Regulation of ERK5 function via its C terminus and its role in HER2+ breast cancer

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Abbreviations

ABL	Abelson murine leukaemia viral oncogene homolog 1
ADCC	Antibody-dependent cell-mediated cytotoxicity
ALA	Alanine
AP-1	Activator protein 1
APS	Ammonium persulfate
ATAC	Acetyltransferase complex
ATCC	American type culture collection
BCR	Breakpoint cluster region
BMK1	Big map kinase 1
BSA	Bovine serum albumin
CD	Common docking domain
CDK	Cyclin-dependent kinase
CHIP-SEQ	Chromatin immunoprecipitation coupled with Sequencing
CMV	Cytomegalovirus
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat
CRUK	Cancer Research UK
DAPI	4', 6-diamidino-2-phenylindole
DFS	Disease Free survival
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethyl sulfoxide
EB	Elution buffer
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N′*,*N′*-tetraacetic acid
ER	Oestrogen receptor
ERK1/2	Extracellular signal-related kinase 1 / 2
ERK5	Extracellular signal-related kinase 5
FACs	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FDA	Food and drug adminstration
FGF	Fibroblast growth factor
FIG	Figure
FL	Full length
FRT	Flippase recognition target
GLU	Glutamic acid
GTCF	Genomics Technologies Core Facility
H3K20	Histone three Lysine 20
H3K36	Histone three Lysine 36
H3K4	Histone three Lysine 4
HBSS	Hank's balanced salt solution
HCL	Hydrochloric acid
HER	Human Epidermal Growth factor receptor
HF	High Fidelity

HIF1α	Hypoxia inducible factor 1α
HRE	Hypoxia response element
KRH	Krepes-ringer hepes
IGFR	Insulin-like growth factor receptor
IL-6	Interleukin-6
IHC	Immunohistochemistry
KM	Kaplan-meier
LB	Luria-Bertani broth
LR	Lapatinib resistant
MAPK	Mitogen activated protein kinase
MEF	Myocyte enhancer factor
MEKK5	Mitogen-activated protein kinase kinase
NLS	Nuclear localisation signal
NRF	Nuclear respiratory factor
NRG	Neuregulin
PAF1	Polyermase associated factor 1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PMSF	phenylmethane sulfonyl fluorideor phenylmethylsulfonyl fluoride
PR	Progesterone receptor
PR 1/2	Proline rich region 1/2
PTEN	Phosphatase and tensin homolog
PVDF	polyvinylidene difluoride
RB	Retinoblastoma
RFS	Relapse-free survival
RIME	Rapid Immunoprecipitation mass spectrometry of endogenous proteins
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RNA POL	RNA polymerase
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SER	Serine
SERM	Selective oestrogen receptor modulators
SGK	Serum and glucocorticoid induced protein kinase
SUPPL	Supplementary
TAD	Transactivation domain
TAE	Tris acetic acid
TAR	Transactive response
TEY	Threonine – Glutamic acid - Tyrosine
THR	Threonine
TLB	Triton lysis buffer
TNBC	Triple negative breast cancer
TNM	Tumour Node Metastasis
TO	Tet operator
TSS	Transcription start site
UK	United Kingdom
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

1. Introduction

1.1 ERK5 basic biology

1.1.1 An introduction to the MAPK: ERK5

Mitogen-activated protein kinase (MAPK) pathways are evolutionarily conserved signalling cascades involved in the regulation of a variety of fundamental cellular processes such as cell proliferation, differentiation, survival and migration (reviewed in [1] [2] [3]). These pathways comprise a three-tier protein kinase cascade which results from the sequential activation of a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPK) and a MAPK, to relay, amplify and integrate many different stimuli, including mitogens, cytokines and various environmental stresses. MAPKs mediate responses to these extracellular cues mainly by regulation of gene expression, but also by post-translational mechanisms involving cytoplasmic targets [4] [5]

At least six distinct groups of MAPKs have been identified. The best known are the four conventional MAPKs: extracellular signal-regulated protein kinases (ERK) 1/2, c-Jun amino-terminal protein kinases (JNK 1/2/3), p38 MAPKs (p38α/β/γ/δ) and ERK5. Each subfamily confers responsiveness to distinct stimuli and elicits specific biological responses. Nonetheless, the situation, in reality, is more complex with evidence of overlapping functions dependent on the biological context. Broadly, ERK1/2 are considered to regulate proliferation and survival [1], whilst p38 MAPKs are critical for immune and inflammatory responses [6]. The JNK subfamily is known to be important for mediating the apoptotic response to cellular stress [7] and cell proliferation [8]. In contrast, ERK5 is much less understood, despite compelling evidence of its functional importance during development and disease. Initially, ERK5 was identified as a stress-activated MAPK, activated by both oxidative and osmotic stresses [9]. However, more recently, alike ERK1/2, ERK5 was also found to regulate cell survival and proliferation in response to mitogens [4] [10].

ERK5 was discovered in 1995 by two independent research groups in California and Michigan who immediately recognised the comparatively large size of this novel member of the MAPK family [11] [12]. Specifically, ERK5 exhibits a molecular weight of approximately 102 kDa, making it twice the molecular weight of conventional MAPKs. Accordingly, Lee *et al.* referred to ERK5 as Big MAP kinase 1 or BMK1 [12]. The different names given to ERK5 illustrates the identity crisis that it suffered until now. Sharing of the name "ERK" was due to the high degree of homology in the N terminal region of ERK5, despite distinct functional domains located in the C terminus, suggesting possible unique physiological and

pathological functions (**Fig 1.1**). Sharing the "ERK" nomenclature has however placed ERK5 very much in the shadow of ERK1/2, given the prominent role of this pathway in cancer. Consequently, the molecular and biochemical characterisation of ERK5 is very limited compared to that of ERK1/2.

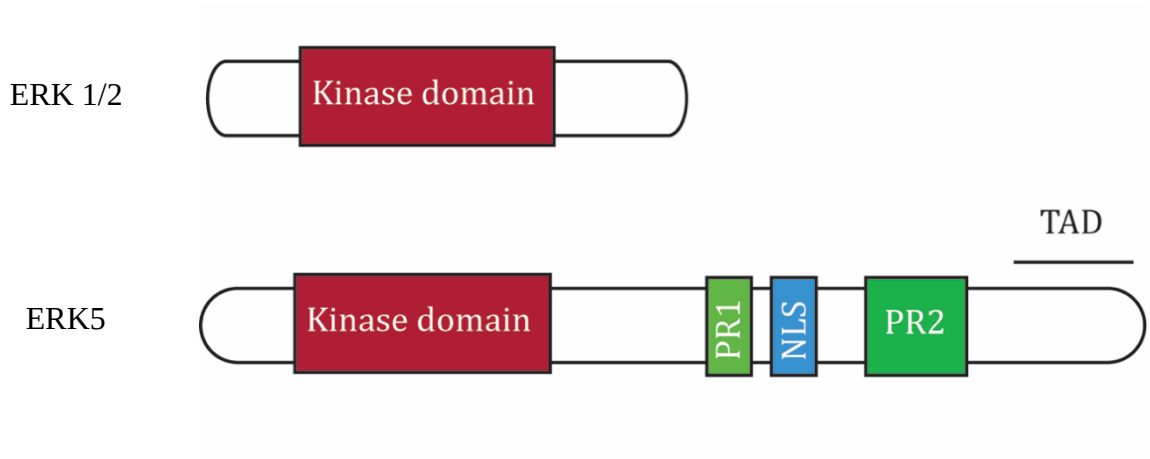

Figure 1.1 The C terminus of ERK5 is unique amongst the MAPK family The four conventional MAPKs include p38, JNK ERK1/2 and ERK5. The terminal MAPKs have a high degree of homology in their N terminal, kinase containing domain. However ERK5 has a unique C terminal extension making it twice the size of the other MAPKs. The C terminus contains regions such as the proline rich domains 1/2 (PR1/2), the nuclear localisation signal (NLS) and the transcriptional activation domain (TAD).

1.1.2 ERK5 structure and functional domains

The human MAPK7 gene which encodes ERK5 is located on the short arm of chromosome 17p11.2 and is 5824 bases long [13]. It contains 6 coding exons, and in mice, three isoforms have been identified (mERK5a, mERK5b and mERK5c) [14]. Slt2p (Mpk1p) and Sma-5 are functional homologs of ERK5 in yeast and C elegans respectively [15] [16]. The open reading frame of 2445-base pairs encodes 816 amino acids in humans (**Fig 1.2**). The N-terminus contains a cytoplasmic targeting domain (aa 1-77). The N-terminal region also features a prominent region encoding the kinase domain (aa 78-406) which can be further divided into regions for specific functions such as MEK5 binding (aa 78-139), MEK5 phosphorylation (aa 218/220) and ERK5 oligomerisation (aa 140-406). Other regions found in the acidic moiety include the common docking (CD) domain (350-406) which is utilised to specifically interact with MAPK phosphatases, substrates and scaffolding proteins [17].

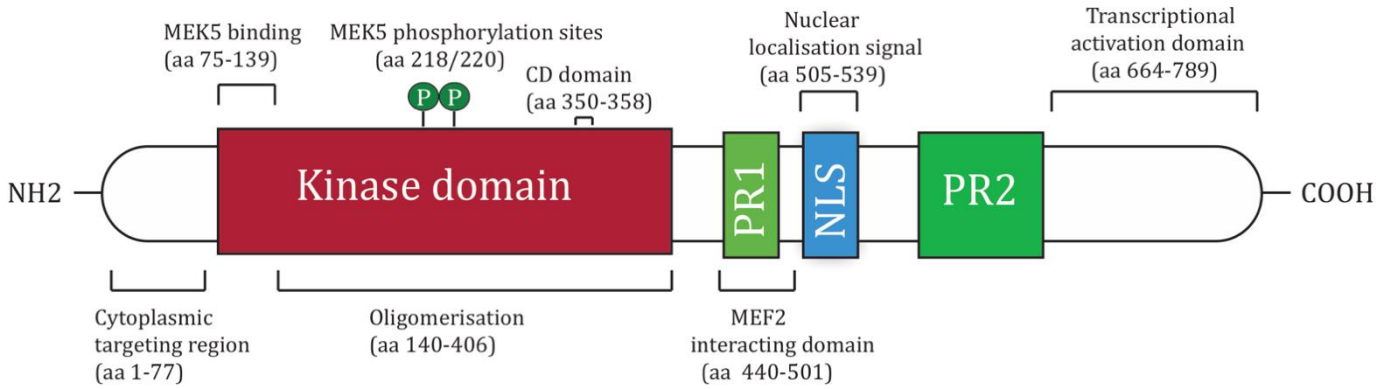

Figure 1.2 Protein structure of ERK5 and functional domains [18]. ERK5 consists of 816 amino acids (aa) and can be divided into functional domains within the N- and C-terminus. The N-terminus contains; cytoplasmic targeting region (aa 1-77), a kinase domain (aa 78-139), which comprises a MEK5 binding region (aa 78-139), a common docking domain (aa 350-358), a large oligomerisation domain (aa 140-406) and the TEY motif (aa 218-220). The large C-terminal tail (400 aa) consists of; two proline-rich domains (PR1 aa 434-485 and PR2 aa 578-701), a MEF2-interacting region (aa 440-501), the nuclear localisation signal (NLS) (aa 505-539) and a transcriptional activation domain (aa 664-789) [18]

The C-terminal tail is a unique feature of ERK5. It shares no homology with other proteins which adds to the enigmatic character of ERK5. As the tail is exclusive to ERK5, it suggests that it enables ERK5 to function in a uniquely compared to conventional MAPKs (**Fig 1.2**). Like the N-terminus, the C-terminus can also be divided into functional domains. It contains an NLS (aa 505-539) which is responsible for the nuclear shuttling activity and therefore controls downstream activation of ERK5 targets within the nuclear and cytoplasmic compartments [19]. There is also an area which is specifically responsible for interaction with MEF2 (aa 440-501) [14]. There are also two proline-rich regions, PR1 (aa 434-465) and PR2 (aa578-701), which confer binding properties to proteins with Src homology 3 (SH3) domains [20]. The tail of the C-terminal tail also possesses a region that functions as a transcriptional activation domain (aa 664-789) [14] [21] [22]. This feature is novel to the MAPK family and is yet to be fully understood [22].

1.1.3 The MEK5/ERK5 pathway

Notable extracellular stimuli that activate ERK5 include vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and inflammatory cytokines, such as interleukin 6 (IL-6) (**Fig 1.3**) [10] [5] [23] [24]. These extracellular stimuli activate a MAPK/ERK kinase kinase (MEKK), namely MEKK2 or MEKK3, which in turn phosphorylate MAPK/ERK kinase 5 (MEK5) at Ser^{311}/Thr^{315} [25] [26]. The formation of a MEKK2/MEK5 complex acts via MEKK2 interaction with the scaffold protein, Lck-associated adaptor (Lad), is required for efficient signal transduction [26]. MEK5 subsequently activates ERK5 by dual phosphorylation at Thr^{218} and Tyr^{220} (TEY motif) present in the N terminal activation loop of the ERK5 catalytic domain. In addition to increasing ERK5 activity, the phosphorylation by MEK5 triggers a conformational change and nuclear translocation of ERK5 [19] [27]. ERK5 is believed to exist in "open" or "closed" conformations (**Fig 1.3**). Quiescent ERK5 exists in a "closed" folded conformational state, defined by an intermolecular interaction between the N- and C-terminal half which constitutes the nuclear export signal (NES) (**Fig 1.3**) [28]. In this conformation, the majority of ERK5 is sequestered in the cytoplasm [19]. Dual phosphorylation at Thr^{218} and Tyr^{220} induces a conformational change to an "open" state (disrupting the N- and the C- terminal intermolecular interaction), allowing autophosphorylation of several residues on the C- terminal tail and exposure of the NLS [28] [19]. In the nucleus, ERK5 regulates transcription via the phosphorylation and activation of various transcription factors through its kinase domain (**Fig 1.3**). ERK5 can also control gene expression via a transactivation domain present in its unique C terminal extension [22].

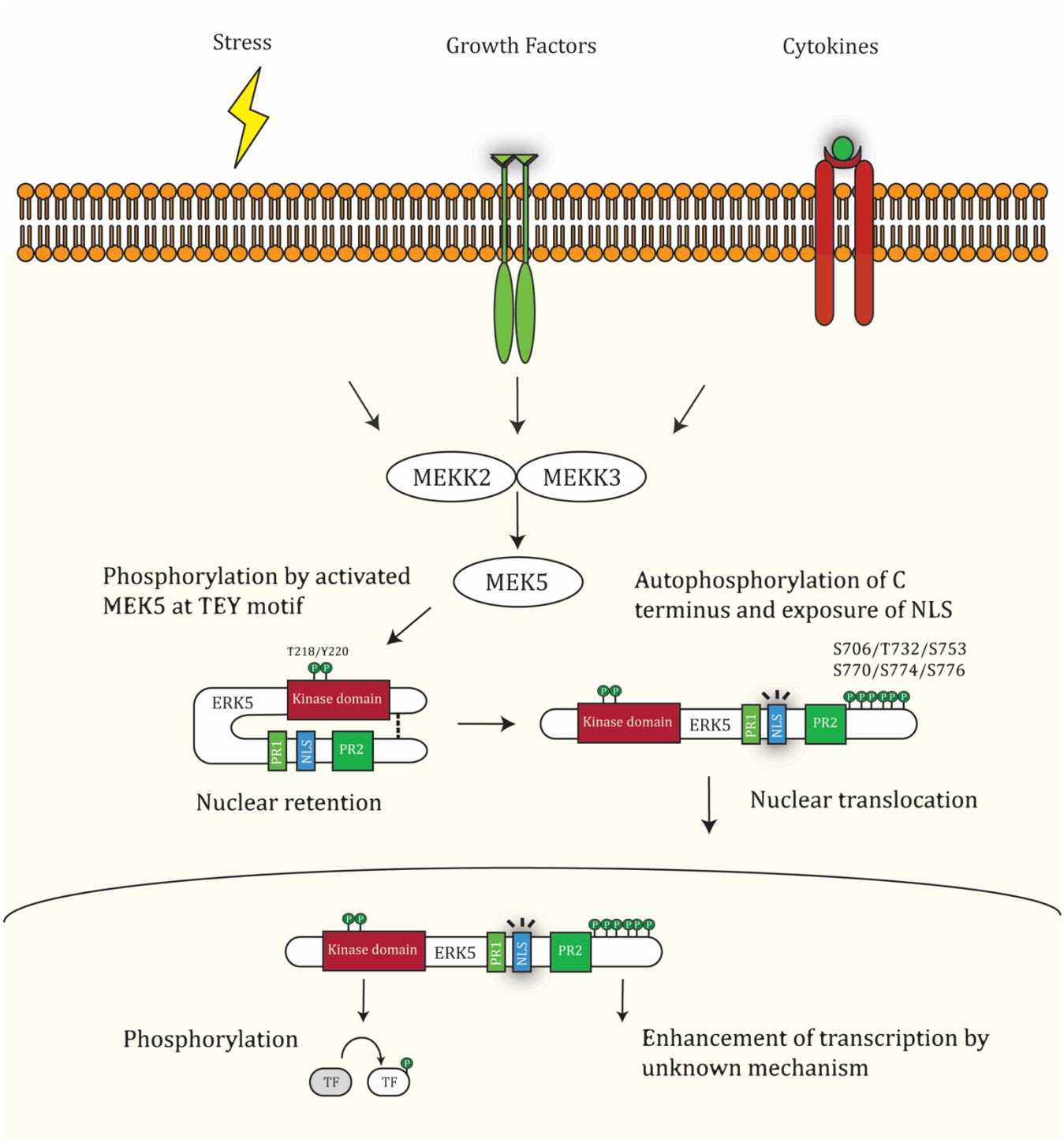

Figure 1.3 The canonical MEK5/ERK5 signalling pathway. A simplified illustration of the linear ERK5 signalling cascade. The signalling axis is activated by a variety of cellular stresses (oxidative, osmotic, laminar flow, U.V), growth factors (EGF, NGF, FGF, VEGF) and cytokines (LIF, IL-6, CSF-1). Activation leads to MEKK2/MEKK3 phosphorylation. MEKK2/MEKK3 phosphorylates MEK5, which in turn phosphorylates ERK5. Upon activation, ERK5 changes conformation and translocates to the nucleus to regulate transcription.

1.1.4 ERK5 and downstream targets

MAPK pathways can regulate gene expression, and a predominant mechanism by which this operates is by altering the activity of transcription factors by phosphorylation. Numerous components of the transcriptional machinery have also been identified as targets of the MAPK pathways. Alike other MAPKs, ERK5 functions primarily by regulating transcription via phosphorylation of transcription factors.

Naturally, the first place to examine the relationship between ERK5 and genetic expression is to identify the transcription factors ERK5 targets and their cognate genes. The most established link is between ERK5 and the myocyte enhancer factor (MEF) transcription factor family [29] [30]. Kato *et al*. showed that MEF2C is a substrate of ERK5 and that ERK5 activates MEF2C by phosphorylating a serine residue at amino acid position 387 of the transcription factor and subsequently increases its transcriptional activity [30]. This effect was linked to the induced expression of the immediate early response gene c-Jun. These genes are implicated in cell proliferation and survival and facilitated transcriptional activation of many late response growth genes. Subsequently, it was shown that MEF2D was a substrate specific to ERK5 while MEF2A and MEF2C were regulated by both ERK5 and p38 [29] [31] [32] [33]. The C-terminal tail was shown to be responsible for enhancing MEF2C activity [14].

In addition to c-Jun, other early response genes have been found to be regulated by ERK5. For example, members of the AP-1 family, c-Fos and Fra-1 are downstream targets of ERK5 [34]. The phosphorylation of these early gene products by ERK5 increased protein stabilisation and furthermore stimulated their transcriptional activity [34]. It was again found that the C-terminal tail was fundamental in regulating these transcriptional activities. This was demonstrated by expressing C terminal truncated mutants and transfecting cell lines with an AP-1 reporter. Furthermore, ERK5 has been found to target the transcription factors c-Myc and Sap1a [35] [36]. Sap1a complexes with c-Fos to positively regulate c-Fos transcriptional activities [37] while c-Myc is implicated in a wide variety of cellular functions and has been classified as an oncogenic transcription factor [38].

1.1.5 ERK5 and modes of transcription

Upon upstream activation by MEK5, ERK5 autophosphorylates various serine and threonine residues in the transactivation domain and consequently enhances ERK5-dependent transcription [22] [21] [39]. At least seven putative autophosphorylation sites have been identified: Ser^{706}, Ser^{730}, Thr^{732}, Ser^{753}, Ser^{769}, Ser^{773}, and Ser^{775}. Phosphorylation at these residues has been demonstrated to stabilise ERK5 in an “open” conformation, promote nuclear transit and enhance ERK5-mediated transcription [39] [27] [21] Despite

previous reports consistently describing enhancement of ERK5 mediated transcription as a consequence of C terminal phosphorylation, the precise molecular mechanism is unknown.

Recent evidence has demonstrated that MAPKs can regulate transcription by becoming tethered to chromatin, associating with chromatin remodelling complexes and/or transcription factors, or binding directly to DNA (**Figure 1.4**) (reviewed in [40]). These studies have demonstrated mechanisms whereby MAPKs regulate transcription beyond phosphorylation of transcription factors. Therefore it is possible that the transactivation domain of ERK5 may enhance transcription by a catalytically independent mechanism. Recent studies have also demonstrated that these C terminal sites can also be targeted by other kinases, namely CDK1 and ERK 1/2 [41] [39]. This opens up the possibility that ERK5 function could be controlled independently of MEK5 via its C terminus.

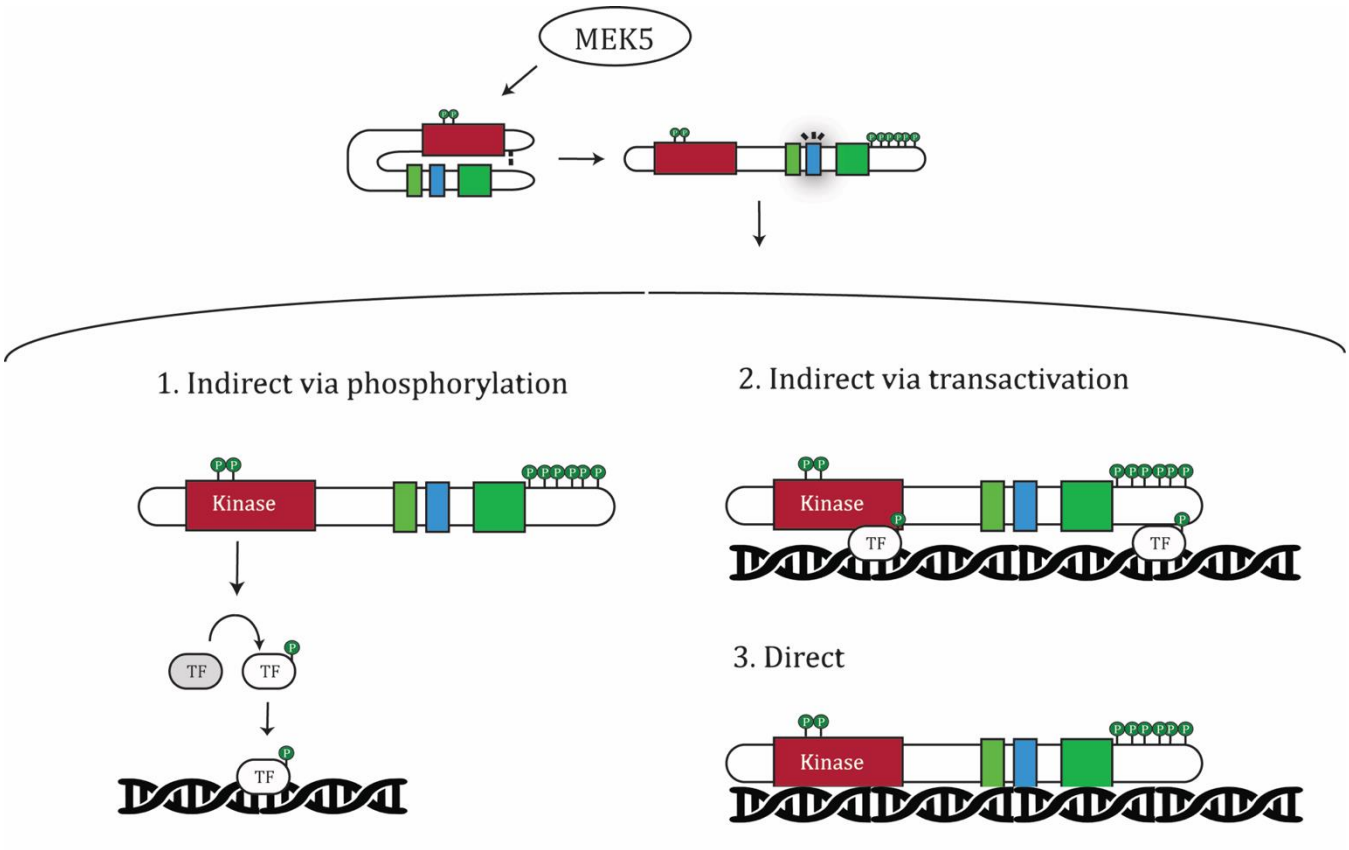

Figure 1.4 Putative modes of transcription by ERK5. 1) Indirect via phosphorylation – considered to be the conventional mechanism whereby MAPKs regulate transcription, via the phosphorylation and activation of transcription factors. 2) Indirect via transactivation – for example by binding to transcription co/factors or via formation of transcriptional complexes. 3) Direct binding to DNA.

1.1.6 ERK5 mouse models

The development of genetically modified mouse models from 2001-2004 led to a greater understanding about the components of the ERK5 pathway during development and homeostasis. Ultimately, these animal models have led to a resurgence of interest in the field of ERK5 and its role in the context of pathological conditions.

Three separate groups generated *erk5*$^{-/-}$ mice and discovered that ERK5 deficiency leads to embryonic lethality at an early stage of development (E 9.5-11.5) due to heart defects [42] [43] [44]. The morphology of endothelial cells which line the vessels and heart were rounded and disorganised. In these mice, angiogenesis did occur but maturation was retarded, and the formation of highly branched networks observed in the wild type animals was absent. Interestingly, Bi *et al*. had previously generated a *mef2c*$^{-/-}$ mouse model which failed to form mature vascular structures (embryonic death E9.5). Alike *erk5*$^{-/-}$ mice; the animals displayed abnormal endocardiogenesis which was combined with a reduction in the production of endothelial directed cytokines such as VEGF and angiopoietin 1 [45].

In 2004, Hayashi *et al*. used a conditional knock out approach to ablate ERK5 in different cell types [46]. The specific removal of ERK5 in endothelial cells caused mice to die at E10, presenting with cardiovascular defects consistent with the phenotype observed in the conventional global ERK5 knockout mice [46]. Ablation of ERK5 in cardiomyocytes (and other cell types) surprisingly did not affect embryonic cardiac development, and the conditional knockout mice proceeded to healthy development. These results demonstrated, for the first time, the critical importance of ERK5 to promote endothelial cell survival *in vivo*.

In addition to the requirement of ERK5 during embryonic angiogenesis, ERK5 is indispensable for angiogenesis in adult mice. Knocking out ERK5 in endothelial cells in adult mice led to lethality 2-4 weeks later [46]. Physiological analysis revealed that blood vessels were leaky, resulting in multiple haemorrhages. The *erk5*$^{-/-}$ endothelial cells lining the blood vessels were phenotypically similar with those found in the *erk5*$^{-/-}$ embryonic model, appearing round, irregularly aligned and apoptotic. *In vitro* deletion of ERK5 also led to the death of endothelial cells, in part due to the downregulation of MEF2C activation. Together, these results demonstrated the critical role for ERK5 in cardiac development and homeostasis.

1.1.7 ERK5 inhibitors

In addition to the development of animal models to investigate ERK5 function, several pharmacological inhibitors have also been synthesised. Whilst whole protein deletion removes total function of ERK5, small molecule inhibitors can block catalytic kinase activity. Here we outlined the different ERK5 inhibitors, their mechanism of action, off-target effects and specificity for MEK5 or ERK5.

