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# The MAP-kinase interacting kinases (Mnks) as targets in cancer

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James Beggs

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Doctoral thesis

UNIVERSITY OF  
**Southampton**

## Abstract

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The Mnks appear to play an important role in tumour development, but are not essential for normal cell growth and development. This makes them attractive targets for designing anti-cancer treatments. The Mnks are directly downstream of the RAS-RAF-MEK-ERK pathway, a pathway that is frequently overactive in cancer cells. The Mnks bind to eIF4G, which is part of the translation initiation complex, and are the only kinases known to phosphorylate the 5' mRNA cap-binding protein eIF4E. Despite numerous studies linking this phosphorylation event to cancer, its precise role in cancer remains unclear. The lack of progress in developing our understanding of the role the Mnks is largely down to the absence of a selective and potent Mnk inhibitor.

Presented here are the results of experiments carried out using a novel Mnk inhibitor, Mnk-I1. These results are also backed up with the results of experiments using cells – Mouse Embryonic Fibroblasts (MEFs) - that have had the Mnks genetically knocked out. What the results show, is that Mnk kinase activity appears to play a key role in cancer cell migration. The mechanism appears to involve an important role for Mnk kinase activity in the translation of vimentin mRNA into protein and in preventing the degradation of the vimentin protein: an established marker of cells that have undergone Epithelial-Mesenchymal Transition (EMT) and become motile.

The results presented in the last chapter focus on whether the Mnks might be suitable targets for overcoming acquired resistance to the MEK inhibitor AZD6244. In the context of a BRAF<sup>600E</sup> amplification, Mnk-I1 appeared to have a small anti-proliferative effect in one cancer cell line tested; however, there was no effect on the proliferation of a cancer cell line with a KRAS<sup>13D</sup> amplification. Included in this set of data is an interesting effect of Mnk-I1 on increasing P-Mnk1 levels.

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## DECLARATION OF AUTHORSHIP

I, James Beggs, declare that the thesis entitled *The MAP-kinase interacting kinases (Mnks) as targets in cancer* and the work presented in the thesis are both my own, unless otherwise stated, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made it clear exactly what was done by others and what I have contributed myself;
- parts of this work have been published as: BEGGS, J. E., TIAN, S., JONES, G. G., XIE, J., IADEVAIA, V., JENEI, V., THOMAS, G. & PROUD, C. G. 2015. The MAP kinase-interacting kinases regulate cell migration, vimentin expression and eIF4E/CYFIP1 binding. *The Biochemical journal*, 467, 63-76.

Signed: .....

Date:.....

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## Abbreviations

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ADP- adenosine diphosphate  
AJC – apical junction complex  
ATP – adenosine triphosphate  
BRSK2 – BR-serine/threonine protein kinase 2  
C6244R – COLO205 AZD6244-resistant cell line  
CD44 – cluster of differentiation 44  
CDK – cyclin-dependent kinase  
CK1 – casein kinase 1  
CYFIP1 – cytoplasmic FMRP-interacting protein  
DKO – double knockout  
DMEM - Dulbecco's modified Eagle's medium  
DMSO - dimethyl sulfoxide  
DTT - Dithiothreitol  
ECM – extracellular matrix  
eIF – eukaryotic initiation factor  
eIF4F – eukaryotic initiation factor 4F complex  
EMT – epithelial-mesenchymal transition  
ERK – extracellular-signal-regulated kinase  
FBS – fetal bovine serum  
FMRP – fragile-X mental retardation protein  
GAPDH - glyceraldehyde-3-phosphate-dehydrogenase  
GDP – guanosine diphosphate  
GTP – guanosine triphosphate  
H – hour  
H6244R – HCT116 AZD6244-resistant cell line  
HER2 – human epidermal growth factor  
IRES – Internal ribosome entry site

JNK – JUN amino-terminal kinase  
KCl – potassium chloride  
MAPK – mitogen-activated protein kinase  
MEF – mouse embryonic fibroblast  
MEK - mitogen-activated protein kinase kinase  
MET – mesenchymal-epithelial transition  
min-minutes  
miRNA – microRNA  
MKK1 – mitogen-activated protein kinase kinase 1  
Mnk – Map-kinase interacting kinase  
mRNA – messenger RNA  
mTOR – mammalian target of rapamycin  
NES – nuclear export signal  
NLS – nuclear localization signal  
NUP50 – nucleoporin 50  
PCR – polymerase chain reaction  
R&D – Research & Development  
ROCK - Rho-associated kinase  
RPMI medium – Roswell Park Memorial Institute medium  
RSK - p90 ribosomal S6 kinase  
RTK – receptor tyrosine kinase  
RT-qPCR – Reverse transcription-quantitative polymerase chain reaction  
TCF – ternary Complex Factor  
TGF – transforming growth factor  
tRNA – aminoacyl-transfer RNA  
TWIST – twist-related protein 1  
UTR – untranslated region  
WASP – Wiskott-Aldrich syndrome protein

WAVE – WASP family verprolin-homologous protein

WT – wild type

YB1 – Y box binding protein 1

ZEB1 – Zinc finger E-box binding homeobox 1



# 1. Introduction

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## 1.1 RAS-RAF-MEK1/2-ERK1/2 Pathway

### 1.1.1 The pathway

The role of the RAS-RAF-MEK-ERK pathway is to relay signals from receptors on the cell surface, intracellularly, to effects on a range of cellular functions, which include cell proliferation, angiogenesis and cell migration (Friday and Adjei, 2008). The influence of the RAS-RAF-MEK-ERK on regulating a wide variety of cellular processes means that when this pathway becomes deregulated it can lead to a range of diseases, including: cancer, neurodegenerative diseases, diabetes and developmental disorders (Plotnikov et al., 2011).

There are many different types of receptors which are capable of activating the RAS-RAF-MEK-ERK pathway, these include: G-protein coupled receptors, receptor tyrosine kinases, integrins and cytokine receptors (Friday and Adjei, 2008). This also means that a wide range of extracellular stimuli, including hormones, growth factors, cell-cell and cell-ECM (Extracellular Matrix) interactions and cytokines, are capable of activating the RAS-RAF-MEK-ERK pathway (Yao and Seger, 2009).

The canonical activation pathway involves the binding of a growth factor to a receptor tyrosine kinase, resulting in auto-phosphorylation of the receptor tyrosine kinase (Friday and Adjei, 2008). The auto-phosphorylation event allows an adaptor protein, such as Grb2 to bind to the phosphorylated site on the intracellular domain of the receptor (Friday and Adjei, 2008). Once the adaptor protein is bound, guanine nucleotide exchange factors, such as

SOS1 or CDC25, can then also associate with the receptor (Friday and Adjei, 2008). The guanine nucleotide exchange factors, once bound to the adaptor proteins, can then activate the RAS protein (Friday and Adjei, 2008). The guanine nucleotide exchange factors cause a conformational change in the RAS protein, which allows for the exchange of a GDP for a GTP (Friday and Adjei, 2008). Once in its GTP-bound state the RAS protein is active it can then activate the RAF protein kinase to start the RAS-RAF-MEK-ERK signalling cascade (Stokoe and McCormick, 1997). RasGAP helps ensure the RAS activity is not too persistent, by promoting the GTPase activity of RAS and therefore causing it to revert to its inactive GDP-bound state (Friday and Adjei, 2008).

The method by which RAS activates RAF is not understood, but is thought to involve several other cofactors (Friday and Adjei, 2008). There are 3 isoforms of RAF: ARAF, BRAF and CRAF (Friday and Adjei, 2008). The three RAF isoforms all phosphorylate the same, only known substrates of RAF: MEK1/2 (Cox et al., 2014). The signaling cascade involves a direct phosphorylation of several serine residues on the MEK1 and MEK2 proteins, by RAF (Friday and Adjei, 2008).

MEK1 and MEK2 are dual specific kinases that are 80% identical (Cox et al., 2014). The active MEK proteins then activate their only known substrates ERK1 and ERK2, by phosphorylating threonine and tyrosine residues on the protein kinases (Cox et al., 2014). ERK1 and ERK2, in humans, are 84% identical in sequence, activated in parallel and share most, if not all, functions, which means they can be referred to as ERK1/2 (Roskoski, 2012). 200-hundred ERK substrates have been identified and they include

regulatory molecules and transcription factors, both in the cytoplasm and the nucleus (Roskoski, 2012).

In a resting cell, the components of the RAS-RAF-MEK-ERK pathway are primarily located in the cytoplasm (Yao and Seger, 2009). Activation of the RAS-RAF-MEK-ERK pathway causes the different components of the pathway to move to different parts of the cell to carry out their specific functions (Yao and Seger, 2009). RAS and RAF move to the plasma membrane and other membranes of the cell, whereas MEK1/2 and ERK1/2 translocate to the nucleus (Yao and Seger, 2009). The particular method by which this translocation occurs is not understood (Yao and Seger, 2009). The NES (Nuclear Export Signal) in the N-terminus of MEK1/2 means that it is rapidly transported out of the nucleus (Yao and Seger, 2009). In contrast, ERK1/2 can remain in the nucleus for up to hours after the initial stimulation of the pathway (Yao and Seger, 2009).

### 1.1.2 ERK1/2 kinases

ERK1/2 catalyze the phosphorylation of 100s of nuclear and cytoplasmic substrates, some of which are discussed here (Roskoski, 2012).

#### 1.1.2.1 Cytoplasmic substrates

The cytoplasmic substrates of ERK1/2 include the RSK family of kinases, cytoskeletal proteins, apoptotic proteins and other regulatory or signaling molecules (Roskoski, 2012).

The RSK proteins regulate cell growth, motility, proliferation and survival (Anjum and Blenis, 2008). The RSK proteins activate a number of transcription factors, including CREB and NF- $\kappa$ B, and also phosphorylate proteins – eIF4B

and ribosomal protein S6 - involved in controlling protein translation (Roskoski, 2012). The RSK proteins, by phosphorylating pro-apoptotic proteins, such as Bad (Bcl-2-associated-death promoter), and preventing them from binding to pro-survival proteins, such as Bcl-xl, promote cell survival (Balmanno and Cook, 2009). This is not the only way ERK activity helps promote cell survival. By phosphorylating the pro-survival protein Mcl-1 directly, ERK stabilizes Mcl-1, whilst at the same time acting through RSK to inhibit an enzyme, GSK3 (Glycogen synthase kinase 3), involved in degrading Mcl1 (Balmanno and Cook, 2009). In addition, RSK also promotes progression through the G1- phase of the cell cycle by phosphorylating, and therefore inhibiting, p27<sup>KIP</sup> (Anjum and Blenis, 2008).

The effect of ERK1/2 signaling on cell motility is unclear. It has been reported that palladin, an actin-binding protein known to inhibit cell motility, is activated by ERK signaling (Asano et al., 2011). On the contrary, another report based on a study in mice linked ERK signaling to an increase in paxillin activity, which via its effects on FAK and cell morphology should increase cell motility (Yoon and Seger, 2006). Similarly, ERK1/2 has been shown to phosphorylate myosin light chain kinase and enhance cell motility in a human cancer cell line (Klemke et al., 1997).

