nM) for 48 h. Cells were then fixed and stained for 20 min with a 25% methanol/0.5% crystal violet solution. Plates were washed in running water, air-dried and the stain dissolved in 10% acetic acid on a shaker before absorbance at 595 nm.

Quantitative PCR

Total cellular mRNA was extracted using the RNeasy kit (Qiagen) and converted to complementary DNA with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). mRNA levels were quantified using a Fast SYBR Green Master Mix (Applied Biosystems) on a 7900HT Fast Real time PCR System (Applied Biosystems). qBase software (San Francisco, CA, USA) was used for data analysis using TATA-binding protein and β -actin as internal controls. The primers used were listed below (F, forward; R, reverse): GCLm: (F): 5'-GGCACAGGTAAAACCAAATA GTAAC-3', (R): 5'-CAAATTGTTTAGCAAATGCAGTCA-3'; GPX2: (F): 5'-TAAGTGGGCTCAGGCCTCTCT-3', (R): 5'-GGTCATAGAAGGACTTGGCAATG-3'; GPX3: (F): 5'-GACAAGAGAAGTCGAAGATG-3', (R): 5'-CTTCCTG TAGTGCATTGAGTT-3'; GSTz1: (F): 5'-TCCTATTCCG AAGCTCCTGC-3', (R): 5'-TTCAGTGCCTGGAAGTCCTT AG-3'; GSTm1: (F): 5'-CTATGATGTCCTTGACCTCCA CCGTATA-3', (R): 5'-ATGTTACGAAAGGATAGTGGG TAGCTGA-3'; Beta-Actin: (F): 5'-TCCTCCTGAGCGCAA GTACTC-3', (R): 5'-CTGCTTGCTGATCCACATCTG-3'; KEAP1: (F): 5'-CAGATTGGCTGTGTGGAGTT-3', (R): 5'-GCTGTTCGCGAGTCGTAAGTTG-3'; SQSTM1: (F): 5'-CTGGGACTGAGAAGGCTCAC-3', (R): 5'-GCAGCTG ATGGTTTGAAAT-3'; TBP: (F): 5'-TGCACAGGAGCCA AGAGTGAA-3', (R): 5'-CACATCACAGCTCCCCACCA-3'; NRF2 primers: (F): 5'-GAGAGCCCAGTCTTCATTGC-3', (R): 5'-TGCTCAATGTCCTGTTGCAT-3'. Primers against the other targets were purchased from Qiagen: GCLc (QT00037310), GGT1 (QT00029470), GPX1 (QT00203392), GSTp1 (QT00086401), GSS (QT00014413) and GSR (QT00038325).

Tissue mRNA extraction and quantitative PCR

The origin of tissues and techniques used are as previously reported [54]. In short, samples were obtained from EGFR-mutant lung adenocarcinoma patients with acquired erlotinib resistance under Human Investigations Protocol #111000928 (Yale Cancer Center, New Haven, CT). Those were reviewed by a pathologist to ensure adequate tumour content. Tumour areas were circled and microdissection performed to enrich for tumour content.

Tissue mRNA extraction and RNA-Seq

The Illumina TruSeq RNA Sample Preparation Kit (Illumina Cambridge Ltd, Essex, UK) was used for RNA tissue extraction and analysis done as previously described [55].

Western blotting

Cells were lysed using 0.5% Triton X-100, 150 mM NaCl, 2 mM EDTA, 10% glycerol supplemented with protease inhibitors cocktail tablets (Roche Diagnostics, Indianapolis, IN, USA), 10 mM β -glycerophosphate, 1 mM Na₃VO₄ and 10 mM NaF. Equal protein amounts were analysed by SDS-PAGE/ western blotting using the antibodies indicated.

Flow cytometry analysis of oxidative species

Cells (15×10^4 /well in a six-well plate) were treated with 10 μ M DAF-FM or dihydroethidine for 30 min, washed with phosphate-buffered saline, trypsinised, pelleted and resuspended in 1 ml of phosphate-buffered saline before flow cytometry using a BD FACSCalibur (DB Biosciences, Oxford, UK). The geometric mean intensity was determined using FlowJo (Tree Star Inc, Ashland, OR, USA).

Animal experiments

A total of 5×10^6 PC9ER or PC9 cells were injected subcutaneously into the flank of female BALB/c nude mice and the tumours grew until they reached 100 mm³. Mice were then randomized into three groups ($n = 10$) and treated by intraperitoneal injection of 25 mg kg⁻¹ per day erlotinib/0.5% w/v methylcellulose and/or 6 mg kg⁻¹ per day EA/1% Tween 80 in distilled water. Such treatments were administered daily from day 7 to 26. Tumours were measured by caliper and volumes calculated as $V = 1/2 \times L \times W^2$ (L ; length, W ; width of tumour). Data analysis was performed by an investigator blinded to the experimental conditions. All experiments complied with ethical regulations as enforced by the local committee.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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Author contributions

MJS, OEP, YLW and HRT conceived and designed the study. HDL did the metabolomics data acquisition and analysis. HDL, YLH and ZGL conducted all the animal experiments and data analysis. WS, EC and RR did the molecular biology data

acquisition and analysis. EB, EFS, GJJEH and JD acquired the mRNA data on gene expression in clinical samples. OEP, YLW, MJS and HRT did the overall data interpretation with others and wrote the paper.

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