Compound	Target	Mechanism of action	MEK5/ERK5 specificity IC_{50}	Off target IC_{50}	Reference
BIX02189	MEK5	Indolinone kinase inhibitor	1.5/59nM	TGFβR1 580nM	[148]
XMD8-92	ERK5	ATP-competitive inhibitor	80nM	BRD4 20nM	N/A
AX15836	ERK5	ATP-competitive inhibitor	8nM	BRD4 3600nM	[132]
JWG-045	ERK5	N/A	20nM	BRD4 2200nM	[120]

1.2 ERK5 and Cancer

1.2.1 MAPKs Cancer

Cancer represents the alteration from normal cellular homeostasis to neoplasia. In the mid-nineteenth century, the German pathologist Rudolf Virchow would comprehensively describe the condition of neoplasia – a new, abnormal and distorted growth, a word which would resonate through the history of cancer [47]. Since then, cancer has been extensively characterised with the advent of technologies which have revealed the truly heterogeneous nature of this disease [48]. Oncologists and academics no longer search for a magic bullet for all cancers, but specific biomarkers which can predict efficacy of treatment for specific subtypes of the disease.

Such understanding has led to the rational design of highly effective treatments which include small molecules targeting mutated proteins, development of antibodies which can target tumours for destruction and combinatorial therapies which target multiple oncogenic nodes [49] [50] [51]. The role of MAPKs, in particular, alterations in the ERK1/2 pathway, feature prominently in the formation of cancer. Notable alterations in the ERK1/2 pathway include mutations in RAS and RAF which are present in 90% of pancreatic cancers and 66% of melanomas respectively (in addition to multiple other cancers) [52]. One

of the most successful targeted therapies, Vemurafenib, targets mutated BRAF and subsequently ERK1/2 activation, leading to suppression of melanoma [50]. Evidence has recently emerged indicating the potential relevance of the ERK5 signalling pathway in cancerous growth.

1.2.2 ERK5 and breast cancer

Clinical data has demonstrated that ERK5 expression is upregulated in breast cancer and this poorly correlated with prognosis (disease-free survival) [53] [54]. Furthermore, numerous papers implicate ERK5 in breast cancer proliferation [55] [56], metastasis [55] [57] [58] and chemotherapeutic resistance [59] [54]. Together, these findings provide a premise to further explore the relationship between ERK5 and tumour development in breast cancer. Here, I will summarise the field of breast cancer, the molecular basis of different breast cancer subtypes and treatment options before exploring the relationship between ERK5 and breast cancer in more detail.

1.2.3 Breast cancer

Globally, breast cancer is the most common cancer in women and is particularly prevalent in Europe and North America [60]. Breast cancer originates in the mammary gland and is the most common cancer in the UK [61]. Improved patient outcomes have been achieved by diagnosing cancer at the earliest stage when a tumour is resectable and curative measures can be directed to a specific site in the body. The development of more efficacious treatments has also reduced breast cancer mortality rates [62]. In particular, advancements in molecular and genomic techniques have allowed a more personalised approach to diagnosis and treatment. Central to the development of these treatments has been to understand breast cancer as a heterogeneous disease which can be classified by pathological, genomic and molecular markers.

1.2.4 Breast cancer classification

Breast cancer can be classified in multiple ways. For instance, a pathological classification can describe breast cancer as non-invasive or invasive. Non-invasive cancers are sometimes described as *in situ* (in the original location). In contrast, invasive breast cancer has the potential to spread beyond the original location, either within the breast or to other parts of the body. Breast cancers can also be categorised by the classical TNM (tumour, node, metastasis) staging and grading (0-IV) systems. Contemporary high throughput approaches have been able to classify into different molecular subgroups, which can predict prognoses and response to specific therapies. In the early 2000s, two pioneering studies by Perou *et al*. and Sorlie *et al*. were able to distinguish five major molecular subsets of breast cancer based on gene

expression clustering [63] [64]. They discovered that these subtypes could be distinguished by the presence or absence of three important receptors: Oestrogen receptor (ER), progesterone receptor (PR) and the human epidermal growth factor 2 (HER2). The Luminal A subtype is the most common subset of patients and has the best prognosis. These breast cancers tend to be oestrogen receptor positive (but can also be progesterone positive) and HER2 negative [65]. Luminal B subtype is distinct from the luminal A subgroup as it is HER2 positive, which contributes to comparative inferior prognoses. The overexpression of the HER2 receptor defines the HER2 subtype; this subtype has a poor prognosis in the absence of treatment. Finally, the triple negative subtype is characterised by the absence of all three receptors.

Table 1.1 Major molecular subtypes of breast cancer identified by gene expression profiling. Abbreviations: ER, oestrogen; PR, progesterone; HER2, human epidermal growth factor receptor 2. [65]

Intrinsic subtype	IHC status	Relative Prognosis	Approximate Prevalence
Luminal A	ER+ and/or PR+/HER2-/Ki67 -	Good	40%
Luminal B	ER+ and/or PR+/HER2+/Ki67+	Intermediate	20%
HER2 overexpressing	ER-/PR-/HER2+	Poor	20%
Basal	ER-/PR-/HER2-	Poor	15%
Normal-like	ER+/PR+/HER2-/Ki67-	Intermediate	5%

Stratification of breast cancer patients based on molecular subtypes rather than other clinicopathological variables (stage, grade, tumour size) has led to a paradigm shift in breast cancer treatment and management. More recent studies such as METABRIC [66] have further revealed stratifications of breast cancer into 10 distinct subtypes by an integrated analysis of copy number alterations and gene expression analysis in 2,000 primary tumours. Collectively, research has allowed a deeper understanding of the heterogeneity of breast cancer and has accordingly changed clinical practice, specifically by allowing the design of tailored treatments for distinct breast cancer subtypes.

1.2.5 Breast cancer treatment

The treatment of breast cancer typically depends on various clinicopathological variables (size, location, tumour size, nodal status and patient's age) in addition to molecular subtype. If the cancer is diagnosed at an early stage, surgical resection is often the most effective therapeutic intervention. Specific medications

are used before or after surgery to reduce tumour size or risk of reoccurrence. These medications, described as adjuvants, can include hormonal therapies, chemotherapy, radiotherapy or targeted therapies. If cancer has spread beyond the original site (i.e. secondary/advanced breast cancer), surgery may not be feasible, and chemotherapy plus hormonal or biologic adjuvants will be used to extend the patient's life. Here I will briefly summarise the different forms of breast cancer treatment.

1.2.6 Hormonal therapy

In the late nineteenth century, it was recognised that breast cancer was often associated with ovarian function and that removal of the ovaries could suppress the growth of metastatic breast cancer [67]. Since then, hormonal manipulation has been a mainstay in the treatment of hormone-dependent breast cancers. Hormonal treatments can include selective oestrogen receptor modulators (SERMs), aromatase inhibitors or oestrogen receptor antagonists. A major advance in the field of oestrogen therapy occurred in the 1970s with the introduction of the first SERM, Tamoxifen. SERMs act by competing with oestrogen for binding to the oestrogen receptor, thus blocking the expression of oestrogen responsive genes driving cancerous growth. Tamoxifen is classified as a SERM rather than an oestrogen receptor antagonist as it can act as an agonist in specific tissues. Tamoxifen is often used as an adjuvant to chemotherapy and/or surgery and has been found to reduce breast cancer reoccurrence significantly [68]. This drug has been the standard endocrine treatment for breast cancer for many years and remains one of the most commonly used and effective treatments. Aromatase inhibitors, a more recent addition to the endocrine agents, operate by blocking the enzymatic conversion of androgens to oestrogens. Aromatase inhibitors currently used in the clinic include Anastrozole and Letrozole [69] [70]. Finally, oestrogen receptor antagonists are agents that exclusively block the oestrogen receptor and do not have any agonistic effects. A notable example is Fulvestrant [71]. Given the fact that most breast cancers are ER+ it is of critical importance to have an array of effective drugs against this subset of breast cancers.

1.2.7 Chemotherapy and Radiotherapy

Before the 1970s, surgery and radiotherapy were the main treatment options for women with breast cancer. The late nineteenth century ushered in the modern era for surgical treatment in breast cancer despite limited understanding of the disease. William Halsted pioneered a surgical procedure now known as a "radical mastectomy", wherein the breast, the breast tissue, the pectoralis muscles and all the axillary lymph nodes were removed [72] [73]. Since then there has been a considerable advancement in surgical techniques, and the extent of invasive mastectomies has been greatly reduced. The discovery of the X-ray in 1895 led to the development of postoperative adjuvant radiotherapy, which has proved to reduce breast

cancer reoccurrence significantly [74]. Clinicians now combine precision surgery with regional radiotherapy to kill cancer cells with high energy X-rays, protons or other particles.

Sidney Farber is typically considered the father of modern chemotherapy due to his discovery that anti folates could achieve temporary remissions in childhood leukaemia in the 1940s [75]. We have since understood that cancer is a disease characterised by uncontrolled proliferation of cells which rely on DNA synthesis, replication and subsequent cell division. Chemotherapeutic agents essentially interfere with the process of normal cell division in multiple ways and are therefore cytotoxic to both normal and cancer cells. Based on this understanding, a chemotherapeutic armoury of drugs has been synthesised over the years and used as systemic treatments to kill rapidly dividing cancer cells. Over the course of the twentieth-century specific therapeutic regimens were designed to increase the probability of durable remissions. Chemotherapy is often employed to treat breast cancer before (neoadjuvant) or after (adjuvant) surgery or in combination with targeted therapies.

1.2.8 Targeted therapies

Despite the relative success of adjuvant chemotherapy and radiotherapy, these types of systemic treatments are considered to lack precision based on the fact that they target both normal and cancer cells. In accordance with our increased understanding of the molecular nature of cancer, academics and clinicians have been able to identify specific molecular targets which are distinct in the cancer cell. The development of these types of therapies is known as targeted or personalised therapy as the treatment is tailored to a specific molecular signature/s in cancer.

Ideal molecular targets often involve targeting a protein which is altered in cancer (mutation and/or expression) and is responsible for driving uncontrolled proliferation. Notable examples include BCR-Abl fusion in chronic myelogenous leukaemia [76], BRAF mutation in melanoma [50] and EGFR mutations in non-small cell lung carcinoma [77]. The treatment of breast cancer also includes an early success story in the field of targeted therapy, with the FDA approval of the monoclonal antibody trastuzumab in 1998 [78]. This drug targets an extracellular epitope on HER2, which is overexpressed in approximately 20% of human breast cancers. Overexpression of HER2, primarily due to gene amplification, leads to constitutive kinase activation and subsequent oncogenic signalling downstream of this tyrosine kinase receptor. Previously this subtype of breast cancer had a particularly poor prognosis before the approval of trastuzumab which has since significantly improved patient outcomes [78]. Trastuzumab is proposed to block HER2 signalling in a number of ways [79]. The most well-known effect of trastuzumab is via the inhibition of the ERK 1/2 and PI3K/Akt pathways downstream of the HER2 receptor [80] [81]. By

binding to the extracellular domain of the HER2 receptor, trastuzumab blocks dimerisation of HER2 and subsequently interferes with trans and autophosphorylation of the receptor, thereby abrogating downstream signalling. Another proposed mechanism involves the internalisation and degradation of HER2 upon trastuzumab binding [82]. Finally, trastuzumab targets HER2 overexpressing cells for destruction by antibody-dependent cellular cytotoxicity (ADCC) [83]. Since the success of trastuzumab, other drugs have been synthesised to block HER2 signalling via different mechanisms. Currently, three HER2-targeted agents, trastuzumab, lapatinib and pertuzumab are available for the treatment of HER2 positive breast cancer. Lapatinib is a tyrosine kinase inhibitor of the HER2 receptor and is often used in patients exhibiting resistance to trastuzumab treatment. Lapatinib gained FDA approval in 2007 based on improvements in patient outcomes compared to the standard of care [84]. The most recent addition to the HER2 directed agents is pertuzumab which gained accelerated approval from the FDA in 2013 after significant improvement in patient outcomes when combined with trastuzumab and docetaxel (vs trastuzumab and docetaxel alone) [85]. Like trastuzumab, pertuzumab binds to an extracellular domain of HER2, but at a different epitope, blocking *ligand-induced* dimerisation of HER2 with other members of the EGFR family (**Fig 1.5**) [86] This is in contrast to trastuzumab which blocks *ligand independent* HER2 dimerisation. The combination is proposed to block HER2 signalling completely.

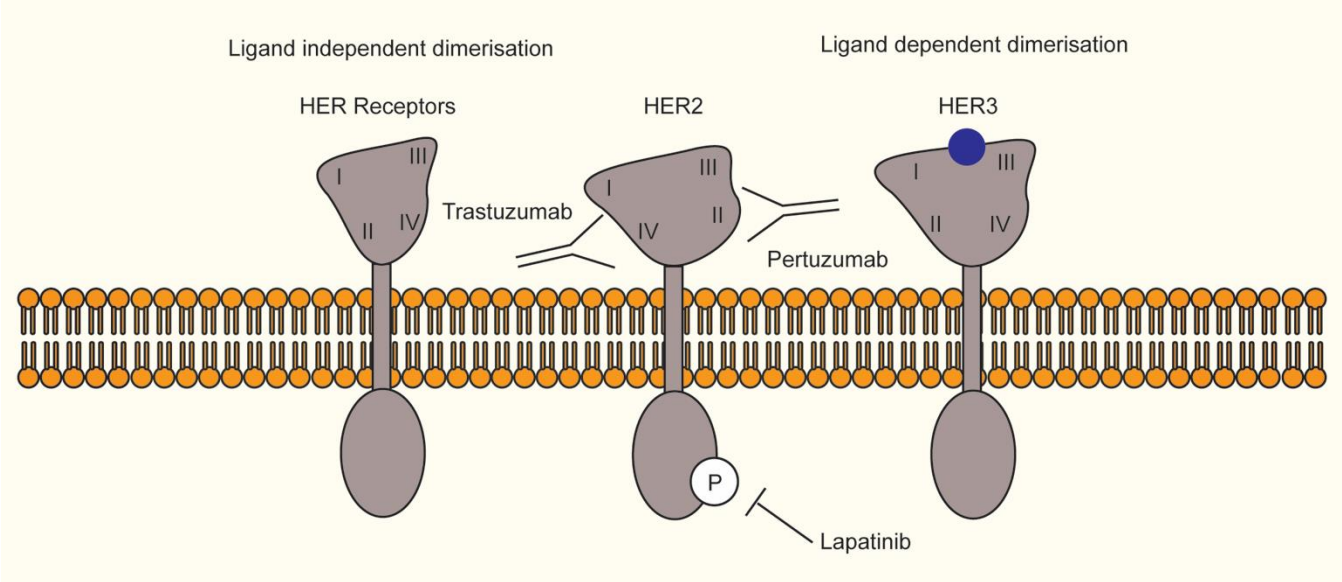

Figure 1.5 Modes of HER2-targeted agents. Trastuzumab binds to domain IV of HER2, preventing constitutive activation of HER2 by blocking ligand independent dimerization, promotes HER2 internalisation and degradation in addition to promoting ADCC. Pertuzumab binds to domain II of HER2 and prevents Heregulin (HRG) mediated HER2/3 dimerisation and signalling. Lapatinib inhibits the tyrosine kinase acitivity of HER2 at the intracellular face of the receptor.

1.2.9 Resistance

Despite advances in early detection and the development of efficacious treatments, resistance to therapy is the most significant clinical obstacle for curing breast cancer. This is highlighted by the fact that approximately 30% of patients with early-stage breast cancer develop resistance, commonly leading to a more aggressive form of the disease [87]. Modes of resistance can be split into two separate categories; intrinsic or acquired. A tumour which has a pre-existing resistance to treatment has *intrinsic* resistance, whereas a tumour which develops resistance over the course of treatment has *acquired* resistance. Resistance mechanisms will also be distinct depending on the breast cancer subtype.

For example, less than 35% of patients with HER2 positive breast cancer initially respond to trastuzumab [88], and 70% of patients who initially respond progress to metastatic disease within a year [89]. Mechanisms of resistance to trastuzumab include steric changes, elevated activation of alternate receptors or activation of signalling pathways that can compensate for the loss of HER2 signalling. Steric changes can include the expression of a truncated p95 isoform, which retains constitutive kinase activity but inhibits the binding of trastuzumab to the extracellular epitope [90]. Elevated activation of alternate receptors include examples such as the Insulin-like Growth Factor Receptor (IGFR) [91] and c-Met [92]. Notable alterations in intracellular signalling pathways include loss of Phosphatase and Tensin homolog (PTEN) and/or PI3K/Akt constitutive activation [93].

1.3 ERK5, HER2 overexpressing cancer cells and resistance

1.3.1 HER2 signalling and ERK5

In 1987 a group of researchers identified that amplification of the *ERBB2* gene (encoding for the HER2 protein) poorly correlated with relapse and survival in breast cancer patients [94]. The last two decades have led to the extensive characterisation of the role of HER2 as an oncogene and the mechanisms underlying HER2 mediated tumourigenesis. Overexpression occurs as a consequence of gene amplification or transcriptional deregulation, leading to a 40-100 fold increase in HER2 protein expression at the cell surface [95] [96]. This leads to constitutive activation of the EGFR family receptors through auto and transphosphorylation at the intracellular domains. Phosphorylated tyrosine residues dock with numerous intracellular signalling molecules, activate downstream signalling cascades, including the ERK 1/2 and PI3K/Akt pathways (**Fig 1.6**). ERK 1/2 signalling contributes to HER2-mediated tumourigenesis by decreasing p27 stability, thereby promoting G1/S cell cycle transition and proliferation [97]. The PI3K/Akt pathway has also been demonstrated to be important in promoting proliferation, survival and invasion [98].

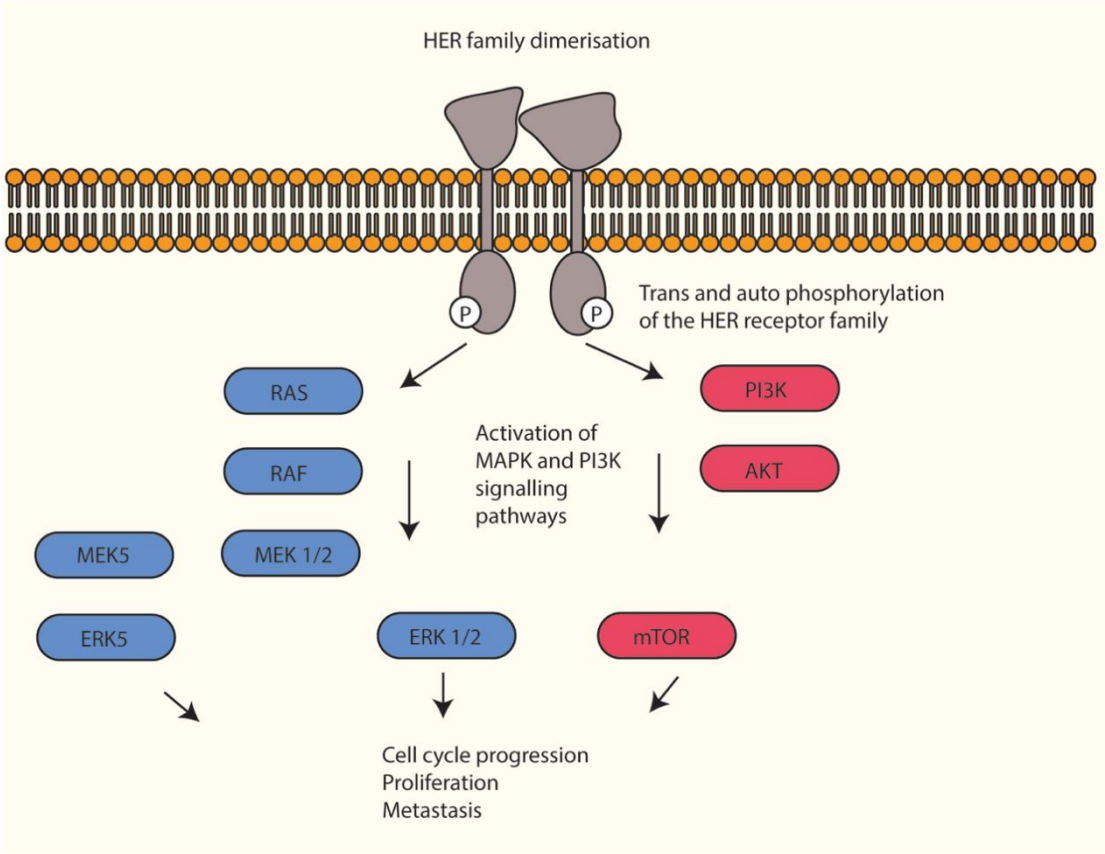

Figure 1.6 HER2 signalling. The HER2 receptor homodimerizes or heterodimerizes with HER2 or HER1/3/4 receptors leading to auto or trans phosphorylation respectively. Constitutive activation of the receptors intitiates downstream signalling cascades including the ERK 1/2, ERK5 and PI3K/Akt pathway leading to tumourigeneis by promoting cell cycle progression, proliferation and metastasis.

In 2002, Esparis-Ogando *et al.* found that the ERK5 pathway was constitutively active in HER2 overexpressing cell lines [99]. ERK5 exhibited a retarded migratory pattern identified by western blotting, indicative of phosphorylation at the N and C terminus of ERK5. ERK5 was also shown to predominantly reside in the nuclei of the HER2 overexpressing breast cancer cell lines, consistent with studies previously looking at ERK5 nucleo-cytoplasmic shuttling upon phosphorylation. They demonstrated the HER2 receptor activated ERK5 by transfecting *ERBB2* cDNA into MCF7 cells which resulted in constitutive activation of ERK5. Furthermore, it was shown that ERK5 participated in serum- induced proliferation of HER2 overexpressing cells. This study was supported by a later study in 2009 which investigated the role of ERK5 in breast cancer *in vivo* [53]. They first demonstrate that tumours resected from a mouse model of HER2 overexpressing breast cancer exhibited increased amounts of phosphorylated ERK5 [100]. Furthermore, they observed that high expression of ERK5 was significantly associated with inferior DFS compared to patients with low expression. The negative impact of ERK5 overexpression on prognoses led the researchers to study how ERK5 regulated the response to treatment. They found that introducing

a dominant negative form of ERK5 facilitated the treatment of trastuzumab, indicating that inhibition of ERK5 could increase the efficacy of HER-2 directed agents. Collectively this data indicated that ERK5 played an important role in HER2 breast cancer and could potentiate the efficacy of HER2-directed agents or help revert HER2 drug resistance [100].

1.3.2 ERK5 and therapeutic resistance

Since the study by Ogando *et al*., several authors have identified a role for ERK5 in therapeutic resistance in breast cancer. Miranda *et al*. demonstrated that TNBC patients cancer with higher expression of ERK5 had inferior rates of relapse following systemic treatments [59]. The link between ERK5 and response to therapy was further substantiated by Ortiz *et al.* who demonstrated that ERK5 inhibition potentiated the efficacy of chemotherapeutic agents in a TNBC cell line (MDA-MB-231) [54]. However, this study was confounded by the fact that the ERK5 inhibitor utilised, TG02, is a multi-kinase inhibitor and is not specific for ERK5.

Further studies implicate ERK5 in the development of resistance in other cancers, such as colorectal cancer and melanoma. A study by Petrus R. de Jong *et al.* suggested ERK 1/2 and ERK5 were interconnected and furthermore, ERK5 signalling was a putative resistance pathway in the context of MEK 1/2 or ERK 1/2 inhibition [101]. The interconnected nature of the ERK 1/2 and ERK5 pathways in the context of resistance is further explored in a study by Tusa *et al.* [102]. Again, this study highlighted the potential of targeting ERK5 as a way to prevent resistance to BRAF / MEK 1/2 inhibition, this time in the context of melanoma. They observed that oncogenic BRAF enhanced ERK5 kinase and transcriptional activities, which subsequently promoted oncogenic growth. Accordingly, combined BRAF and ERK5 inhibition was more effective than the use of a single agent in reducing melanoma growth. An unbiased phosphoproteomic screen also identified ERK5 as a critical driver of proliferation in melanoma cells resistant to treatment [103]. ERK5 was found to have upregulated phosphorylation as a consequence of acquired resistance to ERK1/2 pathway inhibition. Furthermore, a siRNA screen identified ERK5 knockdown having the most significant ability to inhibit proliferation of cells which developed resistance to combined BRAF and MEK 1/2 inhibition [103]. Collectively, these studies provide strong evidence that the ERK5 pathway is involved in the development of resistance, particularly in the context of ERK 1/2 inhibition.

1.4 Aims of the PhD

The aims of my PhD can be explained in two parts. The first part related to understanding how C terminal phosphorylation enhanced ERK5-mediated transcription. The C terminus is a unique feature of ERK5 amongst the MAPK family, and I believe studying this feature will be crucial to understanding its distinct functions in the context of development and disease. For this purpose, I engineered a number of phospho-mimetic mutants that were tested for their ability to control ERK5-mediated transcription using a MEF2 reporter. In parallel, I demonstrated the importance of post-translational modification at the C terminus by utilising two high throughput approaches. Firstly, I developed a Chromatin immunoprecipitation followed by sequencing (ChIPSeq) protocol and proteomic analysis of the interactome upon C terminal modification.

The second part focused on establishing the oncogenic function of ERK5 in the context of HER2+ breast cancer. ERK5 is constitutively phosphorylated in HER2+ breast cancer, implying that ERK5 may be part of oncogenic signalling in this subtype. Therefore to assess its role in oncogenic growth, I employed various ERK5 inhibitors in breast cancer cell lines overexpressing HER2 *in vitro.* I next investigated the role of ERK5 in the development of resistance in HER2+ breast cancer. The biological premise for this investigation is the accumulating evidence for the role of ERK5 in the development of therapeutic resistance (particularly in the context of ERK 1/2 inhibition) and the inverse correlation between ERK5 expression and relapse rates post-treatment in HER2+ breast cancer. I achieved this by utilising HER2 lines resistant to therapy and testing whether ERK5 inhibition could facilitate the action of HER2 directed agents.

Therefore my overall aim of my PhD is to reveal the mechanisms underlying ERK5 mediated transcription and its relevance in HER2+ breast cancer.

My summarised aims are:

- Understand how the C terminus enhances ERK5-mediated transcription
- Establishing the oncogenic function of ERK5 in the context of HER2+ breast cancer
- Exploring the role of ERK5 in the development of resistance in HER2+ breast cancer

2. Materials and Methods

2.1 Cell culture

Flp-In™ HeLa cells were cultured in Dulbecco's Modified Eagles Medium (DMEM, Sigma-Aldrich®). at 37^0C, 5% CO2, containing 10% Foetal calf serum (FCS, Gibco, Paisley, UK), and 1% Penicillin/Streptomcycin (100 μg/ml) (Sigma-Aldrich®). Breast cancer lines: BT-474, MDA-MB-231, SKBR3, MCF-7, MDA-MB-453 were cultured in media with the same additives. Cells were split at approximately 80% confluency using Hank's Balanced Salt Solution (HBSS) (Sigma-Aldrich®) and Trypsin (Sigma-Aldrich®). Cells were counted using a hemocytometer and Tryphan blue stain. Cell lines were preserved using freezing media (FBS +10% Dimethyl sulfoxide -DMSO) (Sigma-Aldrich®) and these cells were used as stock (approximately $5x10^6$ per ml) and stored at -80^0C. The Flp-In™ parental HeLa cell line was used as the host cell line and were given to me by Dr Anthony Tighe (Stephen Taylor Lab) (University of Manchester, Michael Smith Building). The BT-474, MDA-MB-231, SKBR3 and MCF-7 lines were kindly given to me by Dr Hannah Harrison and Henry Pegg (Shore Lab) (University of Manchester, Michael Smith Building).

2.2 ERK5 constructs and phosphomimetics

ERK5 constructs were generated by PCR amplification and were subcloned into the Flp-In™ expression plasmid by digestion with BamHI and Not1. The Flp-In™ expression plasmid had been modified to contain a 3xFlag-Tag sequence and a 5' lamin UTR.