ERK1/2 activity has also been shown to inhibit the nuclear import of importin by phosphorylating nucleoporin50 (NUP50) (Kosako et al., 2009).

#### 1.1.2.2 Nuclear substrates

Inside the nucleus, ERK1/2 directly phosphorylates the ternary complex factor (TCF) family of transcription factors, inducing the expression of immediate-early genes (IEGs) such as c-Fos and c-Myc (Roskoski, 2012). The expression

of c-Fos and c-Myc promotes the expression of the late-response genes, which promote cell survival, cell division and cell motility (Roskoski, 2012). ERK1/2 further contributes to the expression of immediate-early genes by promoting chromatin remodeling by activating the MSK1 and MSK2 kinases (Mitogen- and stress- activated kinases) (Soloaga et al., 2003). The MSK1 and MSK2 kinases are located in the nucleus and are believed to be responsible for the phosphorylation of Histone H3 and HMG-14, which leads to a selective increase in the expression of immediate-early genes, such as c-Fos (Soloaga et al., 2003).

Elk-1 – a member of the Ets family of transcription factors – is the most widely studied ERK1/2 substrate (Yoon and Seger, 2006). ERK1/2 phosphorylates Elk-1 to induce the transcription of c-Fos; however, it is only after sustained ERK activation that c-Fos is stably expressed (Burch et al., 2004) (Yoon and Seger, 2006). The sustained ERK activity is required in order to phosphorylate C-terminal residues on c-Fos, which help stabilize the protein (Burch et al., 2004). Stable expression of c-Fos in the nucleus leads to increased transcription of several genes including Fra-1, but also reduced transcription of cyclin D1 (Burch et al., 2004). After several hours, when ERK activity starts to drop, c-Fos is degraded and replaced by Fra-1 at the cyclin D1 promoter. This results in cyclin D1 expression (Burch et al., 2004). Cyclin D1 expression is an important part of the G1/S transition. Another way ERK activity causes an increase in cyclin D1 expression is by phosphorylating serine 62 on Myc (Seth et al., 1991) (Chambard et al., 2007). This phosphorylation event increases the stability of Myc, which directly increases the transcription of cyclin D1 (Daksis et al., 1994) (Chambard et al., 2007).

ERK signaling has another influence on the cell cycle via its regulation of the assembly of cyclin/CDK (cyclin-dependent kinase) complexes. ERK activity is required for the translocation of CDK2 to the nucleus, where it can be activated (Chambard et al., 2007). ERK is also believed to directly phosphorylate CDK2 leading to its activation (Chambard et al., 2007). The mechanisms linking ERK to both of these methods of regulating CDK2 activity are unknown (Chambard et al., 2007). CDK2, by phosphorylating Rb, forms an important feedback loop which prevents the G1/S transition from reversing (Chambard et al., 2007).

ERK activation is required throughout the G1/S transition (Chambard et al., 2007). The reason is that ERK activity is required to suppress the expression of anti-proliferative genes, such as Tob1 and JunD, which would otherwise prevent cells from entering into S phase (Chambard et al., 2007). In total 175 genes have been shown to be down-regulated, in response to ERK activity, during the G1/S transition (Chambard et al., 2007). C-Fos has been shown to play an important role in this ERK-dependent down-regulation (Chambard et al., 2007).

The signaling specificity, like all kinases, is derived from unique sequences in the N- and C- terminal domains (Roskoski, 2012). A portion of the kinase domain also confers functional specificity on the kinases (Roskoski, 2012).

The ERK substrates include: the transcription activator Elk-1; the transcription factors c-Ets1 and c-Ets2; the serine/threonine kinase p90RSK1; TOB, an anti-proliferative transcription factor; and, the Mnks, which are thought to be involved in regulating translation during tumourigenesis (Friday and Adjei, 2008).

### 1.1.3 Mutations in components involved in cancer

#### 1.1.3.1 RAS

1 in 5 human cancers have a *KRAS* mutation: the most commonly mutated isoform (Baines et al., 2011). In total, up to 30% of all human cancers have a *RAS* mutation (Cox et al., 2014). 86% of these *RAS* mutations occur in the *KRAS* isoform, 8% in *NRAS* and 3.3% in *HRAS* (Cox et al., 2014, Baines et al., 2011). *RAS* mutations are characterised by single-base missense mutations 98% of which occur at G12, G13 or Q61 (Cox et al., 2014). In *K-RAS*, 83% of these mutations occur at the G13 site, with 14% occurring at the G12 site (Cox et al., 2014). The mutations lock *RAS* in its active GTP-bound state (Samatar and Poulikakos, 2014). This means *RAS* and downstream signaling is switched permanently on. How much of a contribution downstream RAF-MEK-ERK signaling makes to the oncogenic potential of these *RAS* mutations is unclear (Samatar and Poulikakos, 2014). *RAS* activates other pathways, such as PI3K and Ral-GEF (Samatar and Poulikakos, 2014). It should, however, start to become clearer now that selective inhibitors of RAF, MEK, ERK, PI3K and AKT have been developed (Samatar and Poulikakos, 2014).

#### 1.1.3.2 RAF

RAF mutations are believed to occur in about 8% of all human cancers, in particular types of cancer such as melanoma the incidence is much greater (59%) (Davies et al., 2002). Of the 3 highly conserved, mammalian, RAF isoforms ARAF, BRAF and CRAF, nearly all the RAF mutations that have been found in human cancer affect the BRAF isoform (Dhomen and Marais, 2007).

The reason that ARAF and CRAF mutations are so rare in cancer is that they require two activating mutations to become oncogenic, whereas BRAF only requires one (Dhomen and Marais, 2007). 90% of BRAF mutations discovered in human cancer involve an amino acid substitution at position 600 of a valine for a glutamate (V600E) (Dhomen and Marais, 2007). The BRAFV600E mutation causes a hyper-activation of the RAS-RAF-MEK-ERK pathway which – via the ERK substrates – causes increased proliferation, cell survival and transformation: 3 hallmarks of cancer (Hanahan and Weinberg, 2011). It is thought that BRAF mutations play an important role in the initiation of tumours, but are not, by themselves, capable of inducing a full progression towards metastatic cancers (Dhomen and Marais, 2007). This theory is supported by the observation of BRAFV600E mutations in 80% of all benign skin lesions and colon polyps (Dhomen and Marais, 2007).

Up to 70% of melanomas contain the BRAF V600E mutation (Dhomen and Marais, 2007). This link with a cancer associated with UV exposure, led to the theory that the BRAF V600E mutation might be caused by UV exposure (Dhomen and Marais, 2007). Whilst this might be the case, it is more likely to be the consequence of an indirect effect of UV (Dhomen and Marais, 2007). The reason a direct effect is unlikely is that the V600E mutation requires a GTG to GAG switch, which is not a typical UV-DNA damage mutation (Dhomen and Marais, 2007). Other RAF mutations also lack the typical UV-DNA damage signature (Dhomen and Marais, 2007). Further evidence against a link between UV and the BRAFV600E mutation is that the mutation has been observed in tissues – ~30% of ovarian and thyroid cancers – where UV would be unable to penetrate (Dhomen and Marais, 2007).



It is clear that there must be some kind of selection pressure that favors the V600E mutation over other BRAF mutations; however, it is not clear what the mechanism is and how this would predispose particular types of cancer to this mutation (Dhomen and Marais, 2007).

#### 1.1.3.3 MEK

MEK1/2 mutations are extremely rare in cancer (Marks et al., 2008) and do not necessarily increase MEK1/2 activity (Emery et al., 2009). Random mutagenesis has been shown to generate MEK mutations, which have important implications for the treatment of some cancers (Emery et al., 2009). MEK mutations MEK1(P124L) and MEK1(Q56P) were shown to cause resistance to single treatments of both AZD6244, a MEK inhibitor, and the BRAF inhibitor PLX4720 (Emery et al., 2009). These mutations did not, however, confer resistance to a combined treatment with AZD6244 and PLX4720 (Emery et al., 2009). A MEK2 mutation, MEK2<sup>Q60P</sup>, has been detected in patient tumour samples (Wagle et al., 2014). Unlike the MEK1 mutations, MEK2<sup>Q60P</sup> conferred resistance to AZD6244, alone, and in combination with a B-RAF inhibitor PLX4720 (Emery et al., 2009, Wagle et al., 2014).

#### 1.1.3.4 ERK

A database of somatic mutations in cancer (COSMIC) shows that mutations at a number of sites across the ERK1/2 proteins have been linked with cancer (Goetz et al., 2014). These include: ERK1R84, ERK1G186, ERK2D321, and ERK2E322 (Goetz et al., 2014). The ERK2<sup>E322K</sup> mutation is often found in cervical and head and neck cancers (Lawrence et al., 2014, Ojesina et al., 2014).

An ERK mutagenesis study showed that ERK mutations have the potential to confer resistance to ERK or RAF/MEK inhibitors (Goetz et al., 2014). The mutations that conferred resistance to ERK inhibitors tended to affect the ATP-binding pocket, whilst the ERK mutations that conferred resistance to RAF/MEK inhibitors were found throughout the ERK1/2 proteins (Goetz et al., 2014).

#### 1.1.4 Anti-cancer targets

There is widespread interest, both from pharmaceutical companies and within the research community, in using the components of the RAS-RAF-MEK-ERK pathway as targets for developing anti-cancer drugs.

##### 1.1.4.1 RAS

Despite being the first oncogene discovered and the most frequently mutated, there is still no effective inhibitor of RAS available (Andrew et al., 2014). The three-dimensional structure of RAS shows how RAS doesn't really lend itself to small molecule inhibition. An approach to inhibit the binding of SOS – the guanine nucleotide exchange factor responsible for activating RAS – found that the binding pocket was too shallow for small molecule inhibitors to bind (Samatar and Poulidakos, 2014). For small molecule inhibitors to bind tightly to a target, they require deep-hydrophobic pockets, but RAS doesn't appear to have any (Cox et al., 2014, Samatar and Poulidakos, 2014).

Instead of targeting RAS directly, alternative methods of indirectly inhibiting RAS activity are being investigated. One of these methods is to inhibit the interaction between RAS and the plasma membrane: where the receptor tyrosine kinases (RTKs), which cause RAS activation, are located (Samatar and Poulidakos, 2014). Farnesyltransferase Inhibitors (FTIs) are designed to do just

that (Samatar and Poulikakos, 2014). FTIs inhibit RAS farnesylation, a post-translational lipid modification required for RAS to attach to the cell membrane (Samatar and Poulikakos, 2014). The problem with FTIs is that they aren't effective at inhibiting the RAS isoforms – KRAS and NRAS – most commonly associated with cancer (Samatar and Poulikakos, 2014). The reason FTIs aren't effective at inhibiting KRAS and NRAS, is that another lipid modification by geranylgeranyltransferase can compensate (Samatar and Poulikakos, 2014). An alternative method of disrupting the subcellular localization of RAS has proven effective at inhibiting KRAS in human cancer cell lines. The method relies on the inhibition of PDE $\delta$  (Zimmermann et al., 2013). PDE $\delta$  has previously been shown to focus the cytoplasmic farnesylated RAS towards the plasma membrane (Chandra et al., 2012). As well as trying to disrupt the interactions that activate RAS, attempts have been made to design inhibitors, which prevent RAS from interacting with, and activating RAF; however, although these inhibitors are still being tested early indications are that they are not very potent (Shima et al., 2013).