The components of the PCR reaction, cycling conditions, primer sequences for the constructs are shown (**Tables 2.1, 2.2 and 2.5**). Pfu DNA polymerase was used with standard buffer. The fragments were ligated using the T4 DNA ligase (New England Biolabs®) The constructs were sequenced by the DNA sequencing facilities at the University of Manchester. The primers used for sequencing are illustrated (**Table 2.7**). The various ERK5 phospho-mimetics were generated by site-directed mutagenesis (QuikChange, Agilent technologies). The components of the mutagenesis reaction, cycling conditions, primer sequences for the constructs are shown (**Tables 2.3, 2.4 and 2.6**) The specific serine and threonine residues were mutated to alanine or glutamic acid using mutagenic primers designed using Quikchange primer design tool found online (**Table 2.6**).

Table 2.1 Components and volumes required for single PCR reaction

Reaction mix	Final concentration
DNA template	100 ng
Forward Primer	0.5 μM
Reverse Primer	0.5 μM
dNTP mix	200 μM
Pfu HF dna polymerase	1 μl
H_20	-
Total volume	40 μl

Table 2.2 PCR cycling conditions

Phase	Temperature (^oC)	Time (seconds)
Initial denaturation	94	90
Denaturation	94	60
Annealing	63	60
Extension	72	60
Repeat 30x	-	-
Final extension	72	600
Finish	4	Short term storage

Table 2.3 Components and volumes required for single mutageneisis reaction

Reaction mix	Final concentration
DNA template	50 ng
Forward Primer (s)	125ng
dNTP mix	1 μl
Pfu HF dna polymerase	1 μl
H_20	-
Total volume	50 μl

Table 2.4 Mutagenesis cycling conditions

Phase	Temperature (^oC)	Time (seconds)
Initial denaturation	95	30
Denaturation	95	30
Annealing	55	60
Extension	68	60/kb of plasmid
Repeat 30x	-	-
Finish	4	Short term storage

Table 2.5 Primer sequences to amplify ERK5 mutants. All primer sequences contain an overhang sequence and the relevant restriction site for subcloning into the expression plasmid. Fw – BamHI / Rv – Not1

ERK5 construct	Forward/ Reverse Primer	Sequence of primer (Overhang/Restriction site/Annealing sequence)	Length of primer (bps)
FL	Fw	GAGA GGATCC ATGGCCGAGCCTCTG	25
	Rv	GAGA GCGGCCGC TCAGGGGTCCTGGAG	27
N1725	Fw	GAGA GGATCC ATGGCCGAGCCTCTG	25
	Rv	GAGA GCGGCCGC GGCCATTCGAGTCCA	27
N1509	Fw	GAGA GGATCC ATGGCCGAGCCTCTG	25
	Rv	GAGA GCGGCCGC AGCCACAGGCTG	24
N1224	Fw	GAGA GGATCC ATGGCCGAGCCTCTG	25
	Rv	GAGA GCGGCCGC AGGCTCAGGAGC	24
C1215	Fw	GAGA GGATCC GGCTGTCCAGAT	22
	Rv	GAGA GCGGCCGC TCAGGGGTCCTGGAG	27

Table 2.6 Mutagenic primers

ERK5 phospho-mimetic	Forward/ Reverse Primer	Sequence of primer (Overhang/Restriction site/Annealing sequence)	Length of primer (bps)
4xA_i (Ser 706)	Fw	CAGCAAGCAGGGCGGCTGCGAGAGAGGCTGA	31
4x$A_{i/ii}$ (Thr732)	Fw	CCTGTGTTCTCAGGCGCACCAAAGGGCAGTG	31
4xA_i (Ser 753)	Fw	AGGAATTCTTAAACCAGGCTTTCGACATGGGCGTG	35
4xA_i (Ser 773)	Fw	GATTCAGCCTCTCTCGCAGCCTCCCTGCTTG	31
4xA_{ii} (Ser 769)	Fw	CAGGATGGCCAGGCAGATGCAGCCTCTCT	29
4xA_{ii} (Ser 773+775)	Fw	TCAGCCTCTCTCGCAGCCGCCCTGCTTGCTG	31
4xE_i (Ser 706)	Fw	GTCAGCAAGCAGGGAGGCCTCGAGAGAGGCTGAATCTGC	39
4xE_i (Ser 753)	Fw	CTGGAGGAATTCTTAAACCAGGAGTTCGACATGGGCGTGGCTG AT	39
4x$E_{i/ii}$ (Thr732)	Fw	CCCTGTGTTCTCAGGCGAGCCAAAGGGCAGTGGGG	35
4xE_i (Ser 753)	Fw	CTGGAGGAATTCTTAAACCAGGAGTTCGACATGGGCGTGGCTG AT	45
4xE_i (Ser 773)	Fw	GCAGATTCAGCCTCTCTCGAGGCCTC CCTGCTTGCTGAC	39
4xE_{ii} (Ser 769)	Fw	CCCCACTGCCCTTTGGCTCGCCTGAGAACACAGGG	35
4xE_{ii} (Ser 773+775)	Fw	GGTCACAAGGTTGACATCAGGCTCCTCTGACATGGAAGACTGA GG	45
D200A	Fw	CACGAGCCATACCAAAGGCACCAATCTTGAGCTCA	35

Table 2.7 ERK5 sequencing primers. Forward primers for the CMV promoter and reverse primer for the BGH region of the plasmid which lie outside the gene of interest (relevant ERK5 mutant).

ERK5 sequencing primers	Forward/ Reverse Primer	Sequence of primer	Length of primer (bps)
CMV primer	Fw	CGCAAATGGGCGGTAGGCGTG	21
600 bp	Fw	TGGTATGGCTCGTGG	15
1200 bp	Fw	CCAGCCTTCTCTACA	15
1800 bp	Rv	TGGATGGTCAGGAGG	15
Reverse BGH	Rv	TAGAAGGCACAGTCGAGG	18
Transactivation domain primer	Fw	CACCCCAGATTGCCACCTC	19

2.3 Agarose gel electrophoresis

Gel electrophoresis was conducted on 1% agarose gels with 5 μl/100 ml ethidium bromide (Sigma-Aldrich® 10 mg/ml) and run at 130V in TAE buffer (40 mM Tris, 20 mM acetic acid, and 1mM EDTA) with a loading dye. A hyperladder 1kb ladder was used to estimate the molecular weight of double stranded DNA. DNA was visualised using a UV transilluminator, extracted and purified using the QIAquick® gel extraction kit (Qiagen).

2.4 Preparation and transformation of bacteria

Competent DH5α strain of *E.coli* were generated using the calcium chloride transformation method and selected for using Ampicillin plates (100 μg/ml). 100 ng- 500 ng of plasmid DNA / 5 μl of ligation mix was added to 100 μl aliquots of *E.coli* and left on ice for 20 minutes. *E.coli* were then heatshocked for 2 minutes at 42°C followed by a period on ice for 2 minutes. 800 μl of Luria-Bertani (LB) broth was added to the E.coli which was then spun at 3000 rpm for 1 minute before extraction of 600 μl and resuspension in the remaining 200 μl. The remaining 200 μl was spread on ampicillin plates and left in the incubator at 37°C overnight.

2.5 Plasmid extraction and purification

Plasmid extraction and purification was completed using the Qiagen Maxi/Mini prep kit according to the manufacturer's protocol. The Maxiprep produced an approximate DNA yield of 1-2.5 μg/μl whilst the Miniprep produced 80-250 ng/μl. DNA concentration was measured using a Nanodrop spectrophotometer and purified DNA was reconstituted to 1 μg/μl with Elution Buffer (EB) (Qiagen).

2.6 Transfection and cellular stimulation

$2x10^5$ cells were seeded in a 6 well plate and were ready for transfection (60-80% confluent) the following day. The jetPEI™ kit according to the manufacturer's protocol. Expression of the ERK5 mutants were induced using 1 μg/ml Tetracycline 16-24 hours before cells were harvested, unless indicated otherwise. Stable cells were selected for using 2 mg/ml Hygromycin B (Invitrogen™ Life Technologies). Where indicated 20 ng/ml EGF was used to stimulate cells 10 or 25 minutes before harvesting.

2.7 Preparation of cell lysates

Cells were washed once with ice cold 1 x Krebs-Ringer HEPES buffered saline (KRH) (125 mM NaCl, 5 mM KCL, 5 mM $MgSO_4$, 1.25 mM $CaCl_2$, 50 mM HEPES pH 7.4) and then placed on ice. Triton Lysis Buffer (TLB) (Tris pH 7.4 4 mM, NaCL 27 mM, EDTA pH 7.4 mM, Triton X-100 400 μM , Glycerol 2%) containing phosphatase/protease inhibitors (Na_3VO_4 1 mM, Beta-glycerophopshate 5 mM, PMSF 1 mM, Aprotonin 10 μg/ml, Leupeptin 10 μg/ml). Volume dispensed on cells depended on cell number but typically 150 μl was used. The lysis reaction was then allowed to proceed for 5 minutes at room temperature before the plates were scraped and lysates were transferred to Eppendorfs and centrifuged for 13,200 rpm for 15 minutes at 4°C. Supernatants were stored at -80°C.

2.8 Protein assay

Protein was quantified using the Bio-Rad DC™ protein assay kit according to the manufacturer's protocol. Samples were typically diluted 1:5 and incubated for 15 minutes after which time the OD_{750} of the samples was measured using a spectrophotometer. A standard curve from bovine serum albumin (BSA) was used to calculate the protein concentrations of the samples.

2.9 Western blotting

2.9.1 SDS-PAGE

Gels for electrophoresis were prepared as follows:

Table 2.8 Resolving gel composition for 7 and 8% gels.

	Percentage of gels	
Reagent	7%	8%
Water	18.45 ml	17.25 ml
1.5 M Tris pH 8.8	9 ml	
Protogel 30%	8.4 ml	9.6 ml
APS 30%	140 μl	
TEMED	14 μl	

Table 2.9 Stacking gel composition

Reagent	Volume
Water	12 ml
0.5 M Tris pH 6.8	5 ml
Protogel 30%	3 ml
APS 30%	200 μl
TEMED	20 μl

The resolving gel was prepared first and poured into a Hoefer Mighty Small™ gel caster overlayed with water saturated isopropanol. The gels were left until polymerisation had occurred. The stacking gel was then prepared and added, combs inserted and the gels left to polymerise. Gels were stored hydrated at 4°C in cling film.

When required for electrophoresis the comb was removed and the gel was washed several times before being attached to the electrophoresis apparatus. The apparatus was filled with running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, 0.03% bromophenol blue). Laemmli buffer (1% SDS, 30 mM Tris-HCL pH 6.8, 2.5% β mercaptoethanol, 10% glycerol) was added to the protein samples and the samples were subsequently heated at 95°C for 10 minutes. Electrophoresis was carried out at 30 mA/gel until the dye reached the bottom of the gel.

2.9.2 Semi-dry immunoblotting

Polyvinylidene difluoride (PVDF) membrane and 4 pieces of blotting paper were cut slightly larger than the gels, the membrane was immersed in methanol for activation. The blot was assembled on the transfer apparatus (Hoefer semiphor transfer units) as follows: 2 pieces of blotting paper, PVDF membrane, electrophoresis gel, 2 pieces of blotting paper and the transfer was carried out for 2 hours at 15v in transfer buffer (Running buffer containing 20% Methanol). Once the transfer was complete the blot was disassembled and the PVDF membrane placed in blocking agent (dried milk diluted 3% with Tris buffered saline and tween – 15 mM Tris, 150 mM NaCl, 0.1% Tween-20) at room temperature for 1 hour. Finally, the membrane was incubated with primary antibody (diluted in blocking agent) overnight at 4°C on a rocking platform. The following day the membrane was washed 3x 15 minutes in TBS-T followed by incubation with secondary antibody (diluted in blocking agent) for 1 hour at room temperature on a rocking platform. The membrane was then washed 3x 15 minutes with TBS-T before application of the appropriate Enhanced Chemiluminescence (ECL) reagent; SuperSignal™ West Femto Maximum Sensitivity Substrate and SuperSignal™ West Pico Chemiluminescent Substrate. The ECL signal was visualilised by exposing autoradiography.

2.10 Antibodies

Table 2.10 Various primary and secondary antibodies used for experiments.

Antibody	Source	Primary /Secondary	Company	Dilution
M2 Antibody	Mouse	Primary	Sigma-Aldrich®	1:2000
Anti ERK5	Rabbit	Primary	Cell Signalling Technology ®	1:1000
Anti β Actin	Mouse	Primary	Sigma-Aldrich®	
Anti Mouse	Sheep	Secondary	GE Healthcare	1:10000
Anti Rabbit	Donkey	Secondary	GE Healthcare	1:10000
Phallodin (Anti mouse)	-	Secondary	Cell Signalling Technology ®	1:2000

Additional antibodies used in each chapter are specified in the individual methods sections.

2.12 Immunofluorescence

Please refer to page 44 for details on the immunofluorescence protocol

2.13 ChIP assay

ChIP_assays were performed according to a published method from the Jason Carroll Lab, Cambridge University with modifications. A protease inhibitor cocktail (cOmplete, EDTA free, Roche) was used to prevent protease degradation. The recipes for the lysis buffers are as follows:

Table 2.11 Recipe for ChIP lysis buffers and

Lysis buffer 1	Lysis Buffer 2	Lysis buffer 3	RIPA buffer x6
50 mM Hepes-KOH, pH7.5 140 mM NaCl 1 mM EDTA 10% Glycerol 0.5% NP-4/Igepal CA-630 0.25% Triton X-100 Distilled water	10 mM Tris-HCL, pH8.0 200 mM NaCl 1 mM EDTA 0.5 mM EGTA Distiled water	10 mM Tris-HCL, pH8.0 100 mM NaCl 1 mM EDTA 0.5 mM EGTA 0.1% Na-Deoxcycholate 0.5% N-lauroylsarcosine Distilled water	50 mM Hepes pH7.6 1 mM EDTA 0.7% Na-Deoxcycholate 1% NP-40 0.5M LiCl

Approximately 1-1.5x10^7 cells were crosslinked using 3.7% paraformaldehyde for 10 minutes after indicated stimulation. Crosslinking was quenched with Glycine 100 mM before lysis. After successive lysis steps the nuclear extract was sonicated using a Bioruptor® (Diagenode) on a 30 second on/off cycle for 30 or 35 minutes using high intensity settings. Sonication was optimised to produce 100-500bp fragments. The input was removed before the cell lysate sample was added to the beads and left to rotate overnight at 4°C. Beads were washed with RIPA buffer before elution with Odom lab elution buffer (50 mM TrisHCL pH 8, 10 mM EDTA, 1% SDS). Samples were then placed in an oven at 65°C for 6 hours for reverse crosslinking. RNAase A (Roche) and Proteinase K (Roche) treatment was then completed before sending the samples off to be sequenced by the Genomics Technologies Core Facility (GTCF) at the University of Manchester.

2.14 Luciferase assay

Please refer to page 45 for details on the luciferase assay protocol

Chapter 3: Threonine 732 is a gatekeeper residue that regulates ERK5 function via its C terminal tail

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ABSTRACT

The extracellular signal-regulated protein kinase 5 (ERK5) is a distinct mitogen-activated protein kinase (MAPK) due to a unique C terminal extension which comprises a transactivation domain. This domain contains several Ser/Thr residues known to be autophosphorylated following upstream activation by MAPK/ERK kinase 5 (MEK5). In this study, we have sought to understand the significance of this unique feedback phosphorylation loop on ERK5 transcriptional activity. Our approach was based on site-directed mutagenesis to determine the impact of mimicking or impairing ERK5 phosphorylation in combination with high throughput analyses. We have identified that one of these sites, Thr^{732}, acted as a functional gatekeeper residue. Specifically, phosphorylation at Thr^{732} was required for enhancing ERK5-mediated transcription, partly through promoting nuclear localisation, but also by facilitating ERK5 recruitment to chromatin. Consistently, using a non-bias quantitative mass spectrometry approach, we found that the ERK5 Glu^{732} mutant exhibited increased affinity for Histone H3 compared to the full length wild-type protein. Overall, these results have provided a novel mechanism underpinning ERK5-mediated transcription via post-translational modifications of its C terminal tail.

INTRODUCTION

Mitogen-activated protein kinase (MAPK) pathways are evolutionarily conserved signalling cascades involved in the regulation of a variety of fundamental cellular processes such as cell proliferation, differentiation, survival and migration (reviewed in [1] [2] [3]). These pathways comprise a three-tier protein kinase cascade which results from the sequential activation of a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPK) and a MAPK, to relay, amplify and integrate many different stimuli, including mitogens, cytokines and various environmental stresses. MAPKs mediate responses to these extracellular cues mainly by regulation of gene expression, but also by post-translational mechanisms involving cytoplasmic targets [4] [5]. The best-known members of the MAPK family are the extracellular signal-regulated protein kinases (ERK) 1 and 2, the c-Jun amino-terminal protein kinases (JNK 1/2/3), and the p38 MAPKs (p38α/β/γ/δ). In contrast, ERK5 is much less understood despite compelling evidence of its functional importance during development and disease. ERK5 was first identified in 1995 by two independent research groups who immediately recognised the comparatively large size of this novel MAPK component [11] [12]. Specifically, ERK5 has an apparent molecular weight of approximately 102 kDa due to a unique C terminal extension, making it twice the molecular weight of conventional MAPKs. The C terminal tail of ERK5 contains two proline-rich domains (aa 434-464 and aa 578-701), a nuclear localisation signal (NLS) (aa 505-539) [19] and a transcriptional activation domain (aa 664-789) [22].

In quiescent cells, ERK5 exists in a "closed" folded conformational state defined by an intermolecular interaction between the N- and C-terminal half which constitutes the nuclear export signal (NES) [28]. In this conformation, the majority of ERK5 is sequestered in the cytoplasm [19]. The phosphorylation of the TEY motif in the N-terminal activation loop of ERK5 by MEK5 disrupts this intermolecular interaction which subsequently induces a conformational change to an "open" state and exposure of the NLS [28] [19]. In addition to controlling nucleo-cytoplasmic shuttling, the phosphorylation of ERK5 by MEK5 increases ERK5 catalytic activity, thereby enhancing transcription via the phosphorylation and activation of a variety of downstream transcription factors. More surprisingly, ERK5-mediated transcription also depends on autophosphorylation at various Ser and Thr residues present in the C terminal transactivation domain [9] [11]. This domain was initially identified by evidence that C terminal truncated ERK5 mutants exhibited impaired transcriptional activity [9]. Further demonstration that mimicking phosphorylation of the transactivation domain by substituting specific Ser/Thr for Glu residues enhanced ERK5-mediated transcription firmly confirmed the functional importance of post-translational modification of the C terminal tail [11]. The recent discovery that CDK1 phosphorylated ERK5 at Thr^{732} and Ser^{753} and that

ERK1/2 phosphorylated Thr^{732}, also raised the possibility that ERK5 transcriptional activity could be enhanced independently of MEK5 [39] [41].

Together, these observations have strengthened the idea that the C terminal of ERK5 is critical for mediating ERK5-dependent transcriptional regulation. This may be particularly significant in human epidermal growth factor receptor 2 (HER2) expressing breast cancer cells that appear to exhibit a form of ERK5 constitutively phosphorylated at the C terminus [99]. This is noteworthy considering that over-expression of ERK5 in this breast cancer subtype correlates with poor prognosis [53]. More recently, phosphorylation at Ser^{753} and Thr^{732} in the C terminus of ERK5 has been shown to promote melanoma growth downstream of oncogenic BRAFV600E [102]. Based on this knowledge we sought to elucidate the precise mechanism underlying ERK5-dependent transcription via post-translational modification of its C terminal tail.

RESULTS

Threonine 732 is critical for mediating C terminal phosphorylation of ERK5. Several studies have previously shown that upon phosphorylation of ERK5 by MEK5 at the TEY motif, ERK5 autophosphorylated multiple Ser and Thr residues in its C terminus [19] [39] [21]. Importantly, these results demonstrated that autophosphorylation was crucial for ERK5 to translocate to the nucleus and mediate gene transcription [11] [12]. At least seven putative autophosphorylation sites have been identified: Ser^{706}, Ser^{730}, Thr^{732}, Ser^{753}, Ser^{769}, Ser^{773}, and Ser^{775}. Thr^{732} and Ser^{753} residues are particularly interesting given that they have been shown to be targeted by other protein kinases, including CDK1 [39] and ERK1/2 [41] [104], thereby suggesting a functional regulation of ERK5 independent of MEK5. To address the biological significance of this unique mechanism, we generated various ERK5 mutants using a site- directed mutagenesis approach (**Suppl Table 1**). cDNAs encoding Flag epitope-tagged human ERK5 full length (ERK5-FL), truncated and phospho-mutants, were subsequently integrated into the genome of HeLa cells using the Flp-In T-Rex system to create isogenic inducible stable recombinant cell lines (**Fig. 1A**). ERK5 expression was induced by incubating the cells with tetracycline (**Fig. 1B**). We utilised HeLa cells as ERK5 had been previously demonstrated to exhibit C terminal phosphorylation upon the addition of EGF and is required for EGF induced proliferation in this cell line.

Initially, we confirmed that ERK5-FL induced in cells expressing a constitutively activated form of MEK5, namely MEK5 dominant active (MEK5DA), or in cells stimulated with epidermal growth factor (EGF), exhibited a mobility shift by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) characteristic

of post-translational modification by phosphorylation **(Fig. 1B**). We further analysed the migration pattern of N and C terminal truncated mutants. Interestingly, ERK5 mutants lacking the C terminal tail (ERK5-ΔC) did not display any evidence of retarded migration under basal or stimulated conditions (**Fig. 1B** and **Fig. Suppl 1A**). In contrast, the analysis of a C terminal fragment (ERK5-ΔN) revealed distinct mobility shifts consistent with the presence of multiple phosphorylation sites in the C terminus of ERK5 (**Fig. Suppl 1A**). To confirm that phosphorylation at the C terminus was a consequence of autophosphorylation, Flp-In Hela cells expressing ERK5-FL were pre-incubated with XMD8-92 in the presence of EGF (5μM, CRUK Manchester Institute, Manchester). As expected, inhibition of ERK5 activity blocked the post-translational modification of the protein in response to EGF stimulation (**Fig. 1C**). More interestingly, the migratory shift of ERK5 caused by EGF treatment was also prevented following the substitution of the Thr^{732} residue by a non-phosphorylatable alanine (T732A) (**Fig. 1C**). Kinetic analysis of EGF stimulation confirmed that phosphorylation of the ERK5-T732A mutant was impaired, rather than delayed (**Fig. 1D**).

The importance of Thr^{732} in ERK5 post-translational modification was further tested by analysing two additional phospho-mutants which had previously been shown to abolish phosphorylation at the C terminal tail [21] [39]. These mutants were generated by replacing Ser^{706}, Thr^{732}, Ser^{753} and Ser^{773} (ERK5-$4xA_i$) or Thr^{732}, Ser^{769}, Ser^{773}, and Ser^{775} (ERK5-$4xA_{ii}$) with alanine residues (**Fig. 1A**). The result showed that the substitution of four alanines produced a similar migratory defect as that observed by the single replacement of Thr^{732} with Ala (**Fig. 1E**). ERK5-4xA mutants migrated with a slightly accelerated mobility compared with ERK5-FL and ERK5-T732A due to the lower molecular weight of Ala compared with that of Ser or Thr residues. Collectively, these observations indicated that Thr^{732} acted as a gatekeeper residue controlling C terminal post-translational modification of ERK5.

Threonine 732 phosphorylation controls ERK5-mediated transcription and ERK5 nuclear localisation. One critical function of the C terminus of ERK5 is to regulate transcription [22]. Accordingly, previous studies had found that mimicking phosphorylation at the C terminus enhanced transcription of ERK5 target genes [39] [21]. Using a similar approach, we tested the ability of various ERK5 mutants to increase transcription using a MEF2 luciferase reporter assay [30]. We verified by immunoblot analysis that tetracycline- induced expression of all mutants to a similar level for comparison (**Fig. 2** and **Fig Suppl 1B**). Initially, we found that induced expression of ERK5-FL or ERK5-ΔC mutants slightly increased transcription via MEF2 to a similar level under basal conditions (**Fig Suppl 1B**). Surprisingly, the ERK5-ΔN fragment displayed a much higher level of transcriptional activity, indicative of the ability of ERK5 to regulate transcription through its C terminal tail independently of its catalytic activity (**Fig Suppl 1B**). To determine the requirement of post-translational modification of the C terminal

tail, we analysed the activity of ERK5-4xA$_i$ and ERK5-T732A, and three additional phospho-mimetics in which Ser^{706}, Thr^{732}, Ser^{753} and Ser^{773} (ERK5-4xE$_i$), Thr^{732}, Ser^{769}, Ser^{773}, and Ser^{775} (ERK5-4xE$_{ii}$), or Thr^{732} alone (ERK5-T732E), were replaced by Glu residues (**Fig. 1A**). The data showed that ERK5-FL, ERK5-ΔC, ERK5-4xA$_i$ and ERK5-T732A exhibited a similar level of transcriptional activity (**Fig. 2A**). In contrast, MEF2-mediated transcription was significantly enhanced in cells expressing ERK5-4xE$_i$, ERK5-4xE$_{ii}$, or ERK5-T732E (**Fig. 2A** and **Fig. Suppl 1C**). Remarkably, the replacement of four Ser by Glu residues did not provide any additional transcriptional enhancement compared to a single Glu substitution at Thr^{732}. Furthermore, mimicking phosphorylation at Ser^{706}, Ser^{753} and Ser^{773}, or Ser^{769}, Ser^{773} and Ser^{775} in the context of an unphosphorylatable Ala^{732} (3xE$_i$-T732A and 3xE$_{ii}$-T732A mutants) did not enhance MEF2-mediated transcription (**Fig. 2B**). These data firmly demonstrated the critical functional importance of Thr^{732} phosphorylation. In parallel, we generated another set of Ala^{732} and Glu^{732} substitution in the context of a kinase-dead mutant (D200A) in order to dissociate the functional requirement of ERK5 autophosphorylation from ERK5 catalytic activity [105]. We found that the catalytic activity of ERK5 was essential in the absence of Thr^{732} phosphorylation (**Fig. 2C**). In contrast, enhanced transcriptional activity exhibited by the T732E mutant was only partially reduced following D200A mutation (**Fig. 2C**), thereby indicating that phosphorylation at Thr^{732} could control transcription independently of ERK5 kinase activity.

To further understand the mechanism underlying the transcriptional function of Thr^{732} phosphorylation, we examined the relationship between ERK5 C terminal phosphorylation and the nuclear translocation of ERK5 (**Fig. 3**). Under basal conditions, ERK5-FL predominantly resided in the cytoplasm of cells, whereas ERK5-ΔC mutants were mainly detected in the nuclear compartment (**Fig. 3A, B** and **Fig. Suppl 1D**). This was consistent with previous studies which showed that the removal of the NES and exposure of the NLS caused nuclear retention of ERK5 [39, 106]. Accordingly, the ERK5-ΔN fragment which contains the NLS (and lacks the NES) displayed an exclusive nuclear localisation (**Fig. Suppl 1D**). Similar to ERK5-FL, ERK5-4xA$_i$ and ERK5-T732A mutants preferentially localised in the cytoplasm (**Fig. 3A** and **B**). In contrast, mimicking phosphorylation at Thr^{732} caused a notably increased proportion of ERK5 in the nucleus. In agreement with our previous observation (**Fig. 2A**), we found no significant advantage of substituting multiple Ser and Thr residues versus the single replacement of Thr^{732} by Glu (**Fig. 3A** and **B**; compare ERK5-4xE$_i$ and ERK5-T732E). As expected, a small but nonetheless significant, proportion of ERK5-FL moved in the nuclei of cells stimulated with EGF (**Fig. 3C** and **D**). Likewise, we observed a slightly higher proportion of nuclear ERK5-T732E in EGF treated compared to unstimulated cells (**Fig. 3C** and **D**). Remarkably, Ala^{732} mutation blocked the nuclear translocation of ERK5 in response to EGF

stimulation (**Fig. 3C** and **D**). Together, these findings supported an important regulatory role of Thr^{732} phosphorylation in ERK5 nuclear shuttling.

High throughput analyses reveal that phosphorylation at T732 facilitates the recruitment of ERK5 to chromatin. From our previous analysis, it was notable that, whilst ERK5-ΔC was mostly nuclear (**Fig. 3A** and **B**), the loss of the C terminal tail did not enhanced transcriptional activity (**Fig. 2A**). This observation led us to suggest that increased transcription caused by phosphorylation at Thr^{732} could not solely be a consequence of increased nuclear shuttling. In light of previous evidence that MAPKs can be found tethered to chromatin [40], we tested the possibility that Thr^{732} phosphorylation enhanced transcription by facilitating the recruitment of ERK5 to specific genomic regions, directly or via interactions with transcriptional factors/co-factors. To explore this hypothesis, we first performed a chromatin fractionation from cells expressing ERK5-FL, ERK5-T732E and ERK5-ΔC to quantify the proportion of chromatin- bound ERK5. We found a clear enrichment of chromatin-bound ERK5-T732E over ERK-FL and ERK5-ΔC (**Fig. 4A**). Subsequently, we performed chromatin immunoprecipitation followed by high throughput sequencing (ChIP-Seq) to identify genomic regions bound to ERK5. To investigate genomic binding interactions independently of nuclear localisation we compared ERK5-T732E with an ERK5 mutant that predominantly resided in the nucleus, namely ERK5-ΔC (**Fig. 3A**). We obtained 1,544 peaks of significant q value (>0.01) in ERK-T732E expressing Flp-In HeLa cells compared with 117 peaks in cells expressing ERK5-ΔC (**Fig. 4B**). This provided the first strong evidence that the C terminus of ERK5 facilitated recruitment to chromatin. Moreover, we discovered in our ChIP dataset that ERK5-T732E binding distribution was enriched in intergenic and promoter regions relative to the whole genome, suggesting that phosphorylation of Thr^{732} enhanced transcription by recruitment to regulatory regions of the genome (**Fig. 4C**). This was supported by further analysis that correlated ERK5-T732E bound promoters with histone marks associated with active transcription (**Fig. 4D**). For example, ERK5-T732E binding correlated with sites such as H3K27ac, H3K9ac, H3K4me3. This was further supported by the observation that ERK5 co-occupied cMYC binding sites, a previously identified ERK5 substrate [35]. A heat map of ERK5 bound promoters demonstrates the same association (**Fig. 4E**).

Next, we sought to characterise the molecular basis underpinning the interaction of ERK5 to genomic DNA. An initial ChIP-Seq experiment performed in the presence of formaldehyde but in the absence of a protein-protein crosslinker failed to detect any ERK5 chromatin interactions. This negative result led us to hypothesise that ERK5 binding to the genome was indirect. Consequently, we utilised an unbiased mass spectrometry-based quantitative proteomics approach to identify potential ERK5 binding partners implicated in genomic interactions. We compared triplicate M2-pull down purifications from ERK5-FL

non induced Flp-In Hela cells (- Tet) and from Flp-In Hela cells induced (+ Tet) to express ERK5-FL or ERK5-T732E (**Fig. 5A**). The protocol was performed according to a previously published study [107]. As demonstrated, the biological triplicates were significantly similar (**Fig. Suppl 3A**). Surprisingly, a similar number of proteins were identified in the immune complexes isolated from induced and non-induced cells (**Fig. Suppl 3B**). Nonetheless, ERK5 was significantly enriched in the induced conditions (**Fig. 5B** and **C**). We also confirmed that ERK5-FL and ERK5-T732E were expressed at a very similar level (**Fig. 5B**). Importantly, we were able to identify phosphorylated ERK5-FL protein at Ser^{730} and Thr^{732} under basal conditions (**Fig. 5B**). Consistent with the absence of stimulation, no phosphorylation was detected on the TEY motif. Further analysis demonstrated that ERK5-FL had significantly more interacting partners than ERK5-T732E (**Fig. 5D**). However, six interactors displayed a significantly higher binding affinity for ERK5-T732E than ERK5-FL (**Fig. 5D**). These were all relevant to transcriptional regulation. In particular, the two most significantly enriched interactors identified in the ERK5-T732E immune complex were Histone 3 and Histone 2A (**Fig. 5D**). The interaction between ERK5 and Histone 3 was confirmed by immunoblot analysis following immunoprecipitation. Consistent with the mass spectrometry data analysis, we found that Glu^{732} mutation markedly increased the binding affinity of ERK5 for Histone 3 (**Fig. 5E**). Together, these findings provided the first strong evidence that phosphorylation at Thr^{732} enhanced ERK5-mediated transcription via facilitating its recruitment to chromatin.

DISCUSSION

This study demonstrated that Thr^{732}, a previously identified phosphorylation site in the C terminus of ERK5 [39] [21], acts as a gatekeeper residue, controlling C terminal mediated nuclear translocation and transcriptional enhancement. Also, we found that phosphorylation at Thr^{732} facilitated C terminal phosphorylation, thereby providing a novel mechanism by which post-translation modification of the C terminal tail controls ERK5 mediated transcription. We also that demonstrate that phosphorylation at Thr^{732} facilitates recruitment to chromatin, which we suggest is the mechanism that underpins transcriptional enhancement.

However, we were not able to find any transcription factors / co-factors that could mediate the binding between ERK5 and chromatin. One possible candidate for further investigation is the TAR DNA-binding protein 43, however, this DNA binding protein is typically involved in transcriptional repression [108]. This opens up the possibility that phosphorylated ERK5 may directly bind to chromatin or we have not detected an intermediary that facilitates ERK5 interaction with chromatin. We were able to correlate

ERK5 binding with occupancy of transcription factors known to be regulated by ERK5 using ChIPSeq, however, these were not detectable via proteomic analysis. We were also unable to detect an interaction with RNA polymerase-associated factor 1 (PAF1) which had previously been shown to act in concert with ERK5 at genomic loci [109] [110]. Kinetic studies demonstrate that kinase recruitment can be transient, perhaps explaining the difficulty in capturing specific interactions [112]. One technique which could be employed to detect such transient interactions would be Rapid Immunoprecipitation Mass spectrometry of Endogenous protein (RIME), which isolates fixed protein complexes and their cognate DNA loci [113].

Recent studies have highlighted Thr^{732} as an important residue implicated in a cross-talk mechanism between ERK1/2 and ERK5 [41] [102]. Despite the increased interest in the function of phosphorylation at Thr^{732}, its role is still poorly understood. Here we provide a possible insight into the function of targeting Thr^{732} for phosphorylation and demonstrate it is critical for regulating ERK5 function. Our results indicate that ERK 1/2 may be able to control ERK5 C terminal function by targeting Thr^{732}, thereby promoting ERK5 nuclear localisation and enhancing ERK5 transcriptional activity by recruitment to chromatin. This data corroborates a recent study which indicates BRAFV600E causes phosphorylation at Thr^{732}, which correlates to an increase in chromatin-bound ERK5 [102].

Several authors have identified that mimicking phosphorylation at multiple C terminal sites promotes nuclear translocation of ERK5 [39] [28]. Similar to the transcriptional function, we identified that mimicking phosphorylation at the single Thr^{732} site was sufficient to promote nuclear translocation. However, unlike the transcriptional function, we do not propose an underlying mechanism in this report. One possible explanation, based on the identification of Thr^{732} as a gatekeeper residue, is that phosphorylation at Thr^{732} facilitates a conformational change upon phosphorylation. This is supported by our observation that ERK5-T732A was unable to translocate to the nucleus upon stimulation, in contrast to ERK5-FL. This hypothesis could be tested by analysing the temporal phosphorylation of the C terminus upon stimulation, to indicate whether phosphorylation at Thr^{732} was a prerequisite for conformational change. Another option would be to analyse whether ERK5-T732A can undergo a conformational change upon stimulation using a biophysical approach.

This unique regulatory mechanism can be used to further interrogate ERK5 function, particularly in understanding its oncogenic function. This is particularly relevant in melanoma and HER2 breast cancer where C terminal phosphorylation has been demonstrated to be involved in cancerous growth [102] [99]. Further work should therefore focus on understanding how Thr^{732} phosphorylation allows ERK5 to be regulated in the context of cancer and clarify its function in a clinically relevant cell line.

MATERIALS AND METHODS

Generation of isogenic Tet-inducible ERK5 expressing Hela cell lines. N-terminal Flag epitope-tagged full length (human) ERK5 (ERK5-FL) cDNA [12], and C and N terminal truncated mutants were sub-cloned into a pCDNA5/FRT/TO vector (Invitrogen) modified to contain an 5' Lamin UTR using Not1 and BamH1 digestion [114]. The various ERK5 phospho-mutants were created by site-directed mutagenesis (QuikChange, Agilent technologies) and sub cloned into the pCDNA5/FRT/TO vector. Primer sequences are indicated in **Suppl Table 2**. All constructs were confirmed by sequencing. The pcDNA5/FRT/TO expression vectors containing ERK5-FL or ERK5 mutants were subsequently co-transfected with pOG44 into the Flp-In Hela host cell line using the jetPEI transfection reagent according to manufacturer's instructions (Polyplus). The Flp recombinase expressed from pOG44 catalyzed homologous recombination between the FRT sites in the host cells and the pcDNA5/FRT/TO expression vectors. Stable recombinant Flp-In Hela cells were selected in hygromycin (2 mg/ml, Invitrogen) for 2 weeks. Resistant colonies were pooled and expanded in DMEM supplemented with 10% fetal bovine serum (FBS, Sigma), 1% penicillin/streptomycin (Sigma). Expression of the constructs was induced by incubating the cells with 1 ug/ml tetracycline (Sigma) for 24 h prior to being harvested.

Immunoblot analysis. Proteins were extracted from cells in radioimmunoprecipitation assay (RIPA) buffer (Sigma) containing EDTA-free protease inhibitor cocktail (Roche). Extracts (50 μg) were resolved by SDS-PAGE and electrophoretically transferred to an Immobilon-P membrane (Millipore, Inc). The membranes were saturated in 3% nonfat dry milk and probed overnight at 4^{o}C with a primary antibody (1:2000) to the Flag-epitope (M2, Sigma). Anti-β actin (1:2000, Sigma) and anti-vinculin (1:2000, Abcam) antibodies were used to monitor protein loading. Immunocomplexes were detected by enhanced chemiluminescence with IgG coupled to horseradish peroxidase as the secondary antibody (GE Healthcare).

Luciferase reporter assays. The reporter luciferase plasmid MEF2-Luc [115] was transiently transfected into Flp-In HeLa cell lines. A pRL-Tk plasmid encoding *Renilla* luciferase was co-transfected to monitor transfection efficiency. Aliquots of cell lysates were assayed for firefly and *Renilla* luciferase activities using the Dual-luciferase reporter assay kit (Promega) on an Orion microplate luminometer.

Immunofluorescence. Flp-In HeLa cell lines cultured on glass coverslips for 72 h in presence of tetracycline were fixed in 4% paraformaldehyde for 20 minutes and permeabilized for 25 min with 0.1% Triton X-100 in PBS. After saturation with 1% BSA for 1 h, cells were incubated with the M2 antibody

(1:200) for 1 hour at room temperature. After 3 washes with PBS, the cells were exposed to a secondary antibody (1:1000) conjugated to Alexa-488 (Invitrogen) in addition to Phalloidin (Sigma) for 1 hour. Nuclei were stained with DAPI (Invitrogen # P36931). Fluorescence images were viewed with a Z1 inverted Axio Observer microscope from ZEISS**.** Fluorescent intensity of nuclear and cytoplasmic signals was quantified using ImageJ software by manual determination of the whole cell intensity divided by the nuclear intensity of ERK5.

Chromatin fractionation and immunoprecipitation (ChIP). Chromatin fractionation was performed according to a previously published protocol [116]. ChIP was performed using a protein-protein crosslinker (ChIP Cross-link Gold, Diagenode), as previously described [117]. The M2 antibody (Sigma) was used to immunoprecipitate protein-DNA complexes or detect chromatin bound ERK5. DNA fragments were isolated after reverse-crosslinked to generate a ChIPSeq library. Paired-end 76b reads were generated on an Illumina HiSeq4000 instrument run by the Genomic Technologies Core Facility (University of Manchester).

Mass spectrometry. Recombinant Flp-In HeLa cell lines were lysed in RIPA buffer (Sigma) containing EDTA-free protease inhibitor cocktail (Roche) 24 hours after tetracycline induction. Mock treated cells with DMSO were used as non-induced controls. Protein extracts (8 mg) were precleared with an anti-mouse IgG (10 ug/mg; Sigma) supplemented with Protein G-Sepharose beads (Invitrogen) and inversion rotated for 5 h at 4 ^{o}C. After centrifugation at 1,000 g for 5 min, cleared lysates were incubated with an anti-Flag M2 affinity gel (Sigma) (10 ug/ml) for 90 min at 4 ^{o}C. Immune complexes were washed five times with ice-cold lysis buffer supplemented with NaCl and analyzed by SDS-PAGE. The gel was then digested with trypsin as previously described [118]. Raw data were analysed with the MaxQuant software suite [119]

Statistical analyses. To compare values between multiple test groups in luciferase reporter experiments, we performed a one-way ANOVA followed by Tukey's test. Data were analyzed using Prism software (GraphPad).

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FIGURE LEGENDS

Figure 1: ERK5 phosphorylation at T732 is a pre-requisite for C terminal phosphorylation. A) A schematic illustration and nomenclature of ERK5-FL, a truncated mutant lacking amino acids 576-816 (ERK5-ΔC), and various mutants in which specific serine and/or threonine residues have been replaced by alanines or glutamic acids. **B-E**) Recombinant Flp-In HeLa cells were incubated with tetracycline for 24 hours. In panel B, mock treated cells with DMSO (-) were used as controls. Where indicated, the cells were transfected with a construct encoding HA-tagged MEK5DA [30] 24 hours before Tet induction, or stimulated 25 min, unless indicated otherwise (panel D), with EGF (20 ng/ml) before harvesting. In panel C, cells were pre-incubated with XMD8-92 (5 μM) for 1 h prior to EGF stimulation. Mock treated cells with DMSO were used as controls (Ctrl). Cell lysates were analyzed by immunoblot using a specific antibody to the Flag-tagged epitope (M2). Antibodies to β actin or vinculin were used to monitor protein loading.

Figure 2: Phosphorylation at T732 enhances ERK5-mediated transcription. A-C) Recombinant Flp-In HeLa cell lines were transfected with a construct encoding a MEF2 luciferase reporter. 24 h later, the cells were incubated with tetracycline for 48 hours to induce expression of ERK-FL, ERK5-ΔC, or specific phospho-deficient or phosphomimetics mutants, as indicated. Non-induced (NI) cells were used as controls. Efficiency of transfection was controlled by co-transfecting a *Renilla* firefly encoding construct. Immunoblot analyses of the cell lysates demonstrate similar level of expression of ERK5-FL and the various mutants. The MEF2 luciferase activity normalized to that of *Renilla* luciferase is expressed as fold to compare relative transcriptional activity under basal condition. The data represent the mean ± SD of three independent experiments performed in duplicate. *, $P < 0.05$ indicates significant differences. **, $P < 0.01$. ***, $P < 0.001$. ns, indicates no statistical difference.

Figure 3: Phosphorylation at T732 contributes to the nuclear translocation of ERK5. Recombinant Flp-In HeLa cell lines were grown on glass coverslips and incubated with tetracycline for 24 hours before fixation. Where indicated, the cells were stimulated with EGF (20 ng/ml) for 25 mins. **A** and **C**) Subcellular localization of ectopically expressed ERK5-FL, ERK5-ΔC, and specific phospho-deficient or phosphomimetics mutants, were visualized using an antibody to the Flag-tagged epitope (M2, green). Phallodin staining (red) was used to detect actin. Nuclei were detected with DAPI (blue). Scale bars: 10 μM. **B** and **D**) The immunofluorescent signal of the Flag epitope was quantitated with ImageJ. The data

expressed as percent of signal in the nucleus correspond to the mean ± SD (N= 30 cells). *, $P < 0.05$ indicates significant differences. ***, P < 0.001. ****, P < 0.0001. ns, indicates no statistical difference.

Figure 4: Phosphorylation of T732 facilitates the recruitment of ERK5 to the chromatin. A) The chromatin fraction was isolated and probed for chromatin associated ERK5-FL, ERK5-T732E or ERK5-ΔC using the M2 antibody. Histone 3 antibody was used to monitor total amount of chromatin. **B)** Recombinant Flp-In HeLa cell lines were incubated with tetracycline for 24 hours prior to protein-protein and protein-DNA crosslinking. Chromatin immunoprecipitation was performed using a specific antibody to the Flag-tagged epitope (M2). The number of significant peaks (>0.01 q value) associated with ERK5-FL and ERK5-ΔC expression was quantified. **C)** Pie charts showing the genomic distribution of ERK5-T732E peaks and the expected ratio of the whole genome. **D**) Profile of H3K27ac, H3K9ac, H3K4me3, H3K27me3 and CMYC at genomic regions in accordance with ERK-T732E binding. **E)** A heat map of ERK5 bound promoters showing the prevalence of various histone modifications and transcription factor co-occupancy, including CJUN, CMYC and ELK.

Figure 5: Identification of ERK5 binding partners by proteomic analysis. A) Illustration of the work flow to analyse the interactome of non-induced, ERK5-FL and ERK5-T732E. B) Graph of the $\log^{10}$ intensity of ERK5 expression in the non induced, ERK5-FL and ERK5-T732E. Table illustrating the identified phosphorylated residues in the ERK5-FL protein. **C and D**) The log ratio for top interactors enriched in the pull downs comparing the non-induced (NI) vs Full Length (FL) (**C**) and Full length vs T732E (**D**) are illustrated in a graph form **E)** Co-immunoprecipitation with an IgG control, ERK5-FL, ERK5-T732E and ERK5-ΔC with Histone 3.

SUPPLEMENTARY INFORMATION

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1: Analysis of C and N terminal truncated ERK5 mutants. Recombinant Flp-In HeLa cells were incubated with tetracycline for 24 hours. In panel A, mock treated cells with DMSO (-) were used as controls. Where indicated, the cells were transfected with a construct encoding HA-tagged MEK5DA 24 hours before Tet induction, or stimulated 25 min with EGF (20 ng/ml) before harvesting. **A**) Lysates of Flp-In HeLa cells expressing ΔC or ΔN truncated mutants were analysed by immunoblot using a specific antibody to the Flag-tagged epitope (M2). An antibody to β actin was used to monitor protein loading. A schematic illustration of the truncated ERK5 mutants is provided. **B** and **C**) Recombinant Flp-In HeLa cell

lines were transfected with a construct encoding a MEF2 luciferase reporter. 24 h later, the cells were incubated with tetracycline for 48 hours to induce expression of ERK5-FL or ERK5 mutants. Immunoblot analyses of the cell lysates demonstrate similar level of expression of ERK5-FL and the truncated mutants. The MEF2 luciferase activity normalized to that of *Renilla* luciferase is expressed as fold to compare relative transcriptional activity under basal condition. The data represent the mean ± SD of three independent experiments performed in duplicate. **, $P < 0.01$ indicates significant differences. ns, indicates no statistical difference. To the left of the dotted line is a reproduction of Fig. 2A for comparison with the ERK5-4xA/E_{ii} mutants. **D**) Recombinant Flp-In HeLa cell lines were grown on glass coverslips and incubated with tetracycline for 24 hours before fixation. Subcellular localization of ectopically expressed ΔC or ΔN truncated mutants was visualized using an antibody to the Flag-tagged epitope (M2, green). Phallodin staining (red) was used to detect actin. Nuclei were detected with DAPI (blue). Scale bars: 10 μM.

Supplementary Figure 2: ChIPseq optimisation. A and **B)** Approximately 1-1.5x10^7 ERK5-FL cells were cross-linked with paraformaldehyde and ChIP cross-link gold. Nuclear extracts were subsequently isolated, sonicated for 30 or 35 minutes and reverse cross-linked for analysis. The DNA was analyzed by gel electrophoresis. The fragment size decreases with time. A 35 minute sonication time was selected to have a chromatin fragments predominantly between 100-500bp. **B)** ChIP was performed using the M2 antibody which enriched ERK5-FL compared to the input.

SUPPLEMENTARY TABLES

Supplementary Table 1: ERK5 and MEK5 constructs used in this study

Mutant	Details	Reference
ERK5-FL		[12]
ERK5-ΔC	Deletion of amino acids 576-816 (-TAD)	[22]
ERK5-ΔC (1-503)	Deletion of amino acids 504-816 (-PR2)	[12] [11]
ERK5-ΔC (1-408)	Deletion of amino acids 409-816 (-NLS)	[19]
ERK5-ΔN (411-816)	Deletion of amino acids 1-410	[12] [11]
ERK5-T732A	Substitution of Thr^{732} to Ala	[39] [41]
ERK5-T732E	Substitution of Thr^{732} to Glu	[5] [6]
ERK5-4xA$_i$	Substitution of Ser^{706}, Thr^{732}, Ser^{753}, Ser^{773} to Ala	[5]
ERK5-4xA$_{ii}$	Substitution of Thr^{732}, Ser^{769}, Ser^{773}, Ser^{775} to Ala	[21]
ERK5-4xE	Substitution of Ser^{706}, Thr^{732}, Ser^{753}, Ser^{773} to Glu	[5]
ERK5-4xE$_{ii}$	Substitution of Thr^{732}, Ser^{769}, Ser^{773}, Ser^{775} to Glu	[7]
ERK5-3xE$_i$-T732A	Substitution of Ser^{706}, Ser^{753}, Ser^{774} to Glu and Thr^{732} to Ala	[5]
ERK5-3xE$_{ii}$-T732A	Substitution of Ser^{769}, Ser^{773}, Ser^{775} to Glu and Thr^{732} to Ala	[7]
ERK5-D200A-T732E	Substitution of Asp^{200} to Ala and Thr^{732} to Glu	[120]
ERK5-D200A-T732A	Substitution of Asp^{200} to Ala and Thr^{732} to Ala	[8]
MEK5DA	Substitution of Ser^{311} and Thr^{315} to Asp	[121]

Supplementary Table 2: Primer sequences for mutagenesis and subcloning. Utilised QuikChange Primer Design (https://www.genomics.agilent.com/primerDesignProgram.jsp)

ERK5 mutant	**Sequence**
ERK5-FL	Fw - GAGA GGATCC ATGGCCGAGCCTCTG Rv - GAGA GCGGCCGC TCAGGGGTCCTGGAG
ERK5- ΔC	Rv - GAGA GCGGCCGC GGCCATTCGAGTCCA
ERK5-ΔC (1-503)	Rv - GAGA GCGGCCGC AGCCACAGGCTG
ERK5-ΔC (1-408)	Rv - GAGA GCGGCCGC AGGCTCAGGAGC
ERK5-ΔN (411-816)	Fw - GAGA GGATCC GGCTGTCCAGAT
ERK5-T732A	CACTGCCCTTTGGTGCGCCTGAGAACACAGG
ERK5-T732E	CCCCACTGCCCTTTGGCTCGCCTGAGAACACAGGG
ERK5-4xA_i	CACTGCCCTTTGGTGCGCCTGAGAACACAGG CAAGCAGGGAGGCTGCGAGAGAGGCTGAATC CCTGTGTTCTCAGGCGCACCAAAGGGCAGTG GATTCAGCCTCTCTCGCAGCCTCCCTGCTTG
ERK5-4xA_{ii}	CAGCAAGCAGGGCGGCTGCGAGAGAGGCTGA CAGGATGGCCAGGCAGATGCAGCCTCTCT CACTGCCCTTTGGTGCGCCTGAGAACACAGG
ERK5-4xE	GTCAGCAAGCAGGGAGGCCTCGAGAGAGGCTGAATCTGC ATCAGCCACGCCCATGTCGAACTCCTGGTTTAAGAATTCCTCCAG CCCTGTGTTCTCAGGCGAGCCAAAGGGCAGTGGGG CCCCACTGCCCTTTGGCTCGCCTGAGAACACAGGG
ERK5-4xE_{ii}	CCCCACTGCCCTTTGGCTCGCCTGAGAACACAGGG CCAGTCAGCAAGCAGCTCGGCCTCGAGAGAGGCC TCATCTGCCTGGCCATCCTGTGGCCC
ERK5-3xE_i-T732A	CACTGCCCTTTGGTGCGCCTGAGAACACAGG GTCAGCAAGCAGGGAGGCCTCGAGAGAGGCTGAATCTGC ATCAGCCACGCCCATGTCGAACTCCTGGTTTAAGAATTCCTCCAG CCCTGTGTTCTCAGGCGAGCCAAAGGGCAGTGGGG
ERK5-3xE_{ii}-T732A	CACTGCCCTTTGGTGCGCCTGAGAACACAGG CCAGTCAGCAAGCAGCTCGGCCTCGAGAGAGGCC TCATCTGCCTGGCCATCCTGTGGCCC
ERK5-D200A-T732E	CACGAGCCATACCAAAGGCACCAATCTTGAGCTCA CCCCACTGCCCTTTGGCTCGCCTGAGAACACAGGG
ERK5-D200A-T732A	CACGAGCCATACCAAAGGCACCAATCTTGAGCTCA CACTGCCCTTTGGTGCGCCTGAGAACACAGG

Supplementary Table 3: Proteins identified by Mass Spectrometry in pull down comparisons

Proteins enriched in ERK5 non induced > ERK5 FL induced

Protein IDs	Protein names	Log ratio
Q13163-4	Dual specificity mitogen-activated protein kinase kinase 5	23.53
X6R6D0	SHC-transforming protein 1	21.57
Q15424-2	Scaffold attachment factor B1	20.83
B4DLC8	Signal transducer and activator of transcription 2	19.98
Q13164	Mitogen-activated protein kinase 7	3.63
P62191	26S protease regulatory subunit 4	4.63
O60701	UDP-glucose 6-dehydrogenase	4.94
P19474	E3 ubiquitin-protein ligase TRIM21	5.00
H0Y4R1	Inosine-5-monophosphate dehydrogenase 2	3.26
P25705	ATP synthase subunit alpha, mitochondrial	3.61
Q01518-2	Adenylyl cyclase-associated protein 1	3.88
P43490	Nicotinamide phosphoribosyltransferase	3.29
P41091	Eukaryotic translation initiation factor 2 subunit 3	2.63
E9PDI2	Adenylyl cyclase-associated protein	2.08
Q9BUA3	Uncharacterized protein C11orf84	2.20

Proteins enriched in ERK5 FL induced > ERK5-T732E

Protein IDs	Protein names	Log ratio
A0A0U1RR32	Histone H2A type 1-J	1.51
Q9NU22	Midasin	1.24
Q14204	Cytoplasmic dynein 1 heavy chain 1	1.86
K7EK07	Histone H3	0.87
A0A087WX29	TAR DNA-binding protein 43	0.96
Q92572	AP-3 complex subunit sigma-1	0.64
Q9BV44	THUMP domain-containing protein 3	0.40