The most promising approach for targeting RAS in cancer is to target the specific mutated version of RAS (Samatar and Poulikakos, 2014). Crystal structures of cysteine-reactive inhibitors bound to KRAS(G12C) revealed a new binding pocket (Ostrem et al., 2013). When inhibitors bound to this pocket, the preference of RAS for GTP was replaced by a preference for GDP and its inactive form (Ostrem et al., 2013). Although the initial set of inhibitors had low potency, they provide a useful starting point in the development of inhibitors that specifically target the different mutant forms of RAS (Ostrem et al., 2013). Given the difficulty in finding a suitable binding pocket on RAS, the discovery of a new binding pocket opens up another approach to inhibiting RAS in tumours (Ostrem et al., 2013).

Elucidating the differences in the roles of the two splice variants of K-RAS - K-RAS-4A and K-RAS-4B – will also be important for guiding effective drug design (Andrew et al., 2014). Evidence to date, based on expression patterns, suggests that K-RAS-4A is more important during tumour initiation – in lung tumours, at least – whilst K-RAS-4B appears to be more important in tumour progression (Andrew et al., 2014).

#### 1.1.4.2 RAF

RAF has proven to be a paradoxical anti-cancer target. The first generation of RAF inhibitors, although effective at inhibiting ERK activity in BRAFV600E mutant cells, actually increased ERK activity in normal cells (Samatar and Poulikakos, 2014). As a result, a small proportion of patients developed benign tumours of the skin (Samatar and Poulikakos, 2014). The reason for this paradoxical effect lies in the way RAF is able to dimerize and transactivate itself (Samatar and Poulikakos, 2014).

In wild type cells, RAF exists in an inactive monomeric form until it is activated by active RAS-GTP (Samatar and Poulikakos, 2014). Once activated by RAS, RAF dimerizes leading to transactivation (Samatar and Poulikakos, 2014). This means that in wild type cells with high levels of active RAS-GTP, RAF exists primarily in active dimers (Samatar and Poulikakos, 2014). If a RAF inhibitor is introduced at concentrations too low to saturate both protomers of the RAF dimers then the inhibitor actually promotes dimerization and transactivation (Samatar and Poulikakos, 2014). The increase in transactivation of the RAF dimers causes an increase in ERK activity, downstream (Samatar and Poulikakos, 2014).

Where the first generation of RAF inhibitors, such as vemurafenib and dabrafenib, are effective is in BRAF<sup>V600E</sup> mutant cells (Samatar and Poulikakos, 2014). In BRAF<sup>V600E</sup> cells with low RAS-GTP, the RAF isoforms exist in their monomeric form (Samatar and Poulikakos, 2014). The difference, compared with normal wild-type RAF, is that BRAF<sup>V600E</sup> is active in its monomeric form (Samatar and Poulikakos, 2014). RAF inhibitors are effective at binding to and inhibiting monomeric BRAF<sup>V600E</sup> (Samatar and Poulikakos, 2014). If these BRAF<sup>V600E</sup> mutant cells acquire an additional mutation that causes increased active RAS-GTP, then the BRAF<sup>V600E</sup> monomers start to dimerise and the RAF inhibitor becomes less effective. (Samatar and Poulikakos, 2014)

The problem this 'RAF inhibitor paradox' presents in the clinic is that whilst some patients, those with BRAF<sup>V600E</sup> mutant tumours, respond very well to first generation RAF inhibitors they are susceptible to unwanted side effects – such as those already mentioned - due to the effect of the RAF inhibitors on increasing ERK activity in normal cells (Su et al., 2012). A problem highlighted by the case of one patient who, following treatment with a RAF inhibitor for their melanoma, developed a form of leukaemia, only for it to reverse upon withdrawal of the RAF inhibitor (Callahan et al., 2012).

It is important to mention that not all RAF mutations remove the need for dimerization in order to become active (Samatar and Poulikakos, 2014). This means that first generation RAF inhibitors, such as vemurafenib and dabrafenib, would be ineffective against tumours containing mutations other than the BRAFV600E mutation (Samatar and Poulikakos, 2014).

So what is being done to get around this RAF inhibitor paradox? One approach has been to design a BRAFV600E inhibitor, LGX818, which binds

to RAF for longer meaning the saturation point can be reached at lower concentrations (Samatar and Poulikakos, 2014). Two other inhibitors, TAK-632 and MLN2480, employ a similar approach with the addition that they are also effective in RAS-mutant tumours and wild type cells (Samatar and Poulikakos, 2014). The problem with this is that by inhibiting ERK activity in normal cells as well, the therapeutic index is reduced. Despite this, MLN2480 has been entered into Phase I clinical trials (Samatar and Poulikakos, 2014). Inhibitors that have a reduced effect on ERK activity in wild type cells, and therefore a wider therapeutic window, are currently in development (Samatar and Poulikakos, 2014).

One way of maximizing the effectiveness of the different RAF inhibitors is to combine them with MEK or ERK inhibitors and to base the different combinations on the mutations present within a particular tumour (Samatar and Poulikakos, 2014). For BRAF mutant tumours with low RAS activity, a combination containing a selective BRAFV600E inhibitor will be more effective whilst in RAS-mutant tumours inhibitors which are effective against RAF dimers will be more effective (Samatar and Poulikakos, 2014).

#### 1.1.4.3 MEK

Activating MEK mutations are rare in human cancer; however, because they lie downstream of RAS and RAF they are attractive drug targets (Marks et al., 2008). Given that ERK1/2 are the only known substrates of MEK1/2, there is a lot of interest in developing MEK1/2 inhibitors as a way of inhibiting ERK1/2 activity (Cox et al., 2014). 15 MEK inhibitors have reached the clinical trial stage (Cox et al., 2014). In contrast to many other kinase inhibitors, MEK inhibitors are highly specific (Cox et al., 2014). MEK inhibitors are designed to target a unique binding pocket next to the ATP-binding site (Ohren et al.,

2004). Once bound, the MEK inhibitor induces a conformational change in the structure of MEK, which locks it in an inactive state (Ohren et al., 2004). This process is called allosteric regulation (Cox et al., 2014).

MEK inhibitors have proven to be particularly effective at treating BRAF-mutant melanoma: the MEK inhibitor Trametinib (GSK112021) has recently been approved by the FDA for the treatment of BRAF-mutant metastatic melanoma (Cox et al., 2014). In RAS-mutant cancers, MEK inhibitors have only been partially effective (Gilmartin et al., 2011). The reason some MEK inhibitors are less effective in RAS-mutant cancers is believed to be due to the different activation state, and method of activation of MEK in RAS-mutant cancer cells (Hatzivassiliou et al., 2013). MEK inhibitors, such as GDC-0623 and G-573, designed to block the feedback activity of MEK on RAF are thought to be more effective in RAS-mutant cancers (Hatzivassiliou et al., 2013).

A dual RAF-MEK inhibitor – R05126766 - has also been developed which has been shown to be more effective at inhibiting ERK activity than a MEK inhibitor (Ishii et al., 2013). The dual RAF-MEK inhibitor works by allosteric regulation, but in a different way to conventional MEK inhibitors (Ishii et al., 2013). Instead, the inhibitor causes a conformational change in the structure of MEK that means it can no longer be phosphorylated or released by RAF (Ishii et al., 2013). By locking MEK and RAF together, the inhibitor blocks both kinases and MEK becomes, in effect, a dominant negative inhibitor of RAF (Ishii et al., 2013). The dual RAF-MEK inhibitor, therefore, overcomes the RAF-MEK feedback, resulting in improved inhibition of ERK activity and its tumorigenic activity (Ishii et al., 2013). The dual RAF-MEK inhibitor highlights

how the MEK-RAF feedback limits the effectiveness of MEK inhibitors (Ishii et al., 2013).

Selumetinib (AZD6244), a highly selective allosteric inhibitor of MEK1 and MEK2, which has entered clinical trials (Samatar and Poulikakos, 2014). So far only modest effects on patients have been observed in phase II clinical trials; however, it is thought that this is because the patients who took part in the trial were not selected on the basis of activating mutations in the ERK signalling pathway (Jänne et al., 2013, Samatar and Poulikakos, 2014).

#### 1.1.4.4 ERK

The early assumption was that ERK inhibitors, given that ERK1/2 is the only substrate of MEK1/2, would not have any additive benefit over MEK inhibitors (Samatar and Poulikakos, 2014). This assumption has now been replaced with intense interest in ERK inhibitors (Samatar and Poulikakos, 2014). There are several reasons that explain why. The contrasting effects in response to inhibitors of different components of the RAS-RAF-MEK-ERK pathway provided a realisation of how complex the pathway is (Samatar and Poulikakos, 2014). Added to this, was the discovery of various feedback loops built in to the pathway (Samatar and Poulikakos, 2014). It was thought that perhaps by inhibiting a protein further downstream it might circumvent the effects of the feedback loops (Samatar and Poulikakos, 2014). Similarly, it was thought that inhibiting ERK1/2 might overcome the resistance to RAF and MEK inhibitors, which has been shown to arise through renewed ERK signaling (Samatar and Poulikakos, 2014). The ERK inhibitor, SCH772984, has been shown to be effective in cancer cell lines that have become resistant to BRAF or MEK inhibitors (Morris et al., 2013). SCH772984 is a 'dual-mechanism' ATP-competitive ERK inhibitor: inhibiting both the kinase activity



of ERK1/2 and its phosphorylation by MEK (Samatar and Poulikakos, 2014). An analogue of SCH772984, MK-8353/SCH900353, is currently being tested in Phase I clinical trials (Samatar and Poulikakos, 2014). Two other ERK inhibitors BVD-523 and RG7842(GDC-0994) have also entered clinical trials (Samatar and Poulikakos, 2014).

### 1.1.5 Resistance

Cancer cells can acquire resistance to RAF, MEK, ERK inhibitors by gaining additional mutations or gene amplifications which drive increased ERK signaling. These can include amplifications of the receptor tyrosine kinases, such as EGFR, to which extracellular growth factors bind in order to stimulate activity down the RAS-RAF-MEK-ERK pathway. Increased RAS activation, either directly - as the result of a RAS mutation - or indirectly - for example, due to down regulation of neurofibromin (NF1), a negative regulator of RAS - can cause a sufficient increase in RAF dimerization and subsequent ERK activity to overcome the effect of inhibition (Samatar and Poulikakos, 2014).

#### 1.1.5.1 RAF inhibitors

Resistance to RAF inhibitors can emerge as the result of mutations upstream of RAF, mutations in RAF itself and mutations in proteins downstream of RAF. The upstream mutations and the mutations affecting RAF itself tend to result in increased RAF dimerization (Samatar and Poulikakos, 2014). One example is the expression of a BRAFV600E splicing variant in which the RAS binding domain is deleted but RAF dimerization is increased (Samatar and Poulikakos, 2014). An example of proteins upstream of RAF driving resistance to RAF inhibitors can be found in the receptor tyrosine kinases (RTKs), which are responsible for initiating the RAS-RAF-MEK-ERK signalling cascade (Samatar and Poulikakos, 2014). The relief of feedback loops involving EGFR

and HER3, due to the inhibitory effects of a RAF inhibitor, have been shown to cause resistance to RAF inhibitors in phase I clinical trials (Prahallad et al., 2012, Montero-Conde et al., 2013). By removing the feedback loop, the RTKs increase their activation of RAS activation and, therefore, RAF dimerization increases, which leads to a quick recovery in ERK signalling (Samatar and Poulikakos, 2014). Mutations which activate RAS can also increase RAF dimerization and, therefore, drive resistance to RAF inhibitors (Samatar and Poulikakos, 2014). Mutations in proteins downstream of RAF tend to cause resistance to RAF inhibitors by causing increased ERK signalling (Samatar and Poulikakos, 2014). Random mutagenesis has been used to generate MEK mutations MEK1(P124L) and MEK1(Q56P), which cause resistance to the BRAF inhibitor PLX4720 (Emery et al., 2009). The particular cell lines harboring these mutations did, however, remain sensitive to combined RAF - PLX4720- and MEK – AZD6244 – inhibition (Emery et al., 2009). A mutation in MEK2, MEK2<sup>Q60P</sup>, has been shown to confer resistance to combined RAF/MEK inhibition (Wagle et al., 2014).

#### 1.1.5.2 MEK inhibitors

MEK1/2 mutations, as previously mentioned, are rare in cancer. There are two reports of MEK mutations conferring resistance to MEK inhibitors: one a study which used random mutagenesis to generate mutations which conferred resistance against the MEK inhibitor AZD6244; a second study detected a MEK2 mutation, MEK2<sup>Q60P</sup>, in patient tumour samples which conferred resistance to AZD6244, alone, and in combination with a B-RAF inhibitor PLX4720 (Emery et al., 2009, Wagle et al., 2014). Resistance to MEK inhibitors can also emerge as the result of mutations affecting other components of the RAS-RAF-MEK-ERK pathway and other pathways (Marks

et al., 2008). Two types of resistance to MEK inhibitors have been reported: intrinsic resistance and acquired resistance.

Intrinsic resistance to MEK inhibitors, as with RAF inhibitors, in BRAFV600E frequently occurs as the result of relief of ERK-dependent negative feedback on RTK signalling (Samatar and Poulikakos, 2014). An example of this is the activation of HER3 signalling in response to AZD6244 in BRAFV600E tumours (Montero-Conde et al., 2013). The increase in HER3 signalling is due to increased expression of HER3, as the result of reduced ERK signaling which reduces the binding of transcriptional repressors, CTBP proteins, to the promoter site in the HER3 gene (Montero-Conde et al., 2013).

Intrinsic resistance to MEK inhibitors can also arise downstream of the RTKs. AZD6244 has been reported to occur as a result of activation of the PKB pathway (Balmanno et al., 2009) (Wee et al., 2009).

Amplifications of KRAS or BRAF have been shown to lead to acquired resistance to the MEK inhibitor AZD6244 (Little et al., 2011, Corcoran et al., 2010). By growing human colorectal cancer (CRC) cell lines containing either a BRAFV600E or KRAS<sup>13D</sup> mutation - but which remain sensitive to the anti-proliferative effects of AZD6244 - in increasing concentrations of AZD6244, the cells became resistant (Little et al., 2011). It was found that this resistance emerged as a result of amplification in the mutation - BRAFV600E or KRAS<sup>13D</sup> - driving the particular cancer cell line (Little et al., 2011). Due to the amplification upstream of MEK, the increased activity down the pathway was able to compensate for the inhibitory effect of AZD6244 on MEK (Little et al., 2011). As a result, enough activity was still getting through to ERK and, consequently, all of its substrates (Little et al., 2011). In one of the

BRAFV600E cell lines COLO205, the resistant derivative (C6244R) developed an IC50 20-times that of the parental COLO205 cell line (Little et al., 2011). The effect was even more striking in the KRAS13D cell line (Little et al., 2011). Even at a concentration 50 times the IC50 of the parental HCT116 cell line, the proliferation of the resistant derivative (H6244R) wasn't inhibited by more than 50% (Little et al., 2011).

#### 1.1.5.3 ERK inhibitors

Feedback loops, as with MEK inhibitors, have proven to be a limitation in the design of effective ERK inhibitors (Cox et al., 2014). 4 ERK inhibitors – BVD-523, MK-8353 and RG7842 (GDC-0994) - have reached the clinical trial stage; however, the effectiveness of these compounds is limited by the way also block the phosphorylation and inactivation of RAF by ERK (Samatar and Poulikakos, 2014, Cox et al., 2014).

### 1.2 The Mnks

#### 1.2.1 Identification and isoforms

The MAP kinase-interacting kinases 1 and 2, a sub-family of serine/threonine kinases, were first discovered in mice in 1997 (Waskiewicz, 1997, Fukunaga and Hunter, 1997); followed, a few years later, by the discovery of 4 human isoforms – Mnk1a, Mnk1b, Mnk2a, Mnk2b (Scheper et al., 2001a, Scheper et al., 2003, O'Loughlen et al., 2004a). Although a number of key binding partners of Mnks have been discovered, it is still unclear exactly what role they play in regulating translation.

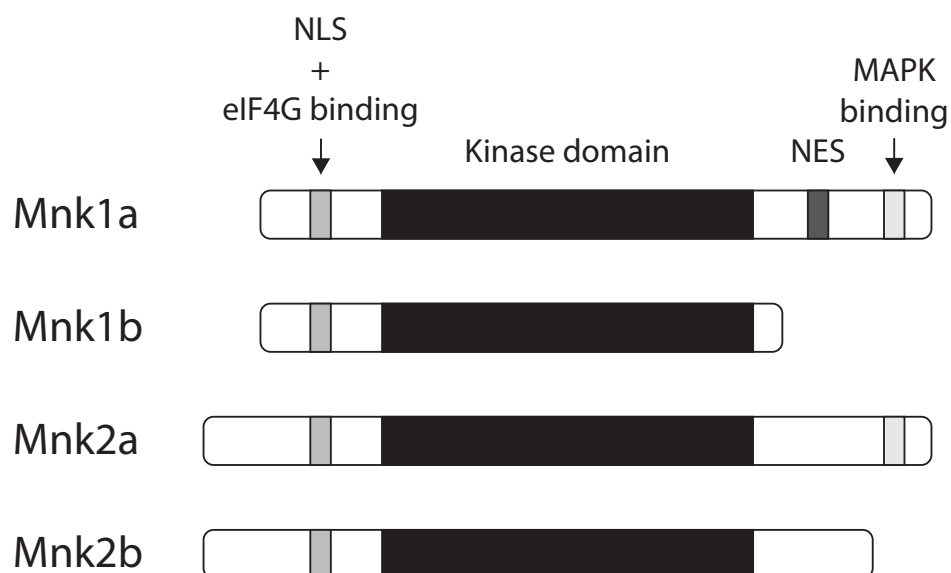


Figure 1.1 A schematic showing the key features of the 4 human Mnk isoforms.

NLS: Nuclear Localisation Sequence; NES: Nuclear Export Sequence. Mice only express the 2 longer Mnk isoforms: Mnk1a and Mnk2a.

The two human Mnk genes, MKNK1 and MKNK2, are expressed throughout the adult body, with particularly high levels of expression in the skeletal muscle and significantly reduced levels in the brain (Cargnello and Roux, 2011). Each human Mnk gene, through variation in splicing, produces a long isoform and a shorter isoform (O'Loghlen et al., 2004b, Slentz-Kesler et al., 2000). Studies on the 4 different human Mnk isoforms have shown that there are some key differences in the roles of the different Mnk isoforms within the cell. The similarity between the 4 isoforms can be found in the N-terminus - containing a Nuclear Localization Signal (NLS) and eIF4G-binding site - and central kinase domain (Hou, 2012). A polybasic region present in the N-terminus is responsible for both eIF4G recognition and, by binding to importin  $\alpha$ , nuclear import (Hou, 2012). The differences occur in the C-

terminal region of the proteins. Mnk1b and Mnk2b have a short C-terminus and lack a MAPK binding site (O'Loughlen et al., 2004b, Slentz-Kesler et al., 2000). The Mnk1a and Mnk2a isoforms have a much larger C-terminus, containing a MAPK kinase-binding domain, facilitating their role in relaying signals from the p38 and MAPK kinase pathways (O'Loughlen et al., 2004b, Slentz-Kesler et al., 2000). The MAPK-binding domain in Mnk1a (Leu-Ala-Arg-Arg) interacts with both p38 and ERK1/2; whereas, the binding domain of Mnk2a (Leu-Ala-Gln-Arg-Arg) only binds to ERK1/2 (Waskiewicz, 1997). Mnk2a, unlike Mnk1a, can stably bind to activated ERK1/2 and is active even during serum starvation (Scheper et al., 2001b). Mnk2a, at least a site (Ser437) in its C-terminus, is also regulated by mTORC1 (Stead and Proud, 2013). The addition of rapamycin, which inhibits mTORC1 kinase activity, increases the level of Mnk2a phosphorylation at Ser437 (Stead and Proud, 2013). This is despite the fact that rapamycin by inhibiting mTORC1-dependent phosphorylation of 4E-BP1 increases eIF4E-eIF4G binding and therefore the phosphorylation of eIF4E by the Mnks (Wang et al., 2007, Pyronnet et al., 1999).

### 1.2.2 Subcellular distribution

Another feature that distinguishes the Mnk1a isoform from the Mnk2a isoform is a CRM1-dependent Nuclear Export Signal (NES) in the C-terminus of the Mnk1a isoform (Parra-Palau et al., 2003). Consequently, Mnk1a can move freely between the nucleus and cytoplasm: this activity can be shown by treating cells with leptomycin B which blocks CRM1- dependent export (Parra-Palau et al., 2003). Without leptomycin, Mnk1 is predominantly localised in the cytoplasm, but the addition of leptomycin causes the accumulation of Mnk1 in the nucleus (Parra-Palau et al., 2003). Mnk2a is also found in the cytoplasm, despite lacking a NES (Scheper et al. 2003). The

unique C terminus of Mnk2a could explain the cytoplasmic location of Mnk2a (Scheper et al. 2003). Mnk2b is partially located in the nucleus where it co-localizes with eIF4E/PML bodies, which has raised speculation that it could have role in phosphorylating eIF4E in the nucleus (Scheper et al. 2003).