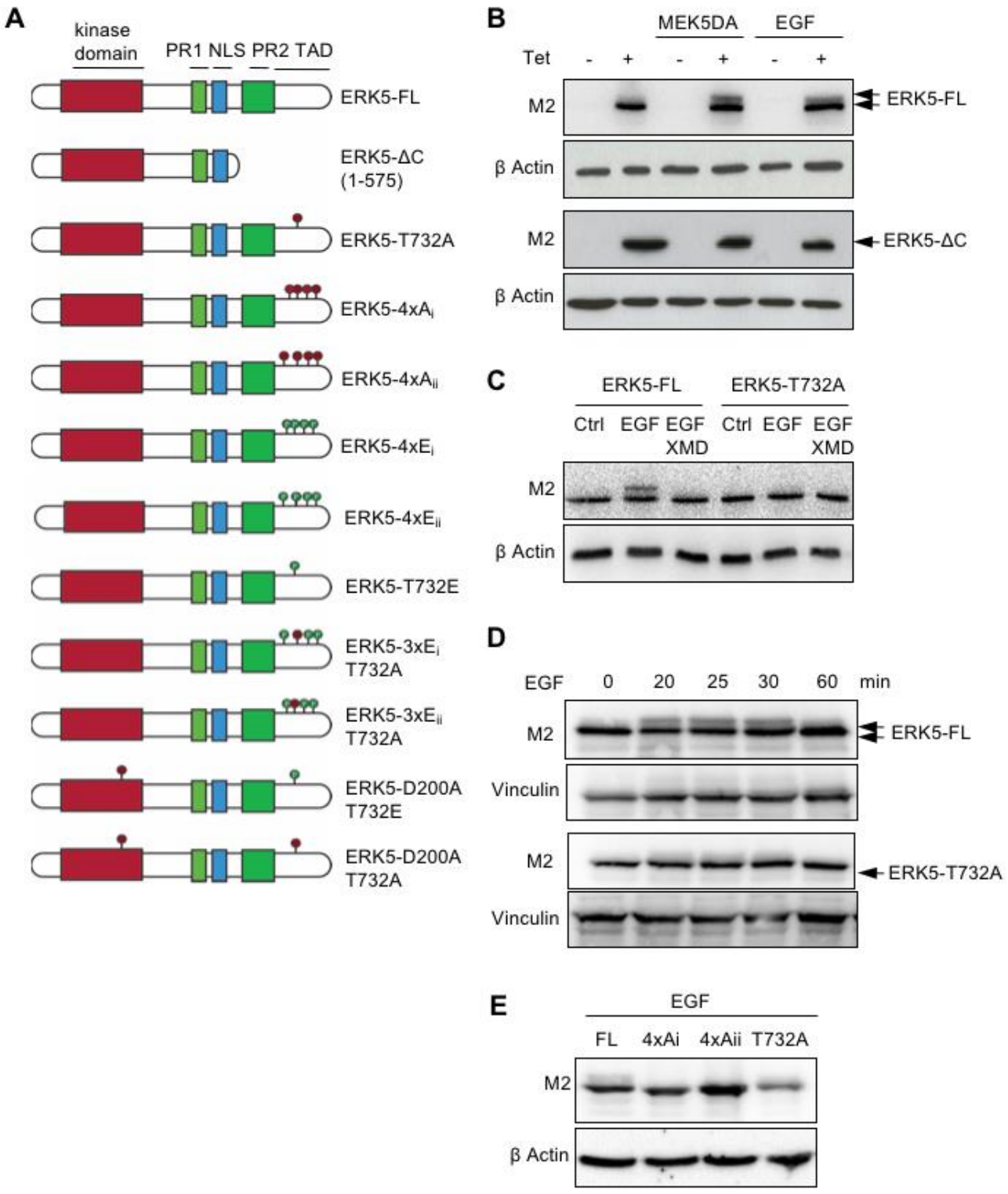

A
kinase domain
PR1 NLS PR2 TAD
ERK5-FL
ERK5-ΔC (1-575)
ERK5-T732A
ERK5-4xA$_i$
ERK5-4xA$_{ii}$
ERK5-4xE$_i$
ERK5-4xE$_{ii}$
ERK5-T732E
ERK5-3xE$_i$ T732A
ERK5-3xE$_{ii}$ T732A
ERK5-D200A T732E
ERK5-D200A T732A
B
MEK5DA
EGF
Tet - + - + - +
M2
ERK5-FL
β Actin
M2
ERK5-ΔC
β Actin
C
ERK5-FL
ERK5-T732A
Ctrl EGF EGF XMD Ctrl EGF EGF XMD
M2
β Actin
D
EGF 0 20 25 30 60 min
M2
ERK5-FL
Vinculin
M2
ERK5-T732A
Vinculin
E
EGF
FL 4xAi 4xAii T732A
M2
β Actin

FIGURE 1

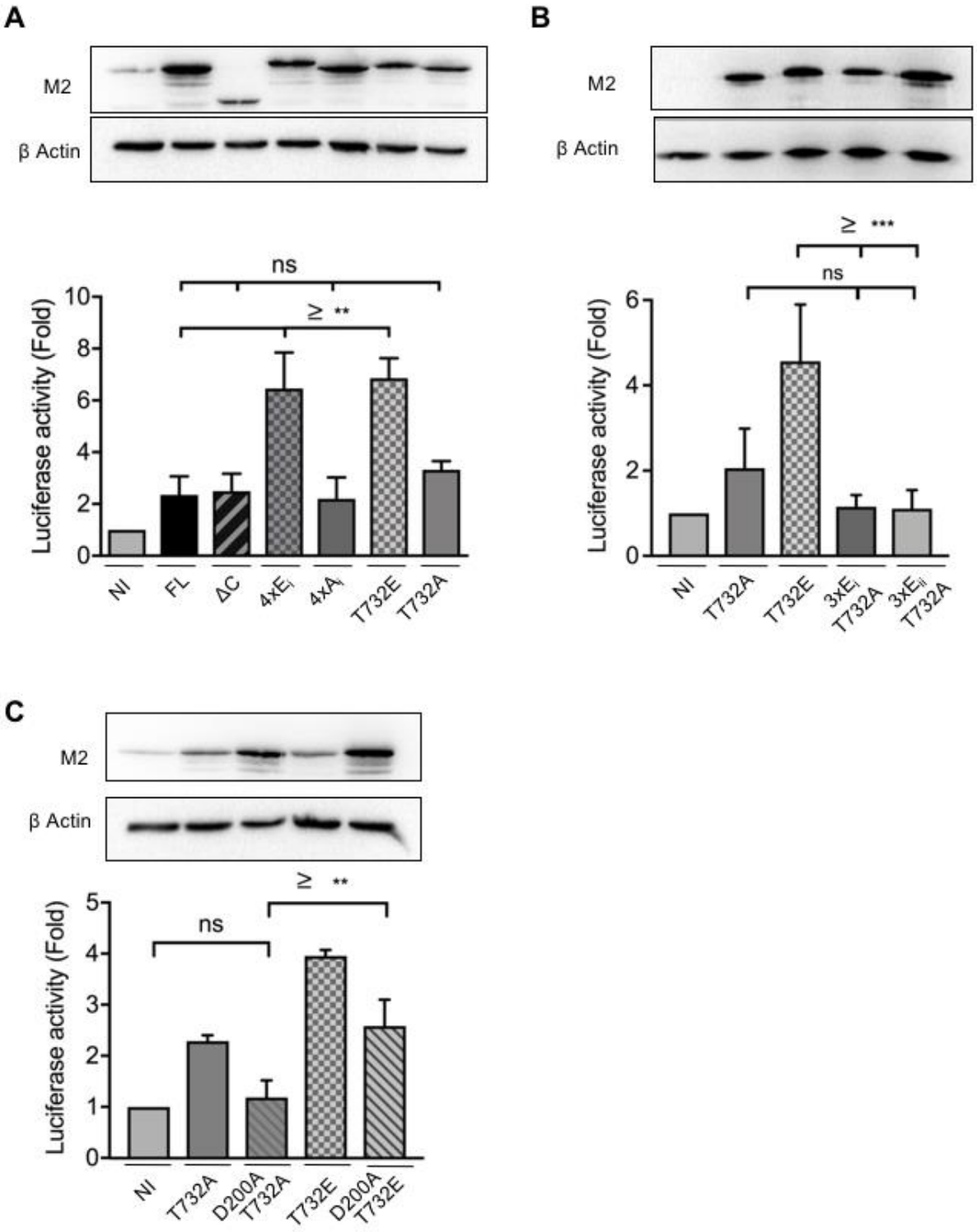

A
M2
β Actin
Luciferase activity (Fold)
ns
≥ **
NI
FL
ΔC
4xE
4xA
T732E
T732A
B
M2
β Actin
Luciferase activity (Fold)
≥ ***
ns
NI
T732A
T732E
3xE
T732A
3xE
T732A
C
M2
β Actin
Luciferase activity (Fold)
≥ **
ns
NI
T732A
D200A T732A
T732E
D200A T732E

FIGURE 2

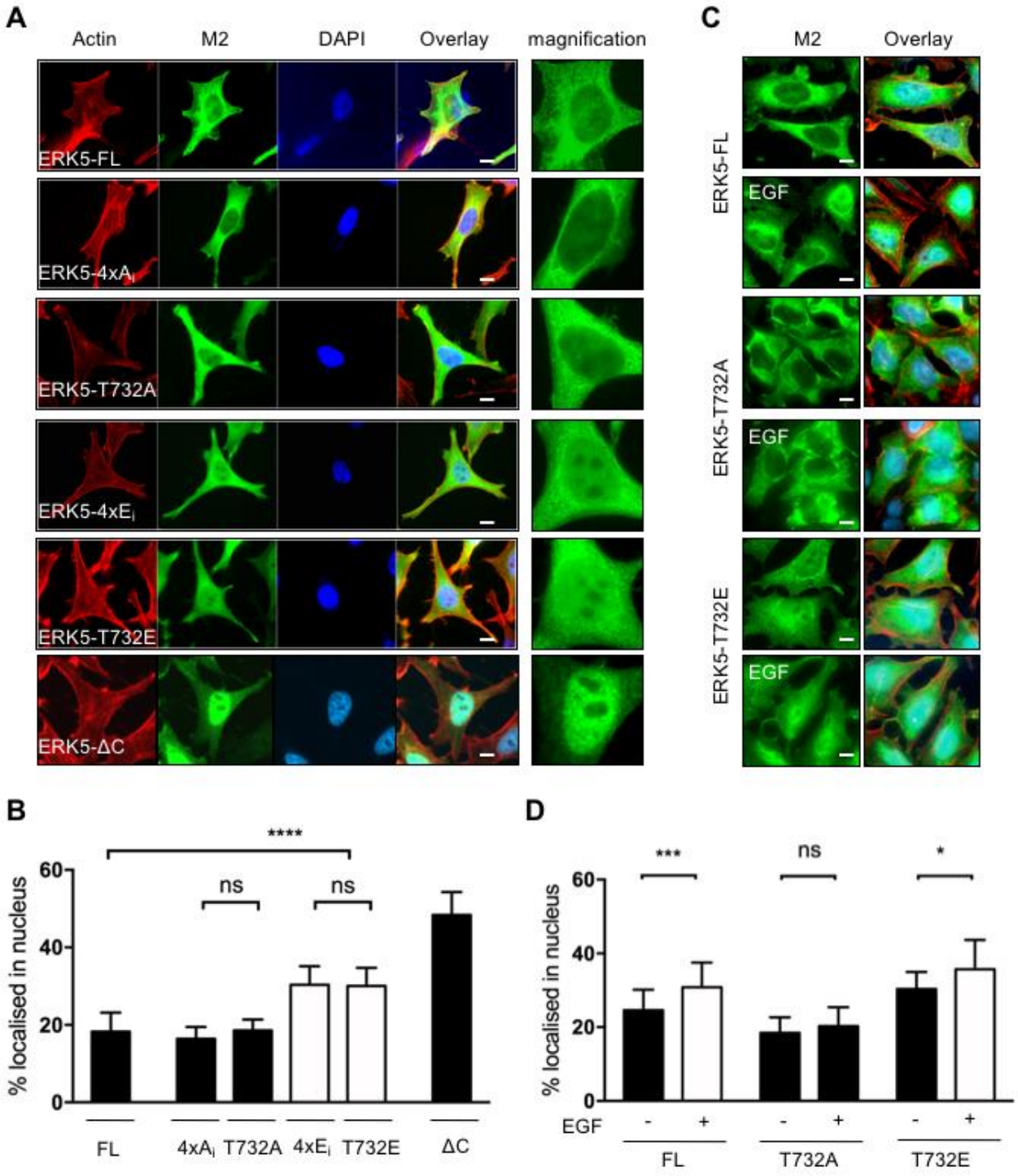

A
Actin
M2
DAPI
Overlay
magnification
ERK5-FL
ERK5-4xA$_i$
ERK5-T732A
ERK5-4xE$_i$
ERK5-T732E
ERK5-ΔC
C
M2
Overlay
ERK5-FL
EGF
ERK5-T732A
EGF
ERK5-T732E
EGF
B
% localised in nucleus
60
40
20
0
****
ns
ns
FL
4xA$_i$
T732A
4xE$_i$
T732E
ΔC
D
% localised in nucleus
60
40
20
0
***
ns
*
EGF
-
+
-
+
-
+
FL
T732A
T732E

FIGURE 3

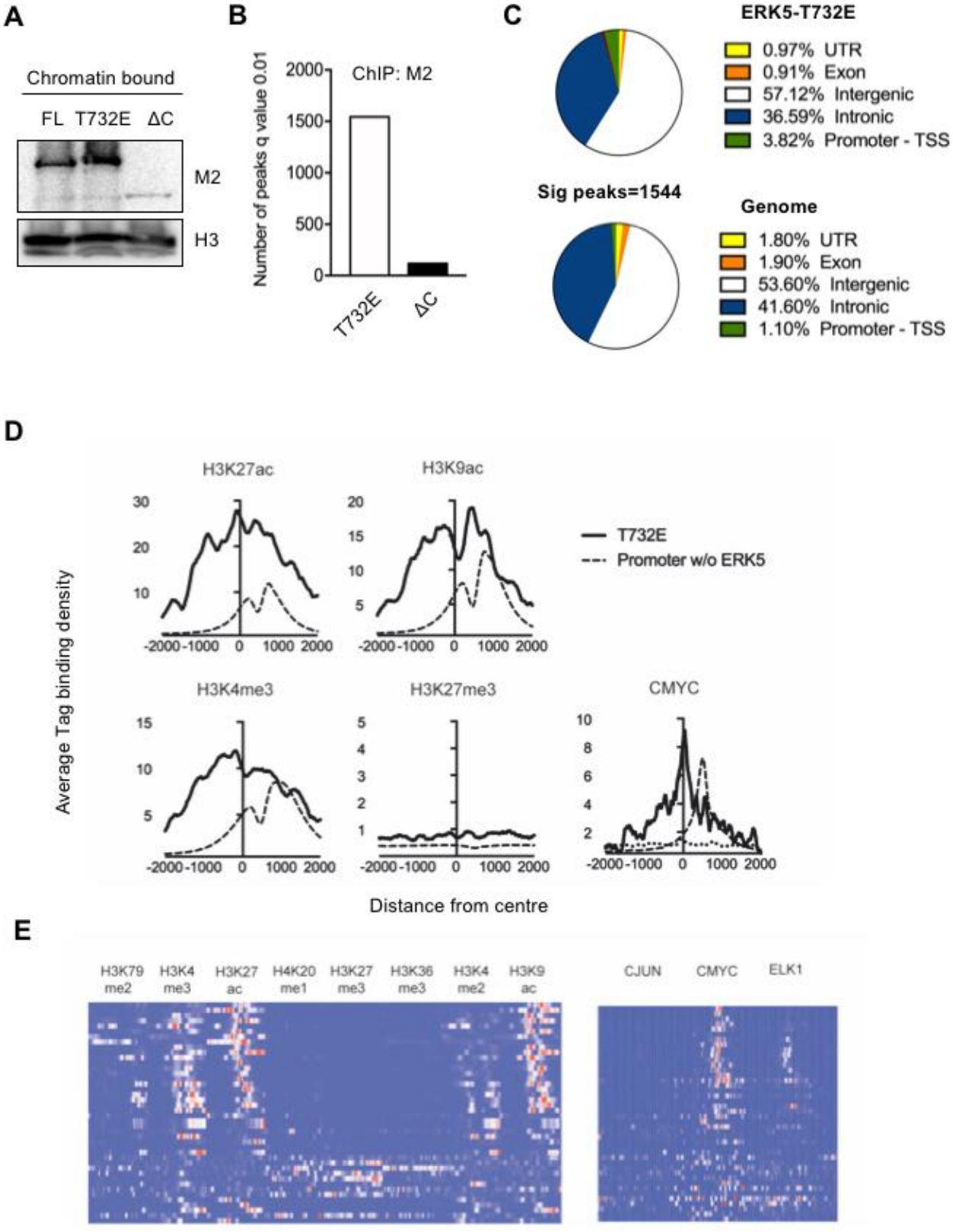
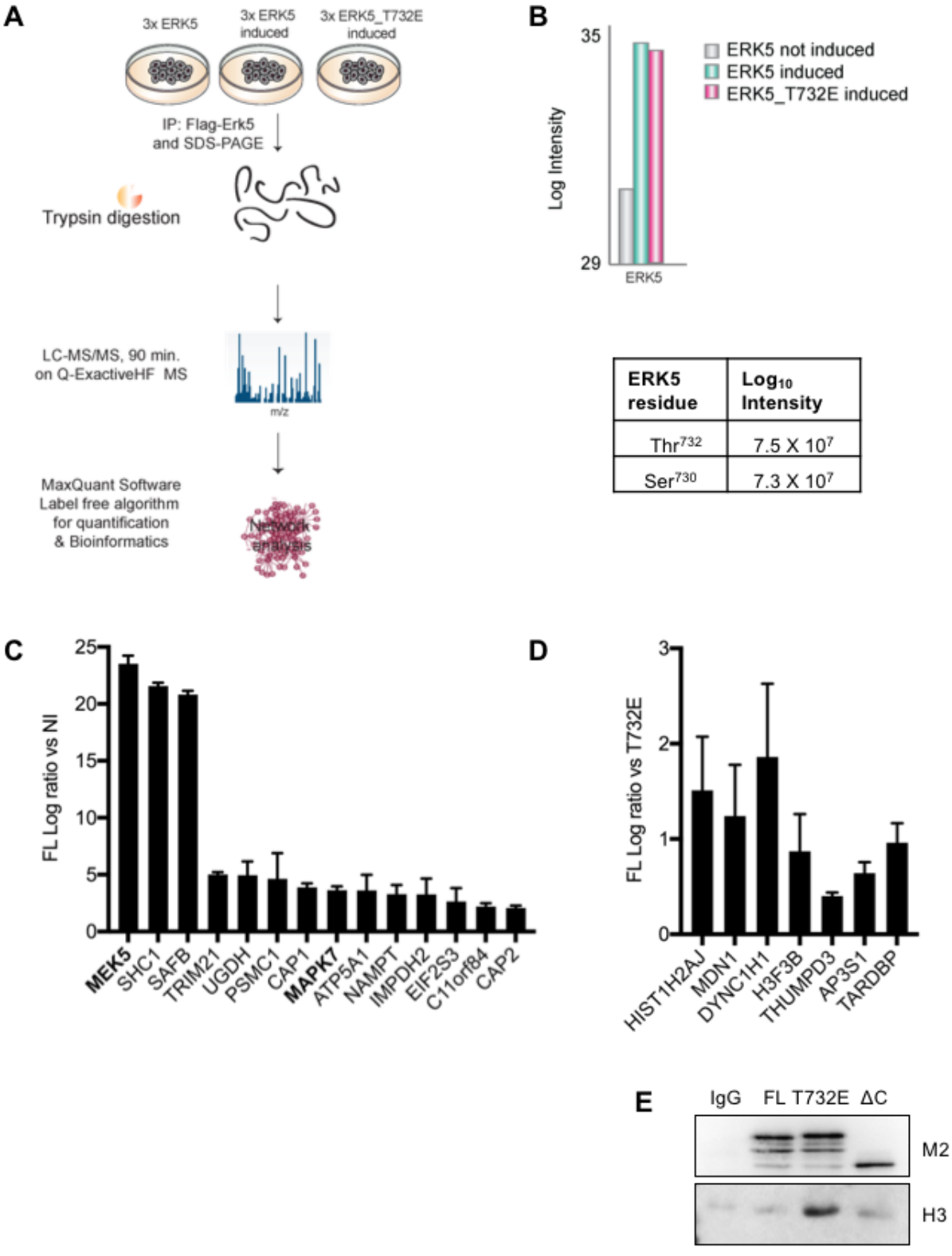
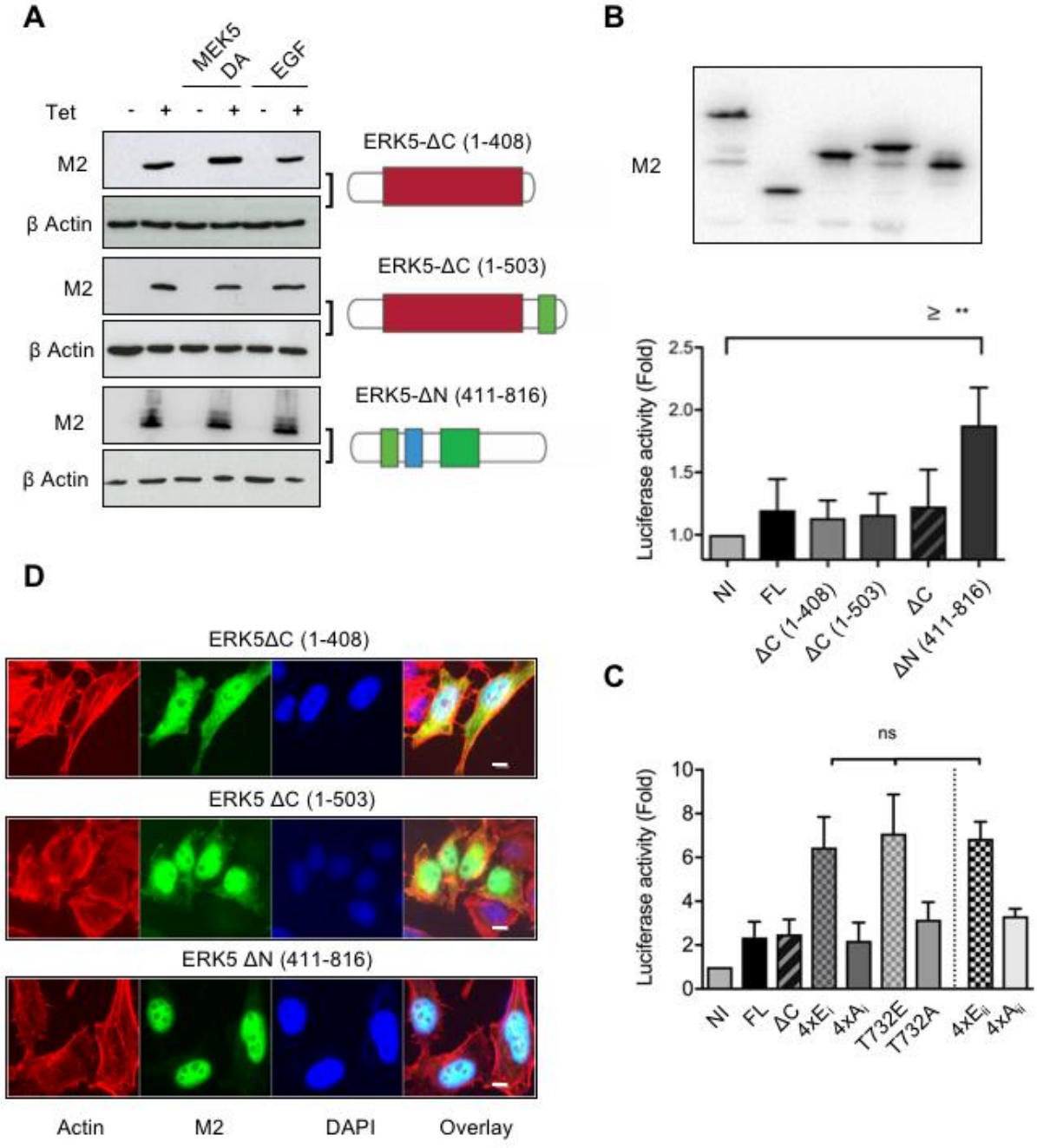

A
Chromatin bound
FL T732E ΔC
M2
H3
B
Number of peaks q value 0.01
2000
1500
1000
500
0
ChIP: M2
T732E
ΔC
C
ERK5-T732E
0.97% UTR
0.91% Exon
57.12% Intergenic
36.59% Intronic
3.82% Promoter - TSS
Sig peaks=1544
Genome
1.80% UTR
1.90% Exon
53.60% Intergenic
41.60% Intronic
1.10% Promoter - TSS
D
H3K27ac
H3K9ac
H3K4me3
H3K27me3
CMYC
T732E
Promoter w/o ERK5
Average Tag binding density
-2000 -1000 0 1000 2000
Distance from centre
E
H3K79 me2
H3K4 me3
H3K27 ac
H4K20 me1
H3K27 me3
H3K36 me3
H3K4 me2
H3K9 ac
CJUN
CMYC
ELK1

FIGURE 4

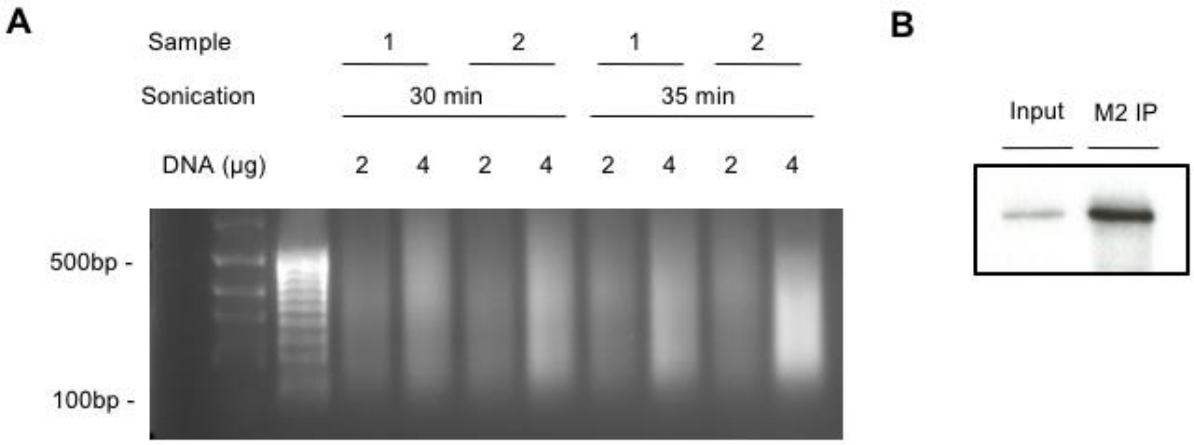


ERK5 residue	Log_{10} Intensity
Thr^{732}	7.5×10^7
Ser^{730}	7.3×10^7

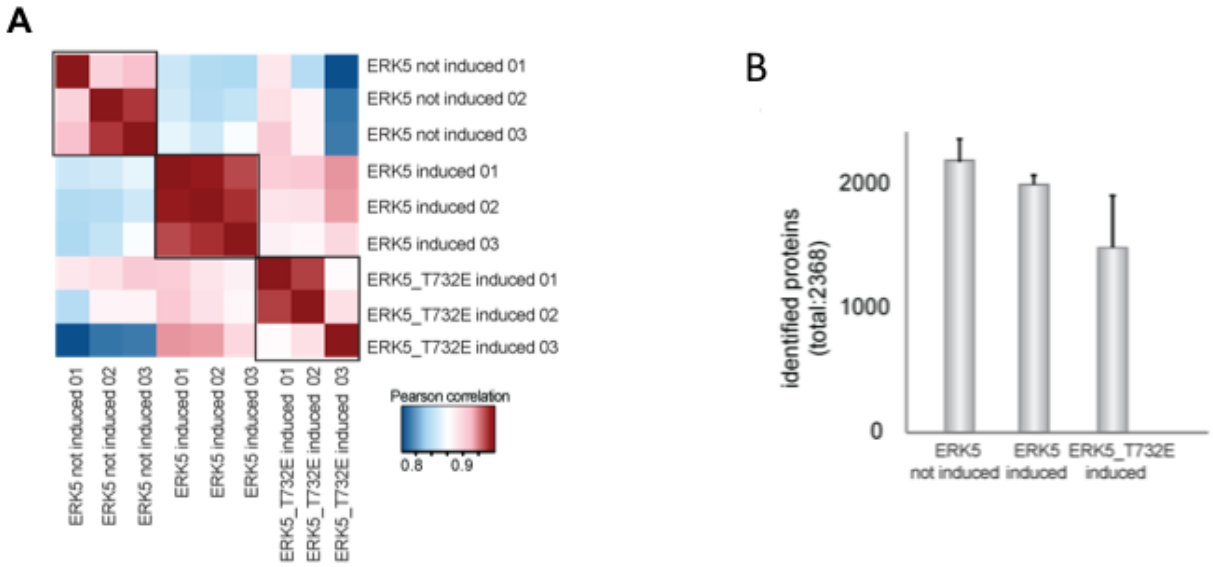
FIGURE 5


A
MEK5 DA
EGF
Tet - + - + - +
M2
β Actin
ERK5-ΔC (1-408)
ERK5-ΔC (1-503)
ERK5-ΔN (411-816)
B
M2
Luciferase activity (Fold)
NI
FL
ΔC (1-408)
ΔC (1-503)
ΔC
ΔN (411-816)
C
ns
Luciferase activity (Fold)
NI
FL
ΔC
4xE
4xA
T732E
T732A
D
ERK5ΔC (1-408)
ERK5 ΔC (1-503)
ERK5 ΔN (411-816)
Actin
M2
DAPI
Overlay

SUPPL FIG 1


A
Sample
1
2
1
2
Sonication
30 min
35 min
DNA (µg)
2
4
2
4
2
4
2
4
500bp -
100bp -
B
Input
M2 IP

SUPPL FIG 2


A
ERK5 not induced 01
ERK5 not induced 02
ERK5 not induced 03
ERK5 induced 01
ERK5 induced 02
ERK5 induced 03
ERK5_T732E induced 01
ERK5_T732E induced 02
ERK5_T732E induced 03
Pearson correlation
0.8
0.9
B
identified proteins (total:2368)
2000
1000
0
ERK5 not induced
ERK5 induced
ERK5_T732E induced

SUPPL FIG 3

Chapter 4: ERK5 drives resistance in HER2+ breast cancer

Adam J Pearson, Jingwei Zhang* & Cathy Tournier

Division of Cancer Sciences, School of Medical Sciences, Faculty of Biology, Medicine and Health, University of Manchester, UK;

ABSTRACT

The advent of HER2 directed therapies has dramatically improved survival rates for women diagnosed with HER2+ breast cancer. Nonetheless, resistance and relapse remain a significant clinical obstacle. Therefore, understanding the molecular basis underlying HER2-mediated breast cancer development and malignancy is critical for developing novel, more effective strategies with prolonged therapeutic effects. One unique feature of HER2+ breast cancer cells is the expression of a constitutively phosphorylated form of the extracellular-regulated protein kinase 5 (ERK5). This is clinically relevant given that elevated expression of ERK5 predicts early relapse rates in women with HER2+ breast cancer post-treatment. Here, we tested the requirement of ERK5 in mediating HER2 resistance to current therapies. We found that combined HER2 and ERK5 inhibition significantly reduced the proliferation of resistant HER2+ breast cancer cells. This correlated with the suppression of phosphorylated Rb, a key mediator of G1/S transition. Together, these results provide the first evidence that targeting ERK5 in combination with HER2 may constitute a viable clinical strategy for breast cancer patients with refractory disease.