### 1.2.3 Mnk regulation

Single Mnk knockout studies in mice showed that Mnk2 is responsible for constitutive eIF4E phosphorylation; whereas, the inducible phosphorylation of eIF4E - in response to increased upstream signalling - can be attributed to Mnk1 activity (Ueda et al., 2004). In the quiescent state, such as when cells are starved, Mnk2 is mainly responsible for the basal activity (Cargnello and Roux, 2011). The differences in the way Mnk1 and Mnk2 are regulated can be explained by their structure.

Mnk1a is mainly responsible for inducible activity as Mnk1b lacks the C-terminal domain containing the ERK/p38 MAPK phosphorylation site, which is responsible for the inducible nature of Mnk1a activity (O'Loughlen et al., 2004a). Compared with Mnk1a, Mnk1b appears to have a higher basal activity (O'Loughlen et al., 2004a). The activity of Mnk1b is independent of cellular stresses and does not correlate with that of ERK1/2 and p38 MAP kinase upstream (O'Loughlen et al., 2004a). The higher basal activity of Mnk1b has been attributed to a unique 12 amino acid sequence – Mnk1bSR - in the C-terminus (O'Loughlen et al., 2007). Mnk2b has very low activity and it is not clear under what conditions it is activated (Scheper et al., 2003).

Mice only express the two longer isoforms, which are often referred to as Mnk1a/Mnk2a, despite not expressing the b-isoforms (Proud, 2015).

#### 1.2.4 Mnk inhibitors

The kinase domain of the Mnk proteins is similar to the rest of the  $\text{Ca}^{2+}$ /calmodulin-modulated protein kinases (CaMK) and the ATP-binding site is highly conserved amongst the protein kinases (Roux and Blenis, 2004) (Sunita et al., 2015). This means that the structure of the Mnks has to be considered carefully when designing Mnk inhibitors, if they are to be at all specific.

The success of any Mnk inhibitor depends on its ability to exploit two key molecular regions: the DFD motif and specific regions of the catalytic domain (EVFTD in Mnk1 and EAFSE in Mnk2) (Hou, 2012). It is these two features, which distinguish the Mnks from other kinases. The DFD motif (Asp191-Phe192-Asp193 in Mnk1 and Asp226-Phe227-Asp228 in Mnk2) has less affinity for ATP than the equivalent DFG motif found in other kinases (Hou, 2012). This reduction in ATP affinity is attributed to the fact that the DFD motif in Mnks is rotated  $180^\circ$  relative to the DFG motif: this unique conformation is often referred to as the DFG/D-OUT conformation in contrast to the DFG/D-IN conformation common to other kinases (Hou, 2012). It is this DFG/D-OUT conformation, which provides an opportunity for the design of specific Mnk inhibitors.

CGP57380 was the first reported Mnk inhibitor (Knauf et al., 2001). CGP57380 was shown to inhibit both Mnk1 and Mnk2 and has subsequently been used in a number of studies investigating Mnk function (Knauf et al., 2001) (Chrestensen et al., 2007) (Bianchini et al., 2008b) (Grzmil et al., 2011). A study by Bain et al. into the selectivity of kinase inhibitors showed that in fact the potency of CGP57380 for the Mnks was relatively low and, perhaps



more worryingly, that it also inhibited 3 other kinases – MKK, CK1 and BRSK2 – with similar potency to the Mnks (Bain et al., 2007).

Cercosporamide, an anti-fungal agent, was later found to inhibit the Mnks (Konicek et al., 2011). Cercosporamide, like CGP57380, targets the ATP binding domain of the Mnks (Hou, 2012). Cercosporamide was used to show that by inhibiting Mnk-dependent eIF4E Ser209 phosphorylation, cell proliferation was reduced and apoptosis increased in cancer cells (Konicek et al., 2011). What limited the development of cercosporamide as a therapeutic, and its value as a research tool, was that it was shown to inhibit a number of kinases such as Jak3, GSK3, ALK4 and Pim1 (Konicek et al., 2011). This means that any anti-proliferative effects, or any other effects, may not be due to cercosporamide-dependent inhibition of the Mnks, but instead may be due to one of these off-target kinases. Similarly, another Mnk inhibitor CGP052088 – related to staurosporine – is thought to inhibit several other kinases (Tschopp et al., 2000) (Hou, 2012).

There are three main types of kinases inhibitors. Type I kinase inhibitors, such as CGP57380 and cercosporamide, target the ATP binding site. The trouble with this is that the ATP binding site is well conserved amongst the kinases, making it difficult to achieve good specificity: however, a selectivity assay for Mnk-I1, which is believed to be a type I Mnk inhibitor, has recently shown Mnk-I1 to be specific for the Mnks (Beggs et al., 2015). Although type I kinase inhibitors may, on the whole, not be the best approach to achieving target specificity the information that is known about their molecular structures and how they interact with the Mnks is useful for designing more potent derivative compounds. This structural information has revealed regions more specific to the Mnks. Two such regions are the Mnk kinase

domain gatekeeper residue – a non-conserved hydrophobic region, independent of ATP binding - and the DFD-binding domain (Hou, 2012).

Type II inhibitors co-target the ATP binding site and an adjacent allosteric site (Hou, 2012). The allosteric site is a hydrophobic pocket formed by the activation loop, which is central to regulating Mnk activity (Hou, 2012). What is useful about the allosteric site is that it is less conserved amongst other kinases than the ATP site (Hou, 2012). As a result, type II inhibitors tend to show more specificity, which has been shown to follow through to fewer side effects in the clinic (Liu and Gray, 2006). The potential in developing inhibitors that target the allosteric site was demonstrated by the 12,000-fold improvement in affinity of p38 MAP kinase inhibitors (Pargellis et al., 2002).

Type III, allosteric Mnk inhibitors, by binding to non-conserved structural motifs outside of the ATP-binding pockets, are thought to yield even greater selectivity for the Mnks (Sunita et al., 2015). They are also non-ATP-competitive and reversible (Sunita et al., 2015). It has been shown that Mnk1 and Mnk2 appear to share a common allosteric binding site, but differ in the structure of their ATP binding site (Sunita et al., 2015). Mnk2 has a larger ATP binding pocket than Mnk1 (Sunita et al., 2015). These structural differences have allowed the design of selective Mnk2 inhibitors, which should help to develop our understanding of the functional differences between the Mnks (Sunita et al., 2015, Teo et al., 2015a, Teo et al., 2015b).

#### 1.2.5 Other Mnk targets

When conducting studies on Mnk1/2, and when considering inhibiting Mnk1/2, it is important to bear in mind that eIF4E is not the only known Mnk substrate. Sprouty2, cPLA2, PSF and hnRNPA1 are all established

phosphorylatable targets of Mnk1/2. What effect the Mnks have on Sprouty2 is debatable. Western blot analysis suggests that Mnk1 maintains Sprouty2 levels by phosphorylating a serine residue, thereby preventing its degradation (DaSilva et al., 2006). A more recent publication suggests the opposite; that Mnk dependent phosphorylation of Sprouty2 promotes its degradation (Edwin et al., 2010). With regards to the role of Sprouty2, it has been suggested that it acts as a negative feedback regulator on receptor tyrosine kinases: such as, EGF and FGF in *Drosophila* and FGF and VEGF in cultured cells. The exact mechanism of this feedback is less understood, but is thought to involve an effect on the availability of regulators of the RTK-Ras-ERK pathway (DaSilva et al., 2006). cPLA2 is responsible for the release of arachidonic acids, a vital part of the inflammatory response. There are two strong pieces of evidence supporting a role for Mnk1 in cPLA2-dependent arachidonic acid release: i) mass-spectrometry shows that Mnk1 phosphorylates cPLA2; ii) dominant-negative Mnk1 inhibits arachidonic acid release (Hefner et al., 2000).

hnRNPA1, a splicing repressor, and PSF are interesting Mnk targets in the context of tumourigenesis. The reason hnRNPA1 is such an interesting Mnk target is that there is inferential evidence linking it to a regulatory effect on tumour metabolism. hnRNPA1 has been shown to encourage the switch from PK-M1 to PK-M2, by repressing the use of exon 9 specific to PK-M1 (Clower et al., 2010). There are several pieces of evidence suggesting PK-M2 to be pivotal in the transitional changes in a cells metabolism as it progresses to a cancerous state (Mazurek, 2011, Cairns et al., 2011). Linking this together, it is possible to infer – by combining the evidence for each of the 3 parts of the possible link – that PK-M2 might link Mnk kinase activity to tumour metabolism, but there is no overriding evidence that draws these inferences

into an explicit Mnk-PK-M2 link. mRNA translation is one of the most energy demanding processes in the cell so it would make sense that cellular metabolism and mRNA translation are in some way linked (Topisirovic and Sonenberg, 2011b). Mnk phosphorylation of hnRNPA1 was shown to reduce its binding to the 3'UTR of mRNA of TNF $\alpha$  (Tumour necrosis factor-alpha) in T cells (Buxade et al., 2005). Phosphorylation of PSF (Polypyrimidine tract-binding protein (PTB)-associated splicing factor), another splicing factor, by the Mnks has been observed to increase its binding to TNF $\alpha$  mRNA (Buxadé et al., 2008). TNF $\alpha$  is involved in the immune response to tumours (Zhang et al., 2002).

One study in glioma has shown that the Mnks, following rapamycin treatment, can increase eIF4E availability through phosphorylation of 4E-BP1 (Grzmil et al., 2011).

## 1.3 Translational control

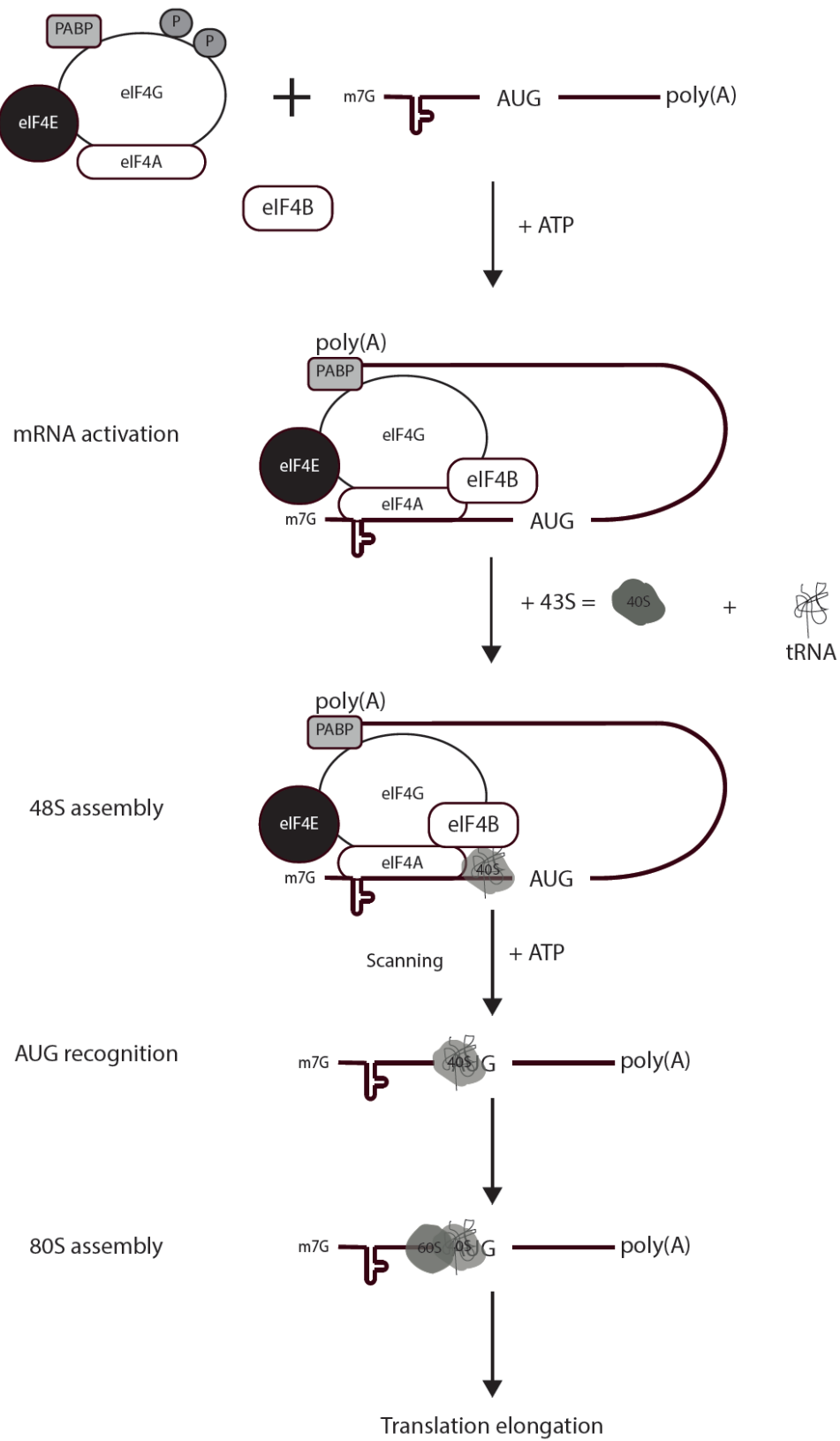
### 1.3.1 Overview

There are two main types of translation in a typical eukaryotic cell: cap-dependent and cap-independent translation. The majority of mRNAs are translated by the cap-dependent mechanism.

### 1.3.2 Cap-dependent translation initiation

Cap-dependent translation initiates when the 5' m<sup>7</sup>G cap of the mRNA binds to the initiation complex eIF4F (Sonenberg and Hinnebusch, 2009). The eIF4F contains eIF4G – the scaffold protein to which Mnks and their substrate eIF4E bind (Sonenberg and Hinnebusch, 2009). eIF4E is the cap-binding protein responsible for bringing eIF4F into contact with the 5' m<sup>7</sup>G cap of the mRNA (Sonenberg and Hinnebusch, 2009). By doing so, eIF4E brings into play another component of eIF4F, eIF4A, which is an RNA helicase: responsible for unwinding any secondary structures that may be present in the 5'UTR of the mRNA (Sonenberg and Hinnebusch, 2009, Parsyan et al., 2011). Once the eIF4F complex is bound to the mRNA, an interaction between eIF4G and eIF3 – part of the 43S ribosomal subunit - facilitates the formation of the 48S initiation complex (Sonenberg and Hinnebusch, 2009). The 43S complex contains the Met-tRNA<sub>i</sub> molecule and the 40S subunit, two components that are important for the transition to translational elongation: the next stage in protein translation (Sonenberg and Hinnebusch, 2009). The 43S complex is recruited to the 5' end cap by the eIF4F complex (Sonenberg and Hinnebusch, 2009). eIF2, in its GTP-bound form, anchors the Met-tRNA<sub>i</sub> to the pre-initiation complex (PIC) (Sonenberg and Hinnebusch, 2009). The PIC then scans along the 5'UTR of an mRNA, until the three base sequence of the start codon (AUG) enters the P (peptidyl) site of the ribosome (Sonenberg and Hinnebusch, 2009). When the AUG start codon enters the P

site, it is recognized by Met-tRNA as being complementary to its anticodon (Sonenberg and Hinnebusch, 2009). This recognition triggers irreversible hydrolysis of the GTP in the eIF2-GTP-Met-tRNA<sub>i</sub> ternary complex (TC) (Sonenberg and Hinnebusch, 2009). The conversion of eIF2-GTP to eIF2-GDP causes it to be released along with other eIFs (Sonenberg and Hinnebusch, 2009). This release allows the large (60S) subunit to join, forming an 80S initiation complex (Sonenberg and Hinnebusch, 2009). Once the 80S initiation complex is formed it can start bringing in aminoacyl-tRNAs into the A (aminoacyl) site and peptide bonds can form (Sonenberg and Hinnebusch, 2009).



\*Based on a diagram featured in Sonenberg and Hinnebusch, 2009

#### 1.3.2.1 PABP

Another protein PABP binds to eIF4G and is responsible for circularizing mRNA through its interaction with the poly(A) tail at the 3' end of mRNA (Sonenberg and Hinnebusch, 2009). It is thought that by bringing together the 5' cap and the poly(A) tail of mRNA into a 'closed loop' it enables re-initiation by post-termination ribosomes (Sonenberg and Hinnebusch, 2009). PABP also enhances the ability of eIF4F to bind to mRNA and, therefore, the binding of 43S to the PIC binding as well (Sonenberg and Hinnebusch, 2009).

#### 1.3.3 Cap-independent translation initiation

When the cap-dependent mechanism is impaired, a small population of mRNAs are translated by the cap-independent mechanism (Sonenberg and Hinnebusch, 2009) (Komar and Hatzoglou, 2011). Although cap-dependent translation forms the focus of the work presented here, it is worth bearing IRES translation in mind as a recently published paper has shown that under certain conditions Mnk might have a role in facilitating IRES activity (Shi et al., 2012). Cellular IRES-translation has not been very well documented and was only accepted very recently (Komar and Hatzoglou, 2011). IRES-translation was first observed in Picorna viruses in 1980s, the term IRES being coined from the discovery of mRNA regions which are capable of recruiting the 40S ribosomal subunit directly to the initiation codon, obviating the 5'end recognition required for cap-dependent translation (Komar and Hatzoglou, 2011, Sonenberg and Hinnebusch, 2009). The growing consensus is that under normal conditions, when cap-dependent translation is fully active, cellular IRES-translation facilitates the translation of mRNAs with long, structured 5'UTRs, which are less favoured when it comes to the 5'cap binding process in the initiation stage of cap-dependent translation (Komar



and Hatzoglou, 2011). Where IRES-translation is believed to be of particular importance is when cap-dependent translation is compromised: for example, when cells become stressed due to a lack of nutrients or oxygen (Komar and Hatzoglou, 2011). This theory ties in with the observation that many proteins involved in overcoming cellular stresses are encoded by mRNAs with IRES regions (Komar and Hatzoglou, 2011).

### 1.1.1 Mnks in translational control

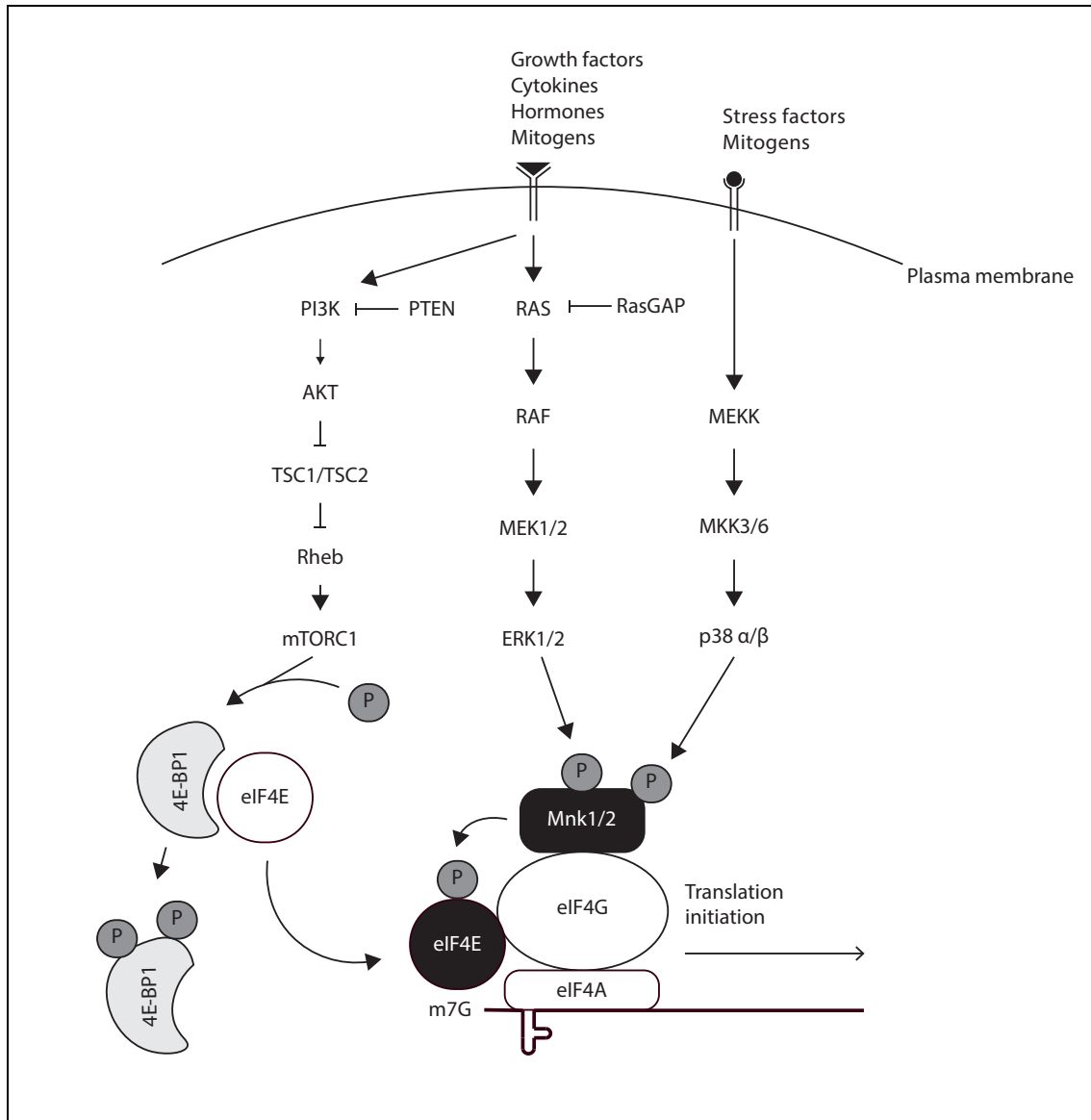


Figure 1.3 The Mnk-eIF4E axis.