INTRODUCTION

Breast cancer is the most common form of cancer in women and the second most common worldwide, following lung cancer [60]. Specifically, the possibility for a woman to get breast cancer in her lifespan is around 12% [122]. Four different molecular subtypes of breast cancer are defined based on the expression level of oestrogen receptor (ER) and progesterone receptor (PR), and human epidermal growth factor receptor type 2 (HER2). However, the mortality from breast cancer in developed countries has decreased significantly due to progress in early detection, but also the discovery of novel therapeutic strategies [62]. In particular, the outcome of patients exhibiting over-expression of HER2 was typically poor until the advent of targeted therapies almost 15 years ago [51]. HER2 is a member of the HER family which comprises the EGF receptor, also referred to as HER1, and HER3 and HER4 [123]. Upon ligand binding to the extracellular domain, the HER proteins undergo dimerisation and transphosphorylation leading to downstream activation of PI3K/Akt and MAPK signalling pathways [124]. HER2 does not have a ligand and relies on homo- or hetero-dimerisation with other HER members to induce cell proliferation. Over-expression of HER2 in breast cancer cells leads to constitutive activation of the receptor, independently of ligand binding to the other HER family members. The two most successful therapies approved in the clinic to block constitutive activation of HER2 in breast cancer include the monoclonal antibody, trastuzumab, also referred to as Herceptin, and the small molecule inhibitor, lapatinib. Several possible mechanisms by which Herceptin might decrease HER2 signaling include prevention of HER2 dimerisation, immune activation or increased endocytic destruction of the receptor [125] [149]. Alternatively, lapatinib acts by directly inhibiting the tyrosine kinase activity of the intracellular domain of HER2 and HER1 [126]. Furthermore, the recent development of antibody-drug conjugates such as Trastuzumab Emtansine (T-DM1) allow targeted delivery of the cytotoxic agent DM1 specifically to tumour cells overexpressing HER2.

Despite the efficacy of these two drugs in a subset of patients, inherent and acquired resistance to HER2 directed therapies is very common [127]. Mechanisms of resistance to trastuzumab include steric changes, such as structural alterations in the HER2 protein; elevated activation of alternate receptors, such as the insulin-like growth factor receptor (IGFR); intracellular activation of alternate signaling pathways to compensate for the loss of HER2 signaling, such as loss of phosphatase and tensin homolog (PTEN) and/or PI3K/Akt constitutive activation (reviewed in [125]).

Mitogen-activated protein kinase (MAPK) signalling pathways have previously been demonstrated to be constitutively active as a result of HER2 overexpression [124]. In particular, the extracellular-regulated protein kinase 5 (ERK5), one of the least well-studied members of the MAPK family, is constitutively

hyperphosphorylated in HER2-expressing breast cancer cells and has also been shown to facilitate the action of trastuzumab [53] [99]. The latter was demonstrated by the introduction of a dominant negative form of ERK5 which potentiated the inhibition of HER2+ mediated growth by trastuzumab. This observation was the first evidence to suggest that ERK5 was important for transducing the oncogenic signal downstream of the HER2 receptor. Further studies have also identified the ERK5 signalling pathway in drug resistance [59] [103] [128]. In particular, a study by Weldon *et al*. demonstrated that MEK5 promoted chemoresistance in MCF-7 cells and the introduction of a dominant negative form of ERK5 would sensitise the cells to treatment-induced death [129]. Furthermore, Song *et al*. demonstrated ERK5 promoted resistance to BRAF-MEK1/2 inhibition in melanoma [103]. In this study, we assessed the suitability of a novel therapeutic strategy based on targeting ERK5 to combat drug resistance in the context of HER2+ breast cancer.

RESULTS

ERK5 hyperactivation associated with poor prognosis in HER2+ breast cancer patients. To assess the impact of ERK5 in breast cancer, we analysed the link between the level of ERK5 expression and relapse-free survival (RFS) in patients using published gene expression datasets from the Kaplan-Meier Plotter (KM Plotter) online tool [130]. Combined analysis of all breast cancer subtypes showed that patients exhibiting high ERK5 expression displayed a modest, nonetheless noticeable, increased probability of survival than patients with low ERK5 expression (**Fig. 1A**). In contrast, high ERK5 expression significantly decreased RFS in HER2+ breast cancer (**Fig. 1A**). This indicated that the level of ERK5 correlated with an inferior outcome for this specific group of patients. Further analysis demonstrated that the HER2+ subtype did not exhibit elevated expression of ERK5 in comparison to HER2- subtypes (www.cbioportal.org) (**Fig. 1B**). Furthermore, no mutations in ERK5 were identified in this patient cohort (www.cbioportal.org).

To establish a molecular link between ERK5 and HER2-mediated signalling we analysed human breast cancer cell lines, namely BT474 (ER+/PR+/HER2+; luminal B) and SKBR3 (ER-/PR-/HER2+), which have been extensively used to model HER2+ overexpressing cancer subtypes [131]. Importantly, both BT474 and SKBR3 are highly proliferative and responsive to trastuzumab and lapatinib treatment. The MDA-MB-231 cell line which represents a basal subtype (ER-/PR-/HER2-) and MCF-7, representing a luminal A subtype (ER+/PR+/HER2-) were used for comparison [131]. Consistent with previously published data, ERK5 expressed in HER2+ cell lines exhibited a mobility shift by SDS-PAGE indicative

of hyper-phosphorylation [21] [99] (**Fig. 1C**). This was not observed in MDA-MB-231 cells (**Fig. 1C**). Likewise, the electrophoretic retarded migration of ERK5 disappeared in BT474 cells incubated with XMD8-92, JWG-045 or AX15836 to inhibit ERK5 (**Fig. 1C-E**) or with an inhibitor of MEK5, namely BIX02189 (**Fig. 1F**). These ERK5/MEK5 inhibitors have different specificities (IC_{50s}), specifically XMD8-92 (80nM) [120], JWG-045 (20nM) [120], AX15836 (8nM) [132] and BIX02189 (1.5nM) [148] Various ERK5/MEK5 inhibitors were used to account for the possibilities of off-target effects. Together, these results indicated that ERK5 was autophosphorylated as a consequence of hyperactivation in breast cancer cells overexpressing HER2.

Pharmacological inhibition of ERK5 signalling does not affect the proliferation of HER2+ breast cancer cells. Next, we investigated the requirement of ERK5 in the proliferation of HER2+ breast cancer cells by testing the effect of ERK5 inhibition. We found that the proliferation of BT474 and SKBR3 was significantly impaired in the presence of XMD8-92 over 5 days (**Fig. 2A**). In contrast, treatment of the cells with AX15836 (2 μM) or JWG-045 (3 μM) had minimal effect (**Fig. 2B**). A similar observation was made when assessing the effect of ERK5 inhibitors on MDA-MB-231 cells (**Fig. 2**). In light of these results and considering the known off target effect of XMD8-92 on BRD4 [132] [120], we concluded that ERK5 signalling was not essential for transducing the mitogenic effect of HER2 in breast cancer cells.

Next, we compared the impact of inhibiting MEK5 for 48 h (BIX; 3 μM) and blocking HER2 activation using lapatinib (Lap; 1 μM), over 48 hours. Two lapatinib resistant HER2+ breast cancer cell lines, namely MD-MBA-453 and BT474-LR were included in the study. We confirmed that ERK5 was specifically hyperphosphorylated in these cell lines over-expressing HER2 (**Fig. 3A**). Interestingly, activity appeared to be further elevated in resistant lines. As expected, the viability of BT474 and SKBR3 cells was significantly reduced in presence of lapatinib, whilst MD-MBA-453 and BT474-LR were relatively more resistant to this treatment (**Fig. 3B and C**). HER2- breast cancer cell lines were the least sensitive to lapatinib (**Fig. 3B and C**). In contrast, MEK5 inhibition had no effect on breast cancer cell viability, except in MDA-MB-453 and MDA-MB-231 where we observe a small reduction. These results indicated that ERK5 signalling was not essential for supporting the survival of breast cancer cells.

Combined inhibition of ERK5 and HER2 suppresses resistant tumour cell proliferation. Given the clinical link between ERK5 expression and early reoccurrence post-treatment (**Fig. 1**), we hypothesised that ERK5 could be involved in the resistance of HER2+ breast cancer to current treatments. Consequently, targeting ERK5 in this subtype of breast cancer may be more efficacious in the context of

resistance. This idea is further supported by the recent demonstration that combined inhibition of ERK5 and BRAF was more effective than BRAF inhibition alone for the treatment of melanoma harbouring the BRAFV600E mutation [104]. Thus, we investigated the potential additive effect of ERK5 inhibition and HER2 directed therapies in HER2+ breast cancer resistant cell lines.

Both sensitive (BT474/SKBR3) and lapatinib resistant (MDA-MB-453, MDA-MB-468 and MDA-MB-361) cell lines were incubated with BIX02189 (BIX; 3 μM) or JWG-045 (JWG; 3 μM) to inhibit MEK5 or ERK5, respectively, together with or without lapatinib (Lap; 1 μM) or herceptin (Her; 100 μg/ml), over 96 hours (**Fig. 4**). Consistent with our previous findings (**Fig. 3B** and **C**), BT474 and SKBR3 cell lines displayed sensitivity to lapatinib (around 80-90% reduction in cell viability), and to a lesser extent to herceptin treatment (around 40-80% reduction in cell viability), but inhibition of ERK5 signalling did not affect their viability (**Fig. 3C, 4A** and **B**). Moreover, MEK5 or ERK5 inhibitors did not enhance lapatinib or herceptin toxicity in these cell lines (**Fig. 4A** and **B**). As expected, MDA-MB-453, MDA-MB-468 and MDA-MB-361 cell lines were generally less sensitive to HER2 targeted therapies than BT474 and SKBR3 (**Fig. 3B**, **4A** and **4C**). Specifically, herceptin induced between 15% and 35% reduction in cell viability and lapatinib between 15% to 65%, depending on the cell types (**Fig. 3C**, **4A** and **4C**). [*We acknowledge that the effect of lapatinib in MDA-MB-453 cells is inconsistent. Figure 3C shows the results from a 48hr timepoint while Figure 4C shows the results from a 96 hour time point at different seeding densities. Furthermore, results for MDA-MB-468 and MDA-MB-361 represent one biological repeat. Figure 4 requires more biological repeats to gain greater confidence in differences observed*]. Similar to previous observations (**Fig. 3C**), we demonstrated that BIX02189 treatment alone would marginally decrease cell viability in MDA-MB-453 cells. The reduction in cell viability in MDA-MB-468 and MDA-MB-361 cells was less evident (**Fig. 4A** and **C**). The treatment of JWG-045 alone produced a similar effect. Likewise, the effect of combining lapatinib with BIX02189 in MDA-MB-453 cells provided a small but significant reduction in cell viability compared to lapatinib alone, while the combined effect of treatment in MDA-MB-468 and MDA-MB-361 cells was not significantly different (**Fig. 4A** and **C**). Interestingly, unlike combined BIX02189 and lapatinib, preliminary observations suggested that combined treatment of JWG-045 with lapatinib provided a synergistic reduction in cell viability in MDA-MB-468 and MDA-MB-361 cells (**Fig. 4A** and **C**). Further work will be required to establish more confidence in this finding. Based on these observations, we decided to explore the cellular basis underlying the additive effect of combining BIX02189 with lapatinib treatment in MDA-MB-453 cells. Therefore we investigated proliferative changes in SKBR3 and MDA-MB-453 cells upon combined treatment with BIX02189 and lapatinib.

Combined treatment of lapatinib and BIX inhibits G1/S transition in resistant cells. We assessed proliferative changes by performing cell cycle analysis in a sensitive cell line (SKBR3) and a resistant line (MDA-MB-453) (**Fig. 5**). We observed that MEK5 inhibition or lapatinib treatment increased the proportion of SKBR3 cells in G0/G1. This correlated with a reduction in the number of cells in S phase. A similar pattern was observed in MDA-MB-453 cells following treatment however with a significant additional reduction in cells in G2/M. There was also evidence that these cells were more resistant to lapatinib. (**Fig. 5**). In particular, lapatinib was much less efficient at decreasing the number of MDA-MB-453 cells in S phase. Interestingly, a significant difference was observed between the effect of BIX and lapatinib on G2/M. Specifically, lapatinib increased, whilst BIX reduced, the number of cells in G2/M in both cell lines (**Fig. 5**). As expected, BIX did not influence the G0/G1 and S phase responses of SKBR3 cells to lapatinib treatment, but slightly reduced the decreased proportion of cells in G2/M (**Fig. 5**). In contrast, BIX enhanced the ability of lapatinib to reduce the proportion of MDA-MB-453 cells in S phase (**Fig. 5**). Combined treatment also caused an increased proportion of cells in G0/G1 to a level similar to that caused by BIX treatment alone (**Fig. 5**). Collectively, this data indicated that combined therapy in resistant cells reduced cell viability by inhibiting G1/S transition.

Combined treatment of lapatinib and BIX inhibits G1/S transition by suppressing phosphorylation of Rb. To explain the molecular basis underlying the additive effect of MEK5 and HER2 inhibition in breast cancer cells resistant to HER2 targeted therapies, we investigated changes in the components of signal transduction pathways involved in controlling cell proliferation. Initially, we confirmed that BIX inhibited ERK5 phosphorylation as indicated by the reduction of the electrophoretic mobility shift of the protein in MDA-MB-453 cells (**Fig. 6**). [*We acknowledge that the shift in SKBR3 cells is not apparent in this experiment. However, we have previously demonstrated that the shift is present in SKBR3 cells in Figure 1C and that BIX02189 can inhibit ERK5 activity in BT474 cells at the 3μM concentration. Requires further repeats*]. We noticed that lapatinib only partially reduced ERK5 phosphorylation in MDA-MB-453 cells (**Fig. 6**). This might indicate that ERK5 activity is partially regulated independently of HER2 signalling in MDA-MB-453 cells. As expected, lapatinib treatment blocked tyrosine phosphorylation of HER2, HER3 and EGFR in both cell lines, without affecting the level of protein expression (**Fig. 6**). However, a residual level of phospho-Tyr EGFR could be detected in MDA-MB-453 cells after lapatinib treatment, potentially suggesting resistance may derive from insensitivity of HER1 to treatment (**Fig. 6**). Consistent with the blockade of HER2/3 and EGFR signalling, ERK1/2 phosphorylation was markedly reduced in SKBR3 treated with lapatinib (**Fig. 6**). Surprisingly, MDA-MB-453 cells exhibited a very low basal level of ERK1/2 phosphorylation which was not affected by lapatinib treatment (**Fig. 6**). In contrast, SKBR3 and MDA-MB-453 cells displayed a similar level of phosphorylated AKT, which was significantly reduced by lapatinib (**Fig. 6**). Interestingly, we could notice a slight increase in the level of phospho-AKT in MDA-MB-453 cells exposed to BIX, indicative of a potential negative feedback loop exerted by ERK5 in resistant cells.

Based on our previous demonstration that BIX and lapatinib exhibited distinct effects on the cell cycle led us to focus our analysis on key mediators of cell proliferation, with a particular focus on regulators of the G1/S transition. The Rb protein plays a pivotal role in the negative control of the cell cycle by suppressing G1/S transition in an unphosphorylated state. As expected, the resistant breast cancer cell line exhibited a higher level of phosphorylated Rb, compared to sensitive cells (**Fig. 6**). This is consistent with an increased proportion of cells in S phase in MDA-MB-453 cells compared to SKBR3 (**Fig. 5**). Interestingly, we observed that BIX inhibition had a more significant effect on expression and activation of Rb in the MDA-MB-453 cells compared to the SKBR3 cells. The addition of BIX alone reduced expression levels of Rb while in combination with lapatinib further reduced levels of phosphorylated Rb. An additive reduction of pRb in the resistant line was consistent with the G1/S arrest observed in our previous findings (**Fig 5**). This was in contrast to the effect of lapatinib in the two cell lines. In the sensitive line, lapatinib reduced expression and phosphorylation of Rb which was not affected by the addition of BIX. To further establish the molecular basis for G1/S arrest we investigated expression and

activity of important regulators of this checkpoint. We therefore examined the changes in the level of G1 checkpoint proteins. Cyclin D1 typically accumulates over G1 and S phase and is reduced during G2/M. In MDA-MB-453 cells we observe a significant increase in cyclin D1 with BIX inhibition which is then reduced upon lapatinib treatment, with or without BIX. This is consistent with a significant reduction of cells in G2/M and an increase of cells in G0/1 with BIX treatment (**Fig 5**). After the addition of lapatinib we see an increase of cells in G2/M but a further increase of cells in G0/G1 (**Fig 5**). This observation correlates with the proportion of cyclin D1 which is present in the different treatment conditions (**Fig 6**). However, the effect in SKBR3 cells is less clear due to low levels of cyclin D1 expressed. BIX02189 does not appear to affect cyclin D1 levels while lapatinib reduced cyclin D1 expression. Surprisingly, we also observed a significant reduction in p21 expression with combination treatment in both cell lines. As p21 is cyclin dependent kinase inhibitor, known to suppress G1/S transition, it is clear that our observations are not controlled regulated by p21. Based on our data, we suggest that combined treatment of BIX and lapatinib reduces cell viability in resistant cells primarily by reducing pRb levels and therefore inducing a G1/S cell cycle block.

DISCUSSION

In this study, we demonstrated that combined MEK5/ERK5 and HER2 inhibition suppresses proliferation in HER2+ breast cancer cells resistant to lapatinib. We have identified that inhibition of proliferation is caused by a G1 arrest as a probable consequence of suppressed Rb phosphorylation. Our evidence indicates that ERK5 regulated Rb expression and activity in MDA-MB-453 cell lines.

Our study corroborates previously published reports that have identified constitutive activation of ERK5 in HER2 overexpressing cell lines [99] [53]. These reports demonstrated that the introduction of a dominant negative form of ERK5 impeded HER2+ mediated growth but did not examine the effect of pharmacological inhibition. For example, in a study by Montero *et al*. ERK5 knockdown was shown to facilitate the action of herceptin in HER2+ overexpressing cells [53]. Our findings, based on using pharmacological inhibitors differ from these results. We demonstrated that inhibition of the MEK5/ERK5 pathway as a monotherapy did not significantly inhibit HER2+ mediated growth. However, we found that ERK5 inhibition could facilitate the action of lapatinib specifically in HER2+ cell lines resistant to therapy. Interestingly, different MEK5/ERK5 inhibitors had distinct effects, dependent on the cell line. We observed an additive effect of BIX inhibition with lapatinib, specifically in MDA-MB-453 lines but not in MDA-MB-468 or MDA-MB-361 lines. However, we observed a strong effect of combined treatment of lapatinib/JWG in MDA-MB-468 and MDA-MB-361 lines. As BIX is a specific MEK5

inhibitor while JWG is a specific ERK5 inhibitor, we hypothesise that ERK5 may be activated independently of MEK5 in these cell lines. Several reports have suggested that ERK1/2 and CDK1 can regulate ERK5 activity independently of MEK5 [39] [41]. Based on these findings it is pertinent to assess whether ERK5 activity is dependent on MEK5 in MDA-MB-468 and MDA-MB-361 lines.

To identify the distinct function of ERK5 in resistant lines, we compared the signal transduction pathways driving HER2 malignancy in sensitive and resistant lines. Previous reports have identified several modes of HER2+ resistance [79]. Significantly, a recent study in Cancer Cell outlined that cyclin D1 and CDK4/6 activity drive resistance by promoting Rb phosphorylation [133]. Our findings also support the key role of phosphorylated Rb in promoting ERK5-mediated HER2+ resistance. This observation is consistent with landmarks studies demonstrating that Rb is a critical mediator of G1/S transition [134]. The next investigations should focus on elucidating the mechanism whereby ERK5 regulates phosphorylation of Rb and clarify this effect using genetic silencing. Given that a number of factors regulate Rb phosphorylation, several candidates should be further investigated. For example, it is possible that ERK5 may regulate Rb via phosphorylation of CDK4/6 or CDK2 [135] [136]. Further candidates for investigation should also include the cyclin-dependent kinase inhibitors that regulate G1/S transition, including p27 [137]. It is also imperative to identify whether ERK5 participates in the regulation of pRB in other lapatinib resistant lines. To further assess whether combined treatment of resistant forms of breast cancer is a viable strategy, it is also required to test this therapeutic strategy *in vivo*.

MATERIALS AND METHODS

Cell culture. Breast cancer cells were cultured in Dulbecco's Modified Eagles Medium (DMEM, Sigma) at 37^0C, 5% CO2, containing 10% Foetal calf serum (FCS, Gibco), and 1% Penicillin/Streptomcycin (100 μg/ml) (Sigma). Cells were split at approximately 60% confluency using Hank's Balanced Salt Solution (HBSS) (Sigma) and Trypsin (Sigma). Cells were counted using a hemocytometer and Tryphan blue stain. Cell lines were preserved using freezing media (FBS containing 10% dimethyl sulfoxide -DMSO) (Sigma) and these cells were used as stock (approximately $5x10^6$ per ml) and stored at -80^0C. The BT474 and SKBR3 cells were gifted to us by Dr Hannah Harrison. We obtained the MDA-MB-453, MDA-MB-361 and MDA-MB-468 cells from ATCC. The BT474-LR cells were generated by the laboratory of M. Cross, University of Liverpool by dose ramping with lapatinib from 0.01μM to 0.1μM over 10 weeks.

Immunoblot analysis. Proteins were extracted from cells in radioimmunoprecipitation assay (RIPA) buffer (Sigma) containing EDTA-free protease inhibitor cocktail (Roche). Extracts (50μg) were resolved by SDS-PAGE and electrophoretically transferred to an Immobilon-P membrane (Millipore, Inc). The membranes were saturated in 3% nonfat dry milk and probed overnight at 4^{o}C with a primary antibody (1:1000) to anti-ERK5 (C.S.T), anti-HER2 (C.S.T), anti-pHER2 (Y1196) (C.S.T), anti-pHER3 (Y1289) (C.S.T), anti-EGFR (C.S.T), anti-pEGFR (Y1172) (C.S.T), anti-ERK 1/2 (C.S.T), anti-pERK 1/2 (C.S.T), anti-Akt (C.S.T), anti-pAkt (S473) (C.S.T), anti-Rb (C.S.T), anti-pRb (S780) (C.S.T), anti-Cyclin E1 (C.S.T), anti-p21 (C.S.T), anti-p27 (C.S.T), anti-STAT3 (C.S.T), anti-pSTAT3 (S727) (C.S.T) and anti-Beta tubulin (C.S.T). Immunocomplexes were detected by enhanced chemiluminescence with IgG coupled to horseradish peroxidase as the secondary antibody (GE Healthcare).

FACS analysis. Cells were collected by centrifugation (1200 rpm, 3 min), washed with 1 ml phosphate buffered saline (PBS, Sigma-Aldrich) and fixed overnight in 70% ethanol diluted in PBS. The following day, the cells were collected by centrifugation (3000 rpm, 2 min), washed twice in 1 ml PBS and re-suspended in 500 ul propidium iodide (PI) diluted in RNase staining solution (C.S.T) for 1 h at room temperature, prior to being analysed by flow cytometer.

Proliferation assays using crystal violet. To assess cell viability in the presence of absence of BIX 01289 (Tocris, 3μM) or lapatinib over 48 hours (C.S.T, 1μM); 60,000 cells (BT474/BT474-LR), 25,000 (SKBR3) and 50,000 (MDA-MB-453), 25,000 MDA-MB-231 and 50,000 MCF-7 cells were seeded per well in a 96 well plate (Corning). After 24 hours media is replaced with fresh DMEM containing Lapatinib (1μM) or BIX 01289 (3μM). For the 96 hour timecourse, the seeding density was halved and new media containing Lapatinib (1μM), BIX 01289 (3μM), JWG-045 (3μM), Herceptin (Roche, 100ug/ml) was replaced. For the 120 hour timecourse, cell density was scaled up for a 12 well plate. Cells were washed with Phosphate buffered saline (PBS, Sigma) once and fixed using 3.7% paraformaldehyde at 4°C overnight. The following day, cells were washed with distilled water and stained using crystal violet. Cells were left to dry and then associated dye is removed using 20% acetic acid and optical density was measured at 590nM as a proxy for cell number. Optical densities were imported into Prism 7 (GraphPad) for statistical analysis and presentation.

ACKNOWLEDGEMENTS

I would like to thank Jingwei Zhang for his critical contributions to this project, particularly during the FACS analysis and immunoblotting. I would like to acknowledge Dr Hannah Harrison from the Paul

Shore lab at the University of Manchester for providing breast cancer cell lines for experimental use. We would also like the thank Dr Michael Cross from the University of Liverpool for providing us with the BT474-LR cells.

FIGURE LEGENDS

Figure 1: ERK5 expression and activation in HER2+ expressing cells A) Kaplan-Meier plots of relapse free survival with respect to ERK5 levels in patients of breast cancer subtypes and HER2+ breast cancer patients. Breast cancer patients of all subtypes (n=3951) and HER2+ breast cancer patients (n=252) with high expression of ERK5 (red) were compared to patients with low expression (black). Data was retrieved from http://kmplot.com/analysis/ **B**) mRNA expression of ERK5 was quantified and compared in patients depending on their HER2 status. Data was retrieved from http://www.cbioportal.org/ **C/D/E**) Cell lysates from MDA-MB-231, BT-474 and SKBR3 were harvested subsequent to preincubation with XMD8-92 (5μM), AX15836 (0-3μM), BIX02189 (0-3μM) and JWG-045 (0-3μM) where indicated.

Figure 2: ERK5 inhibition does not affect cell viability of HER2+ overexpressing cells. A) Cells were seeded in 5x 12 well plate 24 hours prior to treatment. DMSO, XMD8-92 (5μM) JWG-045(3μM) or AX15836 (2μM) treatment was applied where indicated for 120 hours. Cells were fixed and stained with crystal violet every 24 hours. Proliferation data was inserted into a graph form. The data represent the mean ± SD of three independent experiments performed in triplicate for the BT474 and SKBR3 cells and one independent experiment for the MDA-MB-231 cells. *, $P < 0.05$ indicates significant differences. **, $P < 0.01$. ***, $P < 0.001$. ****, $P < 0.0001$. ns, indicates no statistical difference.

Figure 3: ERK5 expression and activity in HER2 negative, HER2 positive and HER2 resistant lines A) Cell lysates from BT-474, MDA-MB-453, BT-LR and MCF-7 and SKBR3 were harvested and probed for ERK5 expression. Beta tubulin was used to monitor protein loading. **B**) Cells were seeded in a 96 well plate 24 hours prior to treatment. DMSO, BIX02189 (3μM) or lapatinib (1μM) treatment was applied where indicated for 48 hours. Cells were fixed and stained with crystal violet after 48 hours of treatment. **C**) The dye was removed, quantified and inserted into a graph.

Figure 4: Combined lapatinib and BIX treatment reduced cell viability in MDA-MB-453 cells. A) Cells were seeded in a 96 well plate 24 hours prior to treatment. DMSO, BIX02189 (3μM), JWG-045

(3μM), lapatinib (1μM), herceptin (100μg/ml) treatment were applied where indicated. Cells were fixed and stained with crystal violet after 96 hours of treatment. The data represents the mean ± SD from two independent experiments performed in duplicate for BT474, SKBR3 and MDA-MB-453 cells while performed once for the MDA-MB-468 or MDA-MB-361 cells. **B/C**) The dye was removed, quantified and inserted into a graph.

Figure 5: Combined lapatinib and BIX treatment caused inhibited G1/S transition Cell cycle analysis was performed by FACs analysis. Data shown are mean ±SD from two independent experiments performed in duplicates. **B)** Percentage decrease of cells in S phase was quantified and illustrated in a graph

Figure 6: Combined lapatinib and BIX treatment suppresses phosphorylation of RB A). Cell lysates from SKBR3 and MDA-MB-453 were harvested subsequent to preincubation with DMSO, BIX02189 (3μM) or lapatinib (1μM) for 24 hours. Cell lysates were probed with the antibodies shown.

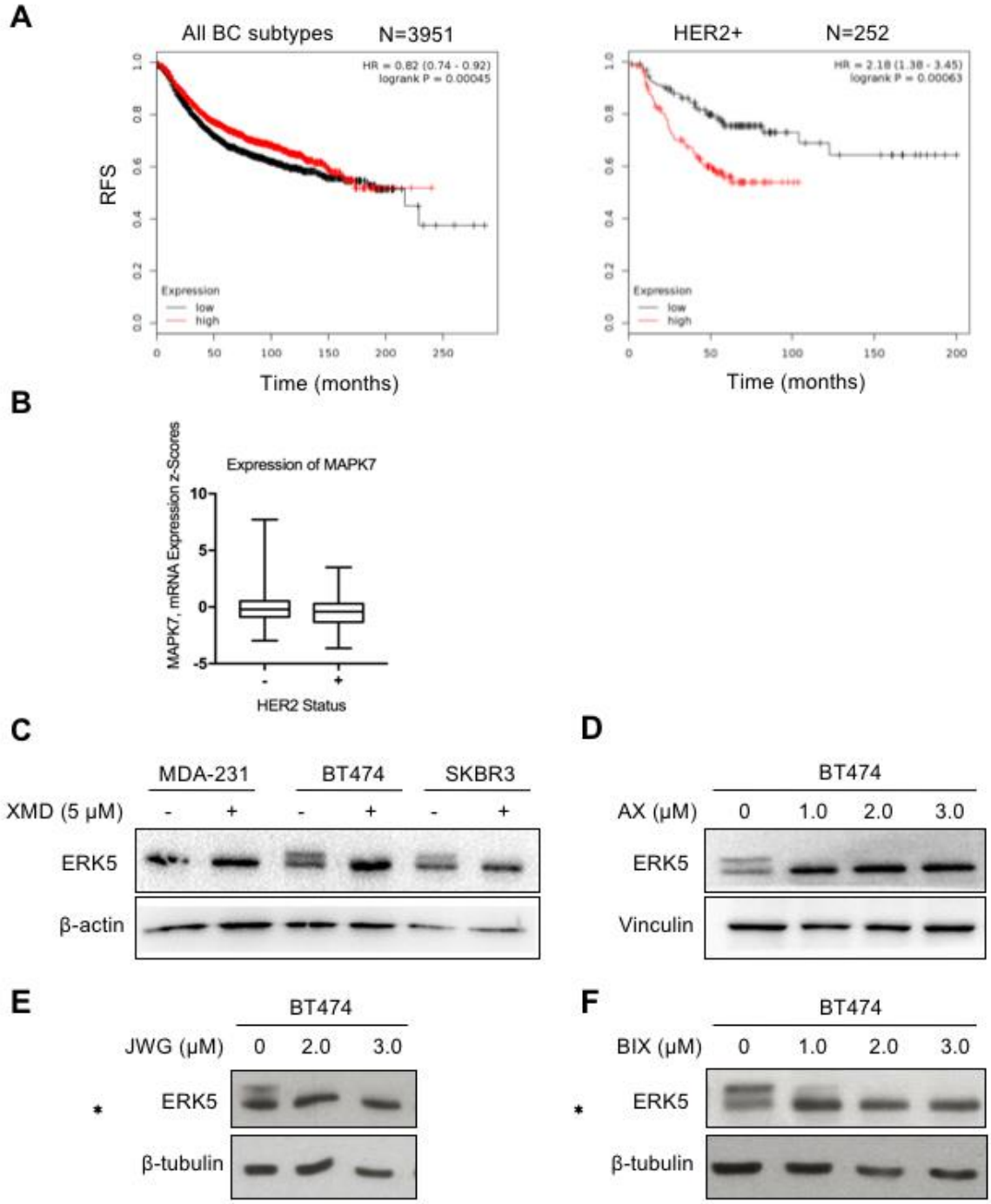

A
All BC subtypes N=3951
HR = 0.82 (0.74 - 0.92)
logrank P = 0.00045
RFS
Expression
low
high
Time (months)
HER2+ N=252
HR = 2.18 (1.38 - 3.45)
logrank P = 0.00063
Expression
low
high
Time (months)
B
Expression of MAPK7
MAPK7, mRNA Expression z-Scores
HER2 Status
C
MDA-231
BT474
SKBR3
XMD (5 μM)
ERK5
β-actin
D
BT474
AX (μM)
0
1.0
2.0
3.0
ERK5
Vinculin
E
BT474
JWG (μM)
0
2.0
3.0
ERK5
β-tubulin
F
BT474
BIX (μM)
0
1.0
2.0
3.0
ERK5
β-tubulin

FIGURE 1

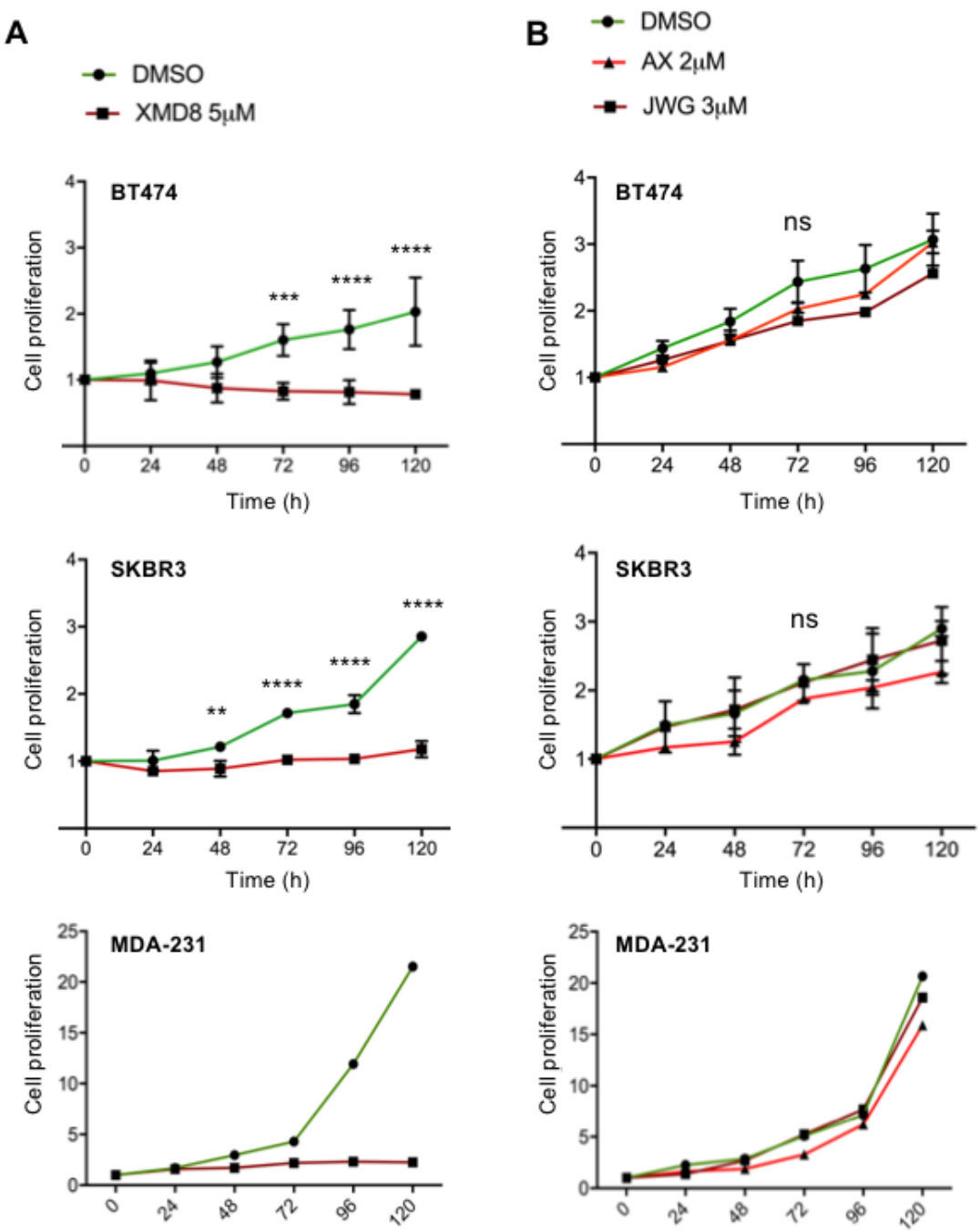

A
DMSO
XMD8 5μM
BT474
Cell proliferation
Time (h)
***
****
****
SKBR3
**
****
****
MDA-231
B
DMSO
AX 2μM
JWG 3μM
BT474
ns
SKBR3
ns
MDA-231

FIGURE 2

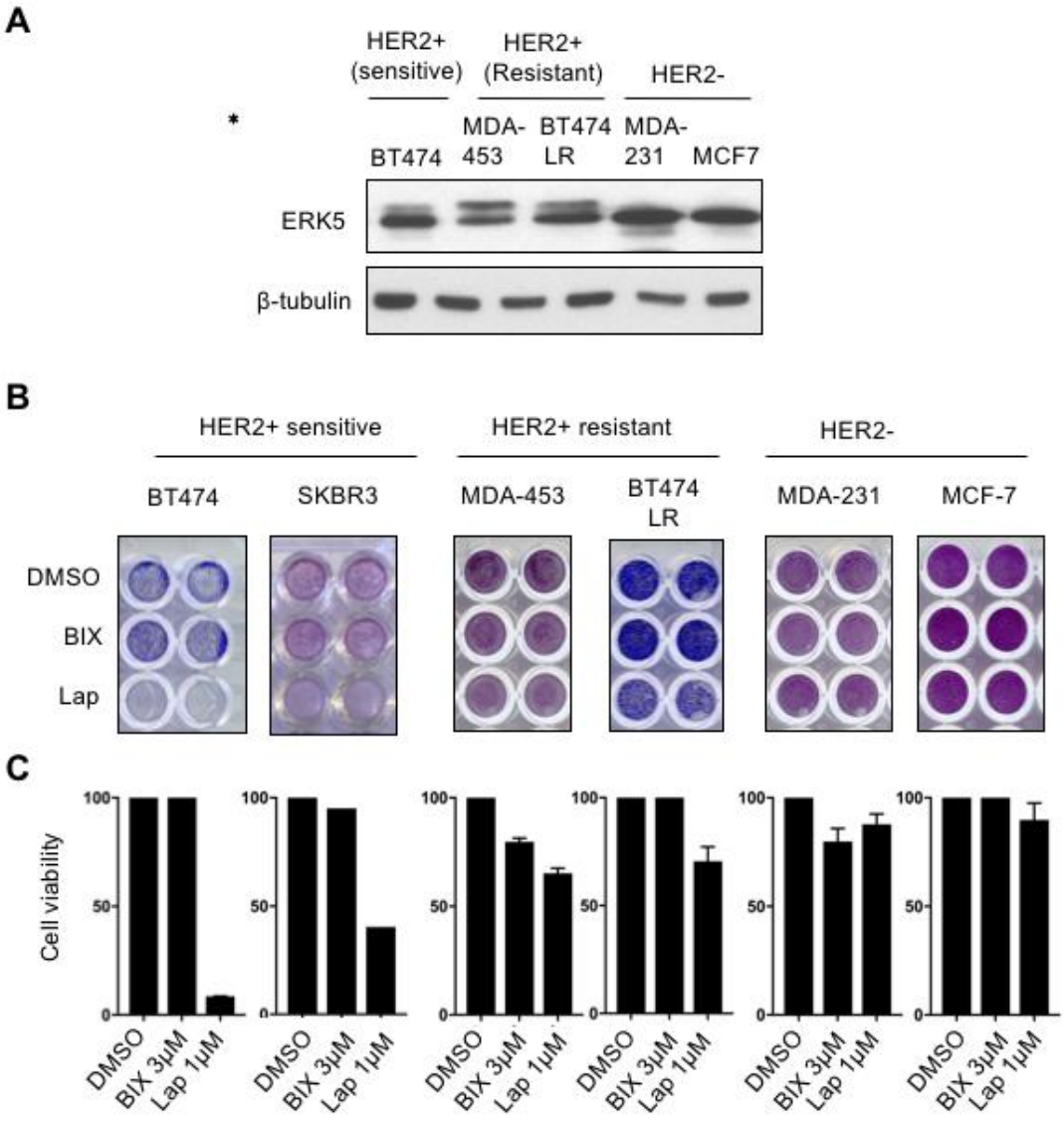

A
HER2+ (sensitive)
HER2+ (Resistant)
HER2-
*
BT474
MDA-453
BT474 LR
MDA-231
MCF7
ERK5
β-tubulin
B
HER2+ sensitive
HER2+ resistant
HER2-
BT474
SKBR3
MDA-453
BT474 LR
MDA-231
MCF-7
DMSO
BIX
Lap
C
Cell viability
100
50
0
DMSO
BIX 3μM
Lap 1μM

FIGURE 3

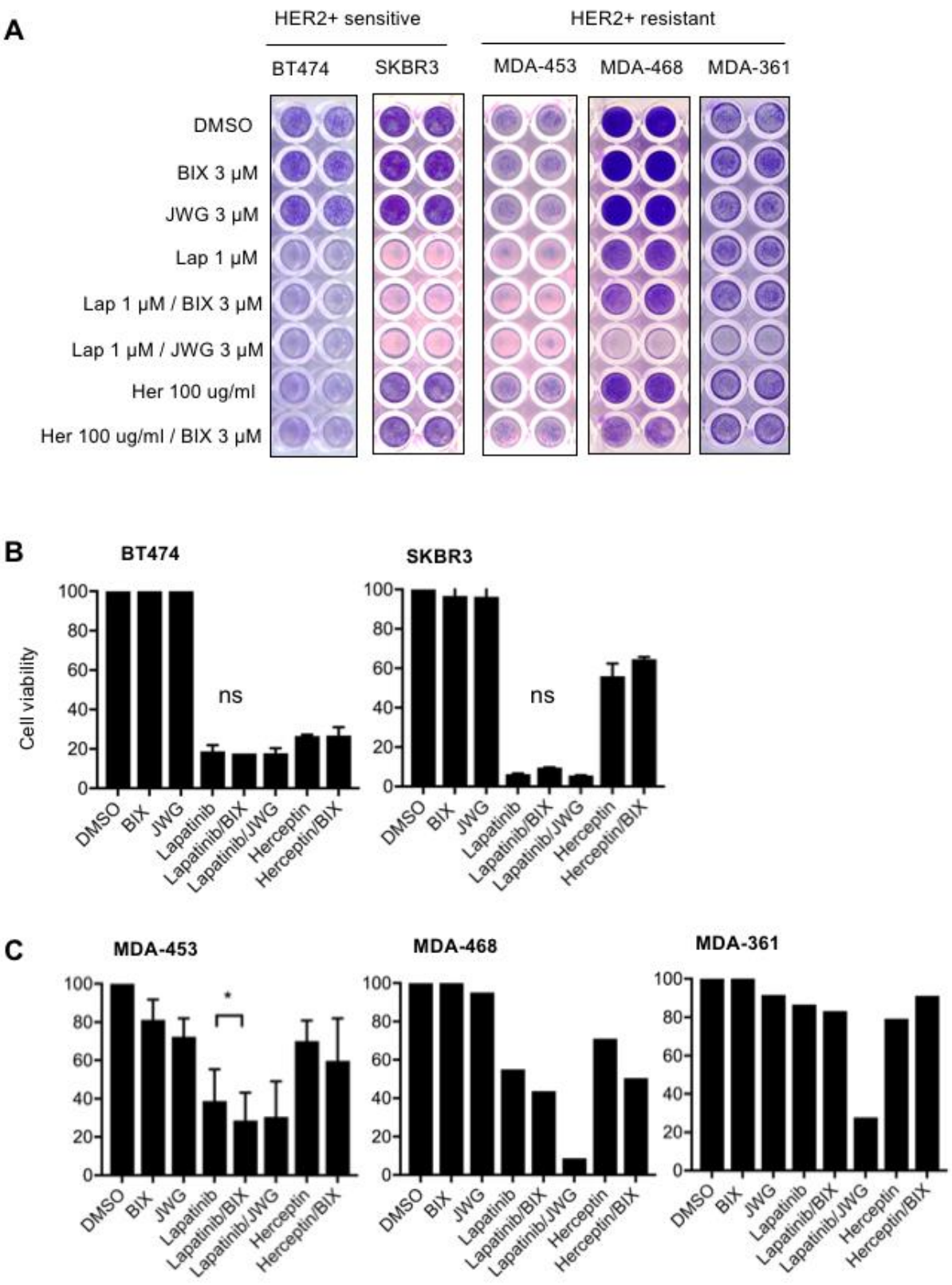
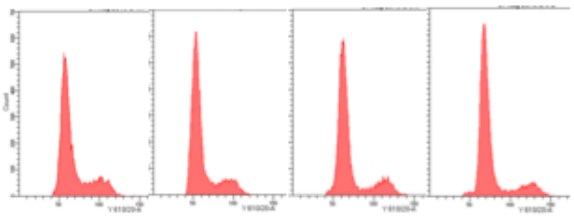

A
HER2+ sensitive
HER2+ resistant
BT474
SKBR3
MDA-453
MDA-468
MDA-361
DMSO
BIX 3 µM
JWG 3 µM
Lap 1 µM
Lap 1 µM / BIX 3 µM
Lap 1 µM / JWG 3 µM
Her 100 ug/ml
Her 100 ug/ml / BIX 3 µM
B
BT474
SKBR3
Cell viability
ns
ns
DMSO
BIX
JWG
Lapatinib
Lapatinib/BIX
Lapatinib/JWG
Herceptin
Herceptin/BIX
C
MDA-453
MDA-468
MDA-361
*

FIGURE 4

A

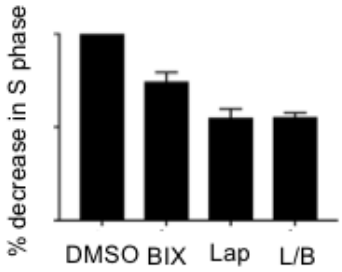
SKBR3



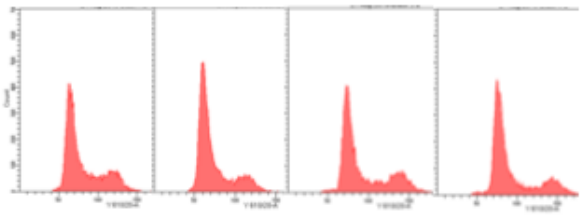
DMSO BIX Lap BIX/Lap

Cell cycle	% cells	% cells	% cells	% cells
G0/G1	63.5 ±0.4	71.1 ±1.6	73.7 ±0.7	75.2 ±0.7
S	28.4 ±0.4	21.1 ±1.6	15.6 ± 0.8	15.7 ±0.8
G2/M	8.1 ±0.7	7.8 ±0	10.8 ± 0.2	9.1 ±0.2

B



MDA453



DMSO BIX Lap BIX/Lap

Cell cycle	% cells	% cells	% cells	% cells
G0/G1	49.1 ±0.1	59.0 ±0.2	52.1 ±1.5	60.3 ±1.4
S	41.6 ±0.3	37.1 ±1.2	34.7 ±1.9	28.6 ±1.7
G2/M	9.3 ±0.4	3.9 ±1.0	13.3 ±0.3	11.1 ±0.3

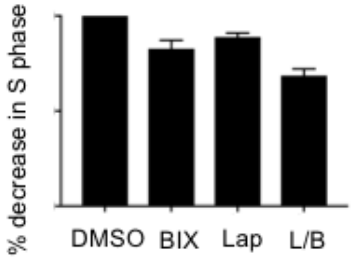


FIGURE 5

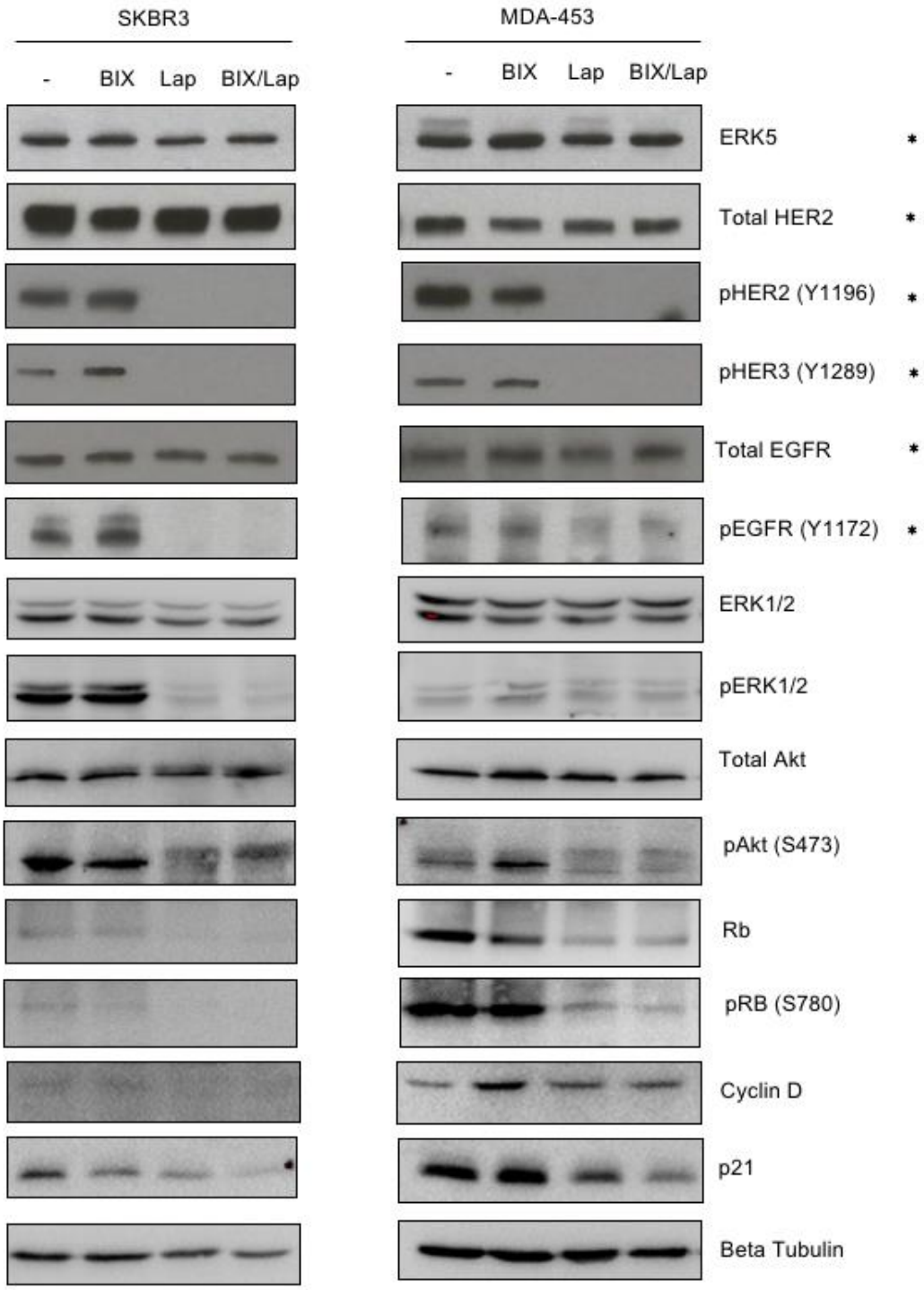

SKBR3
MDA-453
- BIX Lap BIX/Lap
- BIX Lap BIX/Lap
ERK5 *
Total HER2 *
pHER2 (Y1196) *
pHER3 (Y1289) *
Total EGFR *
pEGFR (Y1172) *
ERK1/2
pERK1/2
Total Akt
pAkt (S473)
Rb
pRB (S780)
Cyclin D
p21
Beta Tubulin

FIGURE 6

Discussion

Chapter 3: Threonine 732 is a gatekeeper residue implicated in ERK5-meduated transcription

5.1.1 Summary

Chapter 1 aimed at elucidating the functional relevance of post-translational modification at the C terminus of ERK5. This was based on previous demonstrations that this region contained an NLS and a transactivation domain [21] [39]. Multiple autophosphorylation sites located in the C terminal tail of ERK5 were subsequently identified [105]. By employing a site-directed mutagenesis approach, I found that one of these residues, Thr^{732}, acted a gatekeeper residue required for C terminal phosphorylation, nuclear localisation and ERK5-mediated transcription. Furthermore, I discovered that Thr^{732} phosphorylation increased the affinity of ERK5 for chromatin, thereby providing a mechanism by which the C terminus of ERK5 regulates transcription through the recruitment of ERK5 to specific promoter regions in the genome. Overall, this chapter built upon previous research on ERK5 by demonstrating the critical relevance of Thr^{732} residue and by providing mechanistic evidence to explain how C terminal phosphorylation enhances transcription.