An outline of the key signalling pathways responsible for regulating Mnk kinase activity and phosphorylation of eIF4E. The Mnk-eIF4E axis serves as a convergence point for signals initiated at the surface of the cell – the plasma membrane – by growth factors and other ligands binding to receptors, such as the receptor tyrosine kinases (RTKs). The RAS-RAF-MEK-ERK pathway and the MEK1/2-MKK3/6-p38 α/β pathway activate Mnk activity directly by phosphorylating distinct sites on the Mnks. PI3K-AKT-mTORC1 activity, indirectly, influences Mnk-dependent phosphorylation of eIF4E: phosphorylation of 4E-BP1, by mTORC1, releases eIF4E, which can then bind to eIF4G where it can then be phosphorylated by the Mnks.

The Mnk1/2 – eIF4E/p-eIF4E axis is emerging as a focal point in the regulation of cap-dependent translation initiation and has been heavily implicated in oncogenesis. This can be explained by the fact that it sits at the convergence point of three pathways - MAPK, p38 and mTOR – which are important in responses to determinants for cell growth and proliferation, such as growth factors, and oxygen and nutrient availability. The MNK1/2–eIF4E/p-eIF4E axis relays these signals onto the translation initiation complex. When mutations affecting the different components of these pathways decouple protein production from nutrient and growth factor sensing, they can lead to, or aid, the emergence of tumours. The growing evidence - both from in vitro studies and clinical observations - linking Mnks and eIF4E to tumourigenesis, has been followed by the development of several Mnk inhibitors designed to target this axis in the hope of reverting or hindering tumourigenesis (Zheng et al., 2014). By overcoming some of the problems encountered with early inhibitors, it is hoped that newly developed inhibitors with improved specificity – such as the type III inhibitors, which exploit a highly conserved allosteric site in the Mnks - could be used not only to find out more about the general biological role of the Mnk-eIF4E axis, but also to provide useful drugs for the treatment of cancer (Sunita et al., 2015).

The exact role Mnks play in cap-dependent translation initiation is uncertain. What is known, is that Mnks are essential for eIF4E phosphorylation at Serine 209; however, the consequence of this eIF4E Ser209 phosphorylation on translational output and the activity of eIF4E is contentious (Hay, 2010).

One of the earliest suggestions that phosphorylation is linked to an increase in translation initiation is a 1987 publication by Bonneau and Sonenberg. From their study, they were able to show that phosphorylation of eIF4E –

known then as the Cap-Binding Protein – did not affect its ability to bind to an m<sup>7</sup>-GDP agarose column, an analogue of the mRNA 5' cap (Bonneau and Sonenberg, 1987). However, this was based on the elution of free eIF4E – i.e. not part of the eIF4F complex (Bonneau and Sonenberg, 1987). We now know that phosphorylation of eIF4E by the Mnks only occurs after the formation of the eIF4F complex (Pyronnet et al., 1999). Based on the observation that the amount of phospho-eIF4E is lower during mitosis, when protein synthesis is also reduced, they extrapolated that phosphorylation of eIF4E must be involved in the increase in protein synthesis during interphase (Bonneau and Sonenberg, 1987). This was backed up by the observation that phosphorylation of eIF4E increases significantly, upon translational activation in response to mitogenic stimulation of T-cells (Boal et al., 1993). By separating and then purifying eIF4E and phospho-eIF4E, Minich et al were able to demonstrate that phospho-eIF4E had a stronger binding affinity for m<sup>7</sup>-GTP Sepharose (Minich et al., 1994).

By measuring [<sup>35</sup>S]methionine incorporation, Knauf et al., showed that adding a phosphomimetic active Mnk1 reduced protein synthesis despite increasing phosphorylation of eIF4E (Knauf et al., 2001). When introducing phosphomimetic proteins, it is important to question how closely the model relates to a normal physiological setting. Another concern is over the method itself. Hu and Heikka (2000) showed that the radiolabelling can cause an increase in DNA damage and p53 levels, which can send the cells into senescence or apoptosis. It is possible that the synthesis of a particular subset of proteins is increased as part of the stress response and so any effects of the phosphomimetic Mnk1 may not truly resemble the effects on protein synthesis under normal conditions. Scheper et al. showed conclusively, through the use of surface plasmon resonance, that in the

presence of a highly active Mnk2 the increased phosphorylation of eIF4E reduced its affinity for capped-mRNA (Scheper et al., 2002). This evidence contradicted earlier reports from Minich et al. and Shibata et al. (Minich et al., 1994)(Shibata et al., 1998).

There are several studies that suggest phosphorylation of eIF4E has no effect on eIF4F assembly and translation initiation. McKendrick. et al. showed that non-phosphorylatable mutants (Ser209 alanine mutants) of eIF4E are capable of restoring polysome formation (McKendrick et al., 2001). Using an Mnk inhibitor CGP57380, it was shown that the induction of translation in kidney cells upon the switch from hypertonic stress to isotonic conditions occurs irrespective of eIF4E phosphorylation (Morley and Naegele, 2002). The same inhibitor was used by Knauf et al in an earlier study to show that the initiation complex assembly is not dependent on eIF4E phosphorylation (Knauf et al., 2001).

It is possible that eIF4E phosphorylation does not have any significant effect on global translation. Knocking out both Mnk1 and Mnk2 – the only known eIF4E kinases –does not affect normal cell growth in mice (Ueda et al., 2010a, Ueda et al., 2004). Combined with the recurrent observation that p-eIF4E levels are increased in tumour cells, this has led to the suggestions that the Mnks, in phosphorylating eIF4E, could be important in increasing the translation of a sub-population of mRNAs, which are important in tumourigenesis (Furic et al., 2010).

There are strong suggestions that the effect of eIF4E and its phosphorylation on translation could be mediated through its second function: regulating mRNA nuclear export. 70% of eIF4E is located away from the translational

machinery, in the nucleus (Siddiqui and Borden, 2012). How much mRNA flows into the cytoplasm – where it can then interact with the cytoplasmic population of eIF4E to initiate translation– helps to determine the amount of protein that is made.

The mechanism by which eIF4E regulates nuclear export of mRNA is still not fully understood. What is known is that eIF4E relies on an adaptor protein to interact with the eIF4E-Sensitivity Element (4E-SE) on the mRNA (Siddiqui and Borden, 2012). The only known adaptor protein, which facilitates the interaction between the 3' UTR, 4E-SE and eIF4E, is LRPPRC (Siddiqui and Borden, 2012). eIF4E nuclear export is also dependent on CRM1; however, it is not clear how CRM1 binds to LRPPRC (Siddiqui and Borden, 2012).

When considering the role of eIF4E in cancer, it is interesting how in the cytoplasm eIF4E binds to all mRNA caps; whereas, in the nucleus eIF4E has only been found to associate with a subset of mRNAs. It is suggested that a subset of approximately 700 mRNAs contain the 4E-SE necessary for eIF4E dependent nuclear export (Siddiqui and Borden, 2012). This subset of mRNAs – often referred to as the eIF4E regulon - has been found to include many survival and proliferative proteins, such as: Myc, cyclin D1 and Hdm2 (Siddiqui and Borden, 2012, Phillips and Blaydes, 2008). There is evidence to suggest that nuclear Mnk activity, in phosphorylating eIF4E, can affect the export of particular mRNAs. It has been shown that double-phosphosite mutants (S209 and T210) of eIF4E impair the export of Cyclin D1 mRNA from the nucleus to the cytoplasm and, consequently, its translation into protein (Topisirovic et al., 2004). Similarly, it has been shown that Mnk1 activity on eIF4E is important in controlling HDM2 mRNA export (Phillips and Blaydes, 2008).

In order to interact with the initiation complex – eIF4F - mRNAs must bind to eIF4E, which means they are effectively competing for translation initiation. Depending on the length of their 5'UTR and secondary structures, different mRNAs have different translation efficiencies. Some mRNAs have very long highly-structured 5'UTRs, which due to the ATP-dependent helicase/eIF4A activity that is required during translation initiation, are translated less favorably (Parsyan et al., 2011, Topisirovic and Sonenberg, 2011a). The majority of mRNAs, particularly those involved in maintaining a normal cell physiology, have short unstructured 5'UTRs that are translated more favorably (Topisirovic and Sonenberg, 2011a). It was shown early on that by enhancing activity of the eIF4F complex, through increasing eIF4E availability, the translation of the long structured 5'UTR mRNAs increases disproportionately compared to those mRNAs with short 5'UTRs (Graff, 2003). Whether, eIF4E increases the translation of long 5'UTRs exclusively is uncertain. Wendel et al found that eIF4E phosphorylation correlates with expression and in part translation of Mcl-1 an anti-apoptotic protein with a structured 5'UTR (Wendel et al., 2007b). Mamane et al found that although eIF4E does appear to improve the translation of subsets of mRNAs, this is not selective for long 5'UTRs: a subset of ribosomal mRNAs, which lack any secondary structures in their 5'UTR were also found, through microarray analysis, to be translated more readily in the presence of eIF4E (Mamane et al., 2007). Whether Mnk dependent translation is reserved for mRNAs with long 5' UTRs is uncertain because there is some evidence to suggest that Mnk activity is important for the translation of TOP mRNAs, which by their very nature, contain short 5' and 3' UTRs (Bianchini et al., 2008a). The idea that eIF4E improves the translation efficiency of a subset of mRNA as opposed to having a global effect is supported by studies using polysome

profiles. Polysome profiles measure the average number of ribosomes associated with the mRNA transcripts in a sample. By comparing the number of ribosomes associated with a particular mRNA of interest, it is possible to determine the translational efficiency of the mRNA transcript. The more polysomes associated with an mRNA, the more efficiently that particular mRNA passes through the different stages of translation. This method was used in one study to show that eIF4E phosphorylation is needed for the translation of a number of pro-survival and anti-apoptotic proteins – MMP3, CCL2, VEGFC and BIRC2 – but that introducing a non-phosphorylatable form of eIF4E did not significantly affect the global translation rate (Furic et al., 2010). Another paper used RT-qPCR in conjunction with polysome profiles to show that inhibition of the Mnk, and therefore eIF4E phosphorylation, caused a reduction in the level of vimentin mRNA associated with the polysomal fractions of a polysome gradient (Beggs et al., 2015). It is important to point out that polysome profiles only reflect the translation initiation stage and do not reflect any effects there might be on the elongation stage. eIF4E phosphorylation was shown to be important for translating the mRNA of the  $\beta$ -catenin protein, which is important for self renewal in blast crisis (BC) leukemia stem cells (Lim et al., 2013).