5.1.2 The functional requirement of Thr^{732}

Kasler *et al*. first identified that the C terminal tail of ERK5 contained a potent transactivation domain [22]. This domain was further characterised by the identification of phosphorylation sites present within the tail, which would later be implicated in ERK5 function [21] [39] [105]. In 2007, Morimoto *et al*. carried out an extensive investigation into the phosphorylated residues in the C terminus of ERK5 [21]. Consistent with post-translational modification at these sites, activated ERK5 displayed mobility shift retardation by SDS-PAGE which was completely abolished by blocking phosphorylation at Thr^{732}, Ser^{769}, Ser^{773} and Ser^{775}. Interestingly, the pattern of ERK5 migration by gel electrophoresis was also noticeably impaired by the single replacement of Thr^{732} with an alanine [21]. A further study in 2010 by Elena Diaz-Rodriguez *et al*. also investigated phosphorylation at the C terminus of ERK5 during mitosis [39]. In addition to Thr^{732} and Ser^{773}, they found two novel sites, Ser^{706} and Ser^{753}, phosphorylated during mitosis. Similar to Morimoto *et al*. study, preventing phosphorylation at Ser^{706}, Thr^{732}, Ser^{753}, and Ser^{773} blocked the electrophoretic mobility shift retardation exhibited by activated ERK5. The sites modified in these two studies were the same residues modified in the ERK5-4xA_i [39] and ERK5-4xA_{ii} [21] mutants utilised

in my study. This was achieved by generating various phospho-mutants in which Ala or Glu residues replaced Ser or Thr residues present in the C terminal tail. Initially, I compared the ERK5-T732A and the ERK5-4xA_{ii} mutant. In contrast to their results, I found that both mutants exhibited a similar migratory defect by SDS-PAGE after stimulation. A technical discrepancy worth noting is that ERK5 activated by MEK5 was detected as three bands in both Elena Diaz-Rodriguez's and Morimoto's study, while I could only detect a doublet. This suggested that the gels I utilised for electrophoresis were less resolutive; hence I was unable to detect all phosphorylated forms of ERK5. A future experiment will be to re-analyse my samples using a Phos-Tag that allows specific separation of phosphorylation proteins. Thereby, firmly establishing the requirement of Thr^{732} in post-translational modification of the C terminal tail. Nonetheless, these findings concurred with our report in highlighting Thr^{732} as a critical site of phosphorylation in the C terminus of ERK5.

Several authors have demonstrated that phosphorylation occurs at the C terminal residues via autophosphorylation [21] [105] [138]. However, several recent reports have demonstrated that C terminal phosphorylation can occur independently of MEK5 activation [39] [41] [102]. The first was the demonstration that CDK1 mediated phosphorylation at Thr^{732} and Ser^{753} during mitosis [39]. A further study by Honda *et al*. in 2016 observed that in the presence of oncogenic RAS (V12), ERK2 formed a complex with ERK5 and promoted phosphorylation specifically at Thr^{732} [41]. Accordingly, U0126, a selective MEK1/2 inhibitor, reduced ERK5 phosphorylation at Thr^{732}. A recent study by Tusa *et al*. highlighted the potential translational relevance of this unique regulatory mechanism in melanoma [102]. Specifically, the study demonstrated that blocking ERK5 signalling through inhibition and genetic silencing reduced the oncogenic growth of melanoma cells. The authors revealed that BRAFV600E regulated ERK5 function by phosphorylation at the TEY motif and Thr^{732} and Ser^{753}. Evidence that phosphorylation at Thr^{732} was reduced by incubating the cells with SCH772984, a specific ERK1/2 inhibitor, provided further evidence that ERK1/2 phosphorylated the C terminal tail of ERK5 [102]. Collectively, these reports provide strong evidence that Thr^{732} phosphorylation can occur independently of MEK5, likely by ERK1/2, and further suggests a pathological relevance of phosphorylation at this residue.

Consistent with my findings, the aforementioned studies also further provided the functional relevance of phosphorylation at these sites. However, my results differed when comparing the requirement of distinct phosphorylation sites at the C terminus. While I found that ERK5-T732E was sufficient for inducing maximal ERK5 transcriptional activity, Morimoto *et al*. observed that maximum transcription (measured by AP-1 activity) could only be achieved by mimicking phosphorylation at Thr^{732}, Ser^{769}, Ser^{773} and Ser^{775}

[21]. The authors described two "regions" of phosphorylation of equal importance, i.e. phosphorylation at Thr^{732} and a cluster of C terminal serines. Several reasons could explain this difference. Perhaps, the most obvious is the reporter assay used. In our study, we utilized a MEF2 reporter construct, while Morimoto *et al*. employed an AP-1 reporter construct [21]. Therefore, it is possible that ERK5 exhibits a distinct affinity for different response elements, which are dependent on specific phosphorylated forms of ERK5. This could be tested by repeating my experiments with an AP-1 luciferase construct.

Similar to the Morimoto *et al*. study, Elena Diaz-Rodriguez *et al*. demonstrated that mimicking phosphorylation at Ser^{706}, Thr^{732}, Ser^{753}, and Ser^{773} sites increased ERK5 transcriptional activity via the Nur77 reporter [39]. However, this study did not compare how phosphorylation at individual sites affected transcriptional activity. In our report, we found that phosphorylation of Thr^{732} was critical for transcriptional enhancement mediated by Ser^{706}, Ser^{753}, and Ser^{773}. Accordingly, oncogenic BRAFV600E enhanced ERK5 transcriptional activity via ERK1/2 mediated phosphorylation of Thr^{732} [102]. Our findings firmly establish the functional importance of Thr^{732} in the context of phosphorylation at Ser^{706}, Ser^{753} Ser^{769}, Ser^{773}, Ser^{775}.

It has also been demonstrated that phosphorylation at these residues promoted ERK5 nuclear localisation [39] [19]. Elena Diaz-Rodriguez *et al*. demonstrated that mimicking phosphorylation at Ser^{706}, Ser^{753}, Thr^{732} and Ser^{773} would promote nuclear localisation of ERK5 [39]. Conversely, a mutant of ERK5 in which these residues were replaced by Ala, resided in the cytoplasm. Honda *et al*. found that specifically mimicking phosphorylation at Thr^{732} was sufficient for nuclear retention [41]. Accordingly ERK1/2 mediated ERK5 phosphorylation at Ser^{753} and Thr^{732} downstream of BRAFV600E promoted ERK5 translocation [102]. We also found that phosphorylation at Thr^{732} was sufficient to promote nuclear translocation. Furthermore, blocking phosphorylation at Thr^{732} abrogated nuclear shuttling induced by EGF stimulation. Importantly, and consistent with my results, Elena Diaz-Rodriguez *et al* demonstrated that truncation of the C terminal tail caused nuclear localization but did not enhance transcription, providing evidence that nuclear residency of ERK5 was insufficient to confer increased transcriptional activity [21].

5.1.3 Characterising the ERK5-Chromatin interaction

MAPKs have been shown to interact with chromatin or chromatin-associated substrates in a variety of ways [40]. For example, ERK2 can bind to DNA directly [139], p38 associates with chromatin remodelling complexes (SWI/SNF) [140] and JNK with the acetyltransferase complex (ATAC) [141]. Based on these findings we wanted to explore how ERK5 might regulate transcription through

associations with chromatin. We hypothesised that the transactivation domain in the C terminus might mediate this interaction. As we discovered that phosphorylation at Thr^{732} enhanced transcription, we designed experiments to reveal the mechanism underlying this function using high throughput technologies.

My initial experiments revealed that mimicking phosphorylation at Thr^{732} increased the proportion of chromatin-bound ERK5. This was further confirmed by conducting chromatin immunoprecipitation coupled with high throughput DNA sequencing (ChIP-seq). We found that ERK5-T732E binding correlated with H3K27ac, H3K9ac, H3K4me3, H3K27me3 which are marks typically associated with active transcription [142] [143]. The transcription factor motifs co-occupying ERK5-T732E binding sites also were consistent with previously published reports. For example, cMYC has previously been shown to be bound and regulated by ERK5 [35].

To confirm these interactions we employed an unbiased proteomic approach to identify the relevant transcription factors/co-factors. Unfortunately, we were unable to detect cMYC in our interactome analysis. In the ChIP-seq analysis, we employed two crosslinking reagents, PFA and a protein-protein crosslinker, to increase the ability to capture transient /low-affinity interactions ERK5 participates in. A crosslinker was not utilized during the proteomic analysis, and this may explain the discrepancies in the two experiments. Therefore, it is possible that our proteomic analysis mainly detected the direct interactions with ERK5. A recommended approach to further explore this hypothesis would be the incorporation of the RIME technique. This method uses a fixation technique to stabilise protein complexes and their cognate DNA loci [113]. Using this method we can identify the different interactors which form part of the protein complex with ERK5 and the associated genomic loci. Nonetheless, we were able to identify interactors relevant to transcriptional regulation. Significantly, the top two interactors enriched in the ERK5-T732E compared to the ERK5-FL were Histone 3 and Histone 2A. This result is consistent with the demonstration that ERK5-T732E increased the proportion of chromatin-bound ERK5. Other enriched interactors included midasin, cytoplasmic dynein, TAR DNA binding protein 43 and THUMP domain-containing protein 3. Midasin is a nuclear chaperone protein which could potentially mediate the nuclear shuttling function associated with ERK5-T732E. Furthermore, midasin is associated with the formation of macromolecular complexes which may enable ERK5-T732E to form complexes with the transcriptional machinery [146].

The identification of phosphorylation at Thr^{732} on the C terminus in ERK5-FL by mass spectrometry provided further evidence that Thr^{732} residue is a relevant phospho-residue. Additionally phosphorylation

at Ser^{730} was also detected at comparable intensity. Remarkably, Ser^{730} was identified in the original analysis of ERK5 autophosphorylation sites [105]. As Thr^{732} and Ser^{730} are phosphorylated under basal conditions, it is possible that dual phosphorylation at these site occurs independently of MEK5. This hypothesis is consistent with the findings that Thr^{732} can be targeted by other kinases. Further work needs to be performed to determine the interdependency of Thr^{732} and Ser^{730} phosphorylation and test the impact of dual phosphorylation at these residues on nuclear localisation, transcriptional function and chromatin binding. This will require the generation of additional mutants in which Ser^{730} is replaced by Ala or Glu residues in the context of T732E and T732A mutations.

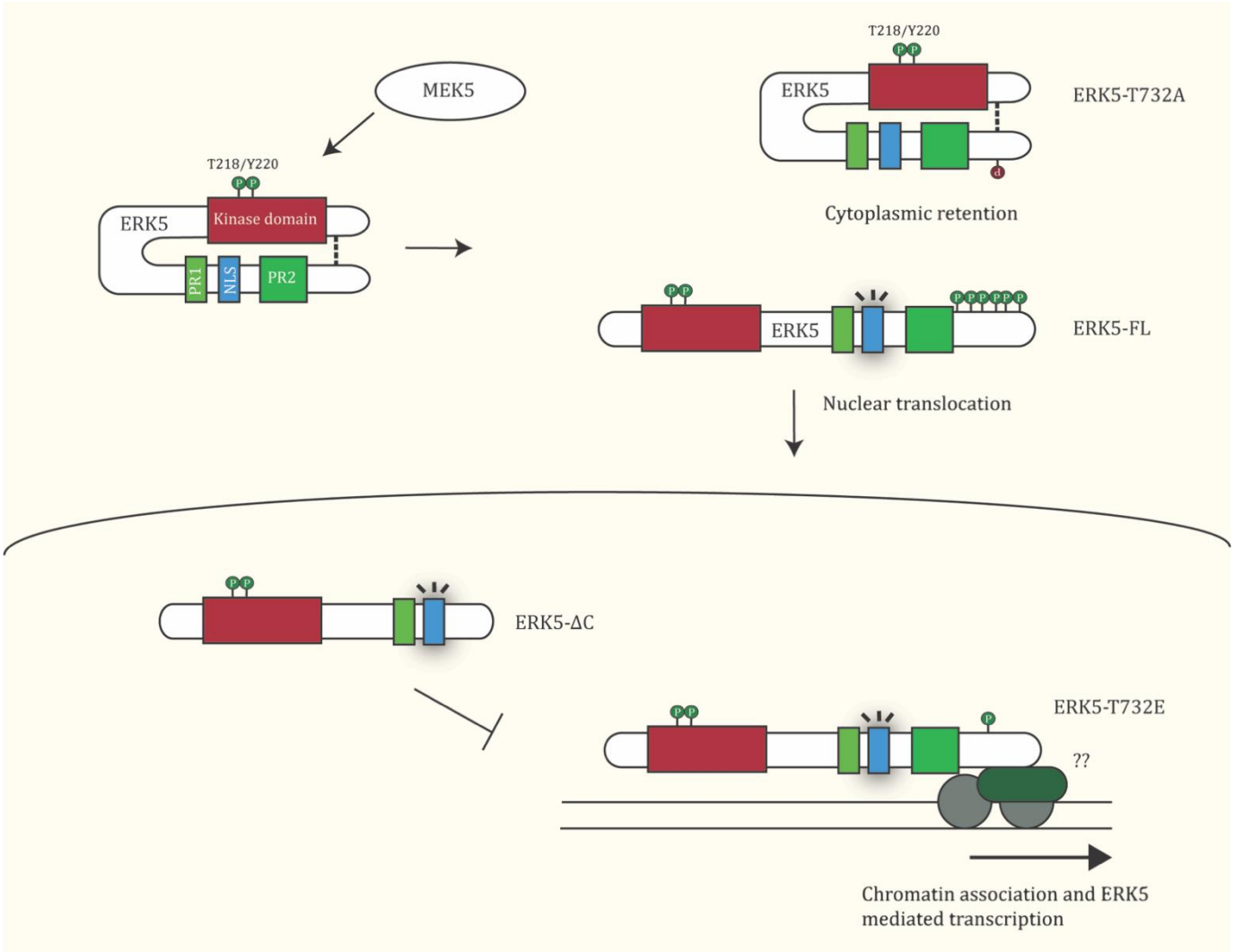


Figure 5.1 The function of Thr^{732} in nuclear translocation and transcriptional enhancement. Upon phosphorylation by MEK5, ERK5 changes into an open conformation and translocates to the nuclear. The ERK5-T732A does not translocate to the nucleus upon upstream stimulation and is retained in the cytoplasm. Upon mimicking phosphorylation at Thr^{732}, ERK5 translocates to the nucleus and enhances transcription through chromatin binding. Despite the nuclear residency of ERK5-ΔC it cannot bind to chromatin and does not enhance transcription.

Chapter 4: ERK5 drives resistance in HER2+ breast cancer

5.2.1 Summary

Chapter 2 focused on establishing the role of ERK5 in HER2+ breast cancer and the development of resistance. Clinical data has previously demonstrated that high ERK5 expression correlates with poor prognosis [53]. Furthermore, *in vitro* analysis has identified that ERK5 was constitutively active in HER2+ breast cancer and genetic silencing of ERK5 facilitated the action of HER2 directed agents [99] [53]. Likewise, our results identified that ERK5 was constitutively active in HER2+ overexpressing cells (SKBR3/BT474). Furthermore, we found that activation was further elevated in HER2+ lines which are resistant to HER2 based therapies. In contrast to previous studies, we observed that pharmacological inhibition of ERK5 did not significantly inhibit growth of cell lines sensitive to HER2 treatment and did not facilitate the action of HER2 directed therapies. However, we did observe that ERK5 inhibition would sensitise HER2+ resistant cell lines to treatment. The additive effect on HER2+ resistant lines could be explained by a further reduction in RB phosphorylation in resistant lines, leading to G1/S arrest and subsequent reduction in cell proliferation. Overall, this chapter reveals an exciting role for ERK5 in the development of resistance to HER2 treatments and provides a potentially viable clinical strategy for targeting refractory disease.

5.2.2 ERK5 in HER2+ breast cancer

Consistent with the findings of Montero *et al*., we observed that high expression of ERK5 dramatically reduced relapse-free survival of patients post-treatment [53]. Furthermore, that ERK5 was constitutively active in our HER2+ overexpressing lines. However, in contrast to previous findings, we did not observe any suppression of HER2+ breast cancer growth when inhibiting ERK5 through pharmacological inhibition [53] [99].

A possible reason for this discrepancy is that the two authors used the introduction of a dominant negative form of ERK5 rather than pharmacological inhibition. Complete disruption of ERK5 activation might produce a greater affect than pharmacological inhibition. However, pharmacological inhibition is a more realistic mode of therapeutic intervention. Only in recent years have commercially available ERK5 inhibitors become available for use. We confirmed that one of these inhibitors, XMD8-92, should no longer be used to investigate ERK5 functionality due to off target effects, as corroborated by previously published studies [120]. A comparison of XMD8-92 with specific ERK5 inhibitors, namely JWG-045 and AX15836 highlighted this effect. Specifically, the proliferative defect observed with XMD8-92 was not

reproducible when utilising specific ERK5 inhibitors. Furthermore, BIX01289, a specific MEK5 inhibitor, also had no significant impact on cell proliferation

5.2.3 ERK5 in the development of resistance

Interestingly, the proportion of phosphorylated ERK5 appeared to be higher in resistant cells. This observation must be confirmed by immunoblot with a phospho-specific antibody as the proportion of phosphorylation ERK5 appeared to be dependent on passage number. It is therefore important to compare the proportion of phosphorylated ERK5 in sensitive and resistant cells at early passage. However, we did observe that inhibiting ERK5 in the context of resistance, in particular in combination with HER2 directed agents, was more effective in reducing cell proliferation. This implied that ERK5 activity may be part of a putative resistance pathway in this subtype of breast cancer. The link between ERK5 and resistance has been previously established in different contexts.

Firstly, a study by Weldon *et al*. identified MEK5 as a protein which strongly promoted chemoresistance in MCF-7 cells, through an unbiased gene microarray analysis [129]. Furthermore, the introduction of a dominant negative form of ERK5 enhanced the sensitivity of MCF-7 cells to treatment-induced death. Further evidence is derived from a study by Ortiz *et al*. who demonstrated that ERK5 inhibition could potentiate the efficacy of chemotherapy in a triple negative breast cancer cell line [54]. Evidence for ERK5-mediated resistance exists in other forms of cancer; particular examples include colorectal cancer and melanoma. For example, a recent study by Petrus R. de Jong *et al.* identified that ERK5 signalling can rescue tumour cell proliferation upon ERK 1/2 abrogation in colorectal cancer [101]. They further indicated that ERK5 signalling could be used as a resistance pathway to circumvent ERK1/2 inhibition. The link has been further substantiated in the context of melanoma [102] [103]. Importantly, ERK5 phosphorylation was found to be significantly upregulated as a consequence of acquired resistance to the ERK1/2 pathway inhibition [103]. Furthermore, the authors revealed that ERK5 as a critical driver of proliferation in melanoma cells resistant to combined BRAF and MEK1/2 inhibition. Accordingly, a study by Tusa *et al*. demonstrated than combined inhibition of ERK5 and BRAFV600E was more effective than BRAF inhibition alone in melanoma cells [102]. Our report identifies elevated ERK5 phosphorylation in HER2+ breast cancer cells resistant to HER2 directed therapy. Lapatinib treatment acts in part by blocking ERK1/2 signalling in sensitive breast cancer cells. Interestingly, the resistant MD-453 cell line did not exhibit constitutive activation of ERK1/2, but displayed elevated ERK5 activity. It is therefore possible that ERK5 activity is increased as a compensatory mechanism for the loss of ERK1/2 signalling in HER2 resistant cells. To firmly establish the role of ERK5 in breast cancer resistance, it is required to firstly

identify elevated ERK5 phosphorylation and subsequently test the combined ERK5 and HER2 inhibition in a larger panel of cell lines exhibiting HER2+ resistance. For example, in MDA-MB-361 and MDA-MB-468 cell lines. Furthermore, the combinatorial effectiveness will have to be clarified *in vivo* using Patient-Derived Xenografts from HER2+ patients with acquired resistance to HER2 directed therapy.

In 2016, Goel *et al*. published a comprehensive study identifying that the cyclin D1-CDK4 pathway mediated resistance in HER2+ breast cancer [133]. This study identified that CDK4/6 inhibitors could re-sensitise resistant cells to HER2 direct therapies by suppression of p-Rb and S6RP. Specifically they demonstrated that dual inhibition of EGFR/HER2 and CDK4/6 suppressed S6RP and phosphorylation of RB, thereby invoking a G1 arrest. Interestingly, targeting ERK5 had a similar effect. we observed a similar reduction in phosphorylated Rb with combined ERK5 and lapatinib treatment. Furthermore, both CDK4/6 inhibition and ERK5 inhibition caused a clear increase in cyclin D1 in resistant lines. Furthermore, Goel *et al*. identified the initiation of a feedback loop upon CDK4/6 inhibition via phosphorylation of Akt, similar to the increased phosphorylated Akt we observed upon ERK5 inhibition. This observation may explain the lack of efficacy in targeting ERK5 alone, similar to targeting CDK4/6 alone, based on a compensatory mechanism sustaining Akt signalling. It is possible that ERK5 regulates CDK4/6 activity in the context of resistance, however this requires further investigation. Min *et al*. demonstrated that using a specific CDK4/6 inhibitor, Palbociclib, phosphorylation of Rb would be suppressed in accordance with an accumulation of Cyclin D1 [147]. These results support the findings in our study and further implicate ERK5 in a cyclin D-CDK4/6 signalling axis which mediates resistance in HER2+ breast cancer. In combined treatment we also observed a decrease of p21, a cyclin dependent kinase inhibitor which suppresses G1/S transition. This finding is inconsistent with the G1/S arrest observed and suggests that further work is required to elucidate the mechanism suppressing G1/S transition via Rb. Collectively, our data provide a rationale for targeting both MEK5/ERK5 and HER2 signalling by suppressing phosphorylation of Rb and Akt signalling.

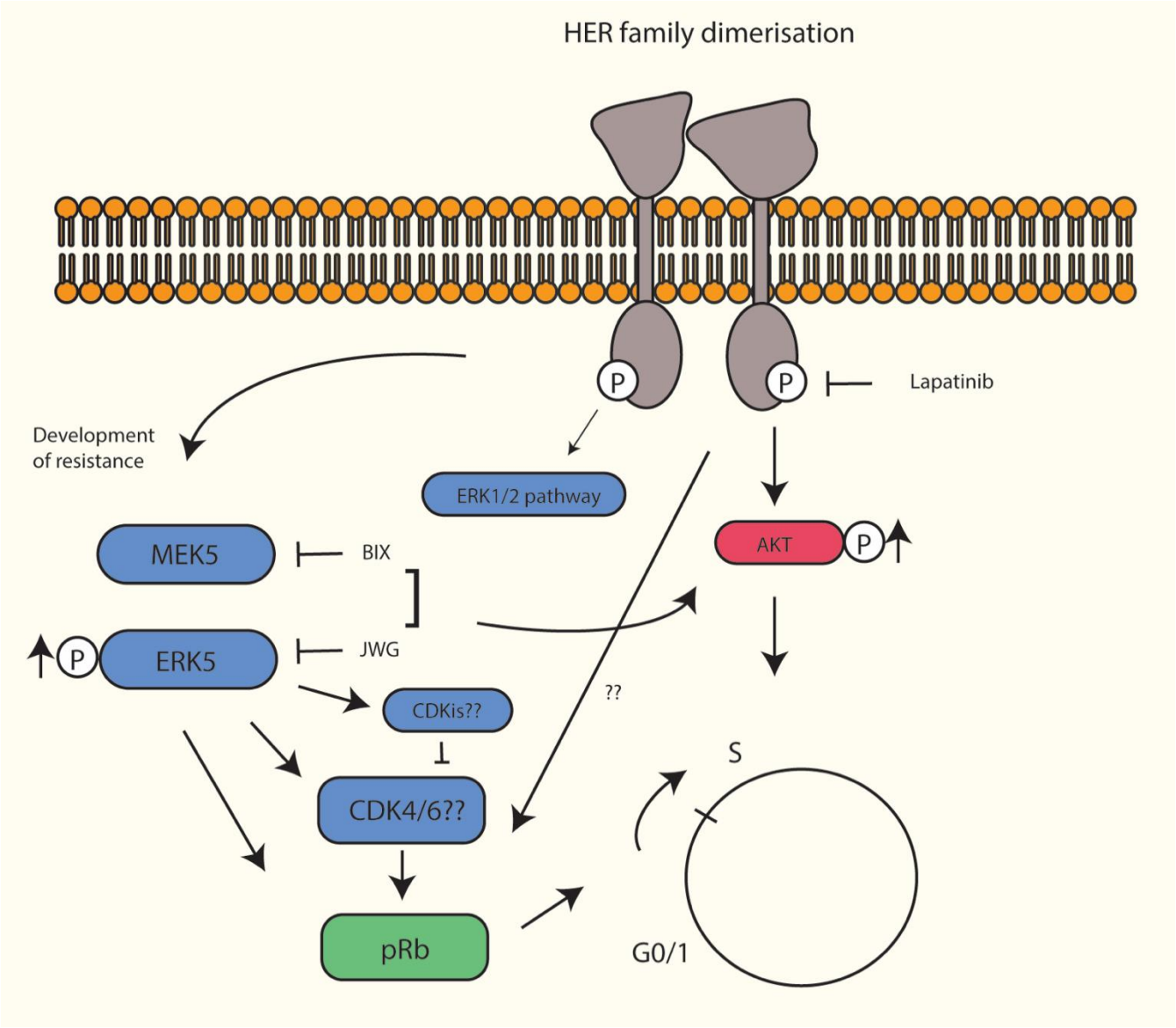


Figure 5.2 The combined inhibition of MEK5/ERK5 and HER2 signalling suppresses phosphorylation of Rb and causes G1 arrest. The development of resistance in HER2+ breast cancer can lead to a loss of ERK1/2 activation and an increase in ERK5 activity. ERK5 promotes phosphorylation of Rb in the context of resistance and regulates Akt activity. Combined inhibition of MEK5/ERK5 signalling and HER2 can suppress phosphorylation of Rb and Akt leading to suppression of resistant cell growth.

5.3 Conclusions

Here we will briefly summarise the conclusions in the two results chapters and connect the results in a manner which provides a basis for future investigations.

Chapter 3

Phosphorylation of Threonine 732

- Acts as a gatekeeper residue facilitating C terminal phosphorylation.
- Is sufficient to enhance transcription observed with mimicking phosphorylation at multiple C terminal sites.
- Is critical for enhancing transcription mediated by other C terminal sites.
- Enhances transcription independently of catalytic activity.
- Is sufficient to promote nuclear translocation observed with mimicking phosphorylation at multiple C terminal sites. Conversely, blocking phosphorylation at Threonine 732 blocks stimulation induced nuclear translocation.
- Facilitates binding to chromatin.

Chapter 4

- ERK5 activity is elevated in HER2+ cells which have development resistance to treatment.
- ERK5 inhibition alone does not significantly affect HER2 mediated growth.
- Combined inhibition of ERK5 and HER2 signaling suppresses tumour cell proliferation in resistant lines by blocking G1/S transition.
- Combined inhibition specifically suppresses phosphorylation of Rb which induces G1 arrest in resistant cells.

5.4 Future perspectives

The two chapters concern fundamental ERK5 biology (chapter 3) and the translational relevance in HER2+ breast cancer (chapter 4). Despite the different approaches in the two sections, there are ways clear ways the two studies could be connected and built upon. The first chapter reveals that phosphorylation at Thr^{732} controls ERK5 C terminal phosphorylation and consequent function, via transcriptional enhancement and nuclear translocation. Chapter 2 highlights that ERK5 is constitutively active in HER2+ cancer and exhibited a mobility shift detected by SDS-PAGE, indicative of phosphorylation at the C terminus. Furthermore, that ERK5 hyperphosphorylation is present in HER2+ resistant cell lines. Therefore, it would be interesting to demonstrate whether blocking phosphorylation at Thr^{732} would abrogate the development of resistance in HER2+ cell lines. This could be investigated by designing a CRISPR Knock-in, modifying the Thr^{732} residue to block phosphorylation of ERK5 in a HER2+ resistant line. This is based on the assumption that ERK5 contributes to resistance by functioning in the nucleus or by promoting oncogenic signalling via ERK5-mediated transcription.

Further investigations will study whether genetic silencing of ERK5 in these lines can re-sensitise resistant lines to lapatinib treatment. Previous reports have also utilised an anti-p-T732 antibody which will be required to analyse the presence of phosphorylation at this site in the context of resistance. The identification of phosphorylation at Thr^{732} as a target for kinases such as ERK1/2 and CDK1 also highlighted the significance of this site in regulating ERK5 function, independently of MEK5. An intriguing possibility is that kinases can circumvent MEK5 regulation of ERK5 by targeting Thr^{732} directly, in both a physiological and pathological context. Further work is needed to investigate the relevance of Thr^{732} in the context of cancer. It is likely that targeting this site in the context of resistance in melanoma and HER2+ cancer will reveal it's oncogenic relevance. Once established, this site could be targeted to combat the development of resistance in breast cancer and melanoma by specifically blocking ERK5 oncogenic function.

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