### 1.1.1 mTOR

When investigating the contribution the Mnk-eIF4E axis makes to translational control, it is important to consider the contribution of another signalling pathway: the PI3K-mTOR pathway (Grzmil et al., 2011). The PI3K-mTORC1 pathway collects signals coming in from growth factors and hormones and converts it into an effect on eIF4E availability. Growth factor signals are received at the plasma membrane by receptor tyrosine kinase receptors, which then phosphorylate and activate PI3K. PI3K activation leads



to the activation of Akt/PKB. Akt inhibits the Rheb-GAP TSC2. The Rheb-GTP binds to and activates mTORC1. mTORC1 phosphorylates several proteins: the most important, in terms of Mnk activity, is 4E-BP1. 4E-BP1 has the same eIF4E-binding region as eIF4G: YXXXXLϕ (Joshi et al., 2004). What this means is that when hypo-phosphorylated, 4E-BP1 competes with eIF4G – the translation initiation complex scaffold – for eIF4E binding. Following, for example, the binding of a growth factor to a receptor tyrosine kinase on the cell membrane, the PI3K-Akt-mTOR signalling cascade is activated resulting in mTORC1 kinase dependent phosphorylation of 4E-BP1. Hyper-phosphorylated 4E-BP1 is unable to bind to eIF4E. In short, this means that the output of a growth factor receptor or hormone binding to a receptor on the plasma membrane is an increase in available eIF4E. Interestingly, recent evidence suggests mTORC1 may not be the only kinase responsible for phosphorylating 4E-BP1. Having confirmed the effect of mTOR kinase inhibitors, one study showed that in a colorectal cancer cell line, an absence of mTORC1 kinase activity did not preclude the phosphorylation of 4E-BP1 (Zhang and Zheng, 2012). Another study has showed that the Mnks can phosphorylate 4E-BP1, which means it could be the activity of the Mnks that is responsible for this observed phosphorylation of 4E-BP1 in the absence of mTORC1 kinase activity (Zhang and Zheng, 2012, Grzmil et al., 2011).

One of mTORC1's other targets, S6K1 and S6K2, by inactivating IRS1, upstream of mTOR, is central in an established feedback-loop: as mTORC1 through regulating translation is so pivotal in maintaining cellular homeostasis, it makes sense, from an evolutionary point of view, that such self-regulation exists (Laplane and Sabatini, 2012, Hou, 2012). S6K1 also has quite a broad input in terms of cell physiology, impacting translation –

although not clear, transcription, metabolism and cell growth (Laplane and Sabatini, 2012, Hou, 2012).

mTOR provides the catalytic domain for a second complex: mTORC2. The function and regulation of mTORC2 still remains ambiguous, owing to the lack of an effective mTORC2 selective inhibitor (Laplane and Sabatini, 2012, Hou, 2012). What is known about mTORC2, is that it is not regulated in the same way as mTORC1. It is inferred, from its role in regulating Akt, SGK and PKC, that growth factors regulate mTORC2; however, the mechanism is unclear (Laplane and Sabatini, 2012, Hou, 2012). One role of mTORC2 is to phosphorylate AGC kinases, such as Akt, which regulate cell survival and the rate at which cells pass through the cell cycle (Feldman et al., 2009, Zoncu et al., 2011). The role of mTORC2 as a central regulator of the cytoskeleton is also well established (Feldman et al., 2009, Zoncu et al., 2011).

Nutrient availability, cellular energy and oxygen availability - crucial factors in a cells decision to divide - all feed into the PI3K-Akt-mTOR pathway at different levels (Laplane and Sabatini, 2012, Hou, 2012). Nutrient availability and, more specifically, amino acid availability is sensed by the Rag proteins on the lysosomal surface, which then activates inactive-mTORC1 present on the lysosome surface (Laplane and Sabatini, 2012, Hou, 2012). Cellular energy levels (ATP levels) are coupled to AMPK activity, which when in the presence of low ATP and thus high AMP activates TSC2 to bring about inhibition of mTORC1 activity (Laplane and Sabatini, 2012, Hou, 2012). When oxygen levels are low – hypoxia – HIF-1 becomes stabilised and, consequently, increases the transcription of REDD1, which similar to the response to low energy levels, inhibits mTORC1 via activation of TSC2 (Laplane and Sabatini, 2012, Hou, 2012). Indeed, hypoxia has been shown to

activate AMPK, independently of HIF-1 to bring about an induction of the energy stress response (Populo et al., 2012).

TOP mRNAs are the best characterised of targets of mTOR, in terms of translational control (Bianchini et al., 2008a). TOP mRNAs are a group of mRNAs which contain short 5'UTR and 3'UTRs (Bianchini et al., 2008a). It has been shown that association of TOP mRNAs with polysomes, in response to growth factor stimulation, is dependent on mTOR activity (Bianchini et al., 2008a).

#### 1.3.4 Mechanisms for regulating translation

Most of the mechanisms for regulating protein translation act at the initiation stage (Sonenberg and Hinnebusch, 2009). There are two key mechanisms of regulating translation initiation. The first involves the initiation factors themselves and their availability and phosphorylation, which tends to have a more general effect on translation initiation; the second mechanism is more selective and involves proteins and microRNAs (miRNAs) which recognize specific mRNAs (Jackson et al., 2010).

##### 1.3.4.1 eIF2

The overall effect of phosphorylation of eIF2 is a reduction in translation initiation, but the translation of two particular mRNAs that code for the transcription factors ATF4 and ATF5 increases (Jackson et al., 2010). Phosphorylated eIF2, once released from the initiation complex, sequesters and inhibits the activity of eIF2B (Jackson et al., 2010). As a result, the amount of eIF2 that associates with the initiation complexes drops causing a reduction in overall mRNA translation (Jackson et al., 2010). mRNAs with a particular configuration of two uORFs are an exception, the translation of

these mRNAs is increased (Jackson et al., 2010). ATF4 and ATF5 are the best characterized examples in mammals (Jackson et al., 2010). The particular uORF configuration consists of a short uORF1 and a longer uORF2 which overlaps the *ATF4/5* ORF (Jackson et al., 2010).

#### 1.3.4.2 eIF4E-4E-BP1 homeostasis

The availability of components of the eIF4F complex is an important mechanism in regulating translation initiation. eIF4E is an important part of the eIF4F complex. The availability of eIF4E is governed by 4E-BP1, which sequesters eIF4E away from eIF4G and the rest of the eIF4F complex (Richter and Sonenberg, 2005). By disrupting the interaction between eIF4E and eIF4G, and therefore the association of eIF4E with the Mnks, 4E-BP also represses eIF4E phosphorylation (Müller et al., 2013). When 4E-BP1 is phosphorylated by mTORC1 it releases eIF4E. The phosphorylation status of 4E-BP1 and its regulation of eIF4E availability is believed to be under homeostatic control and has important implications for some methods of studying the role of eIF4E in translational control (Yanagiya et al., 2012).

Knock down or knock out studies are a useful tool for studying the functional role of a gene and the protein it codes for. By seeing what happens to a system when a protein of interest is removed it is possible, using a reliable readout, to deduce what its function is. However, there is a potential caveat to studies that use this method to study eIF4E function. This caveat is highlighted in a study by Yanagiya et al. The study showed that knockdown of eIF4E had no significant effect on methionine incorporation - a common measure of protein synthesis (Yanagiya et al., 2012). On this basis, it would be reasonable to deduce that eIF4E has no role in translation. Luckily, we have over 30 years of research supporting a role for eIF4E in translation,

(Altmann et al., 1985). The reason why knocking out eIF4E had no effect on protein synthesis is that eIF4E is under tight homeostatic control. The availability of eIF4E is regulated by a family of 4E-binding proteins (4E-BPs). When eIF4E is knocked down, 4E-BP1 no longer has anything to bind to and consequently gets degraded: by an E3 ubiquitin ligase, the KLHL25-CUL3 complex. Knockdown, using shRNA - the method used in this study - is rarely absolute and so there will be a residual amount of eIF4E – as a western blot included in their study testifies. An interpretation of this finding is that eIF4E and 4E-BP1 levels are part of a homeostatic mechanism (Hinnebusch, 2012). The determining factor in deciding whether 4E-BP1 is degraded is its phosphorylation status. To bind to eIF4E, 4E-BP1 has to be hypophosphorylated. If there is no eIF4E around then the unbound, hypophosphorylated 4E-BP1 is a target for E3 ubiquitin ligase mediated degradation. Hyper-phosphorylated 4E-BP1, which also exists in an unbound state, is resistant to degradation (Yanagiya et al., 2012). When eIF4E is knocked down the amount of eIF4E sequestered by 4E-BP1 would just equilibrate so that the relative amount of free unbound eIF4E is held at a constant. eIF4E is thought to be in excess of eIF4G in the particular cell type, HeLa, used for this study: this explains why when eIF4E was knocked down there was still enough residual eIF4E to form eIF4E-eIF4G complexes (Yanagiya et al., 2012).

#### 1.3.4.3 CYFIP1-FMRP

One mechanism of translational control that is gaining a better understanding is the CYFIP1-eIF4E complex. CYFIP1/Sra1 competes with 4E-BP1 for binding to eIF4E, via a similar domain to that of both 4E-BP1 and eIF4G (Napoli et al., 2008). The difference with 4E-BP1 is that CYFIP1 binding

does not exclude eIF4G; it has been shown that the presence of eIF4G does not affect the ability of CYFIP1 to bind to eIF4E (Napoli et al., 2008). CYFIP1 binds another protein, the Fragile-X Mental Retardation Protein (FMRP), which was first thought to suppress global mRNA translation, but recently it has emerged that FMRP may also induce the translation of particular mRNAs (Napoli et al., 2008, Lucá et al., 2013). FMRP is believed to help recruit CYFIP1 to mRNAs and help stabilize the interaction between CYFIP1 and mRNAs (Napoli et al., 2008). Without FMRP, neuronal cells are unable to develop mature synapses and causes Fragile-X Syndrome (FXS).

It has recently been suggested that FMRP controls the translation of mRNAs into proteins involved in the process of EMT (Epithelial-Mesenchymal-Transition) in cancer cells. A study showed that removing FMRP causes an increase in expression of E-cadherin and a decrease in vimentin expression, suggesting that FMRP is required for vimentin expression (Lucá et al., 2013). It is not clear whether this is due to an effect on translation because the total mRNAs levels for each of these proteins was affected in the same way that their overall expression was affected, suggesting it could be a transcriptional effect (Lucá et al., 2013). Another result, from the same study, showed that FMRP could be regulating the stability of the vimentin mRNA (Napoli et al., 2008). The results presented in chapter 5 of this thesis and in another publication, suggest that FMRP represses the translation of vimentin through its interaction with CYFIP1 (Beggs et al., 2015). These results showed that either inhibiting Mnk kinase activity or knocking out the Mnks (in particular Mnk2) increases CYFIP1-eIF4E binding (Beggs et al., 2015). The ability of FMRP to associate with mRNAs depends on the binding of CYFIP1 to eIF4E (Napoli et al., 2008). It appears that when eIF4E is phosphorylated by the Mnks it inhibits CYFIP1-eIF4E binding and, therefore, the association of

FMRP with mRNA. The same set of results also show that inhibiting the Mnk1s reduced the translation efficiency of vimentin mRNA (Beggs et al., 2015). From this, it is possible to infer that the increased CYFIP1-FMRP binding to mRNA, due to the inhibition of eIF4E phosphorylation by the Mnk1s, is responsible for repressing the translation of vimentin mRNA. This finding contradicts the study by Luca et al., which showed that FMRP increases vimentin expression, but they both agree that the CYFIP1-FMRP complex has an important role in regulating vimentin expression (Beggs et al., 2015, Luca et al., 2013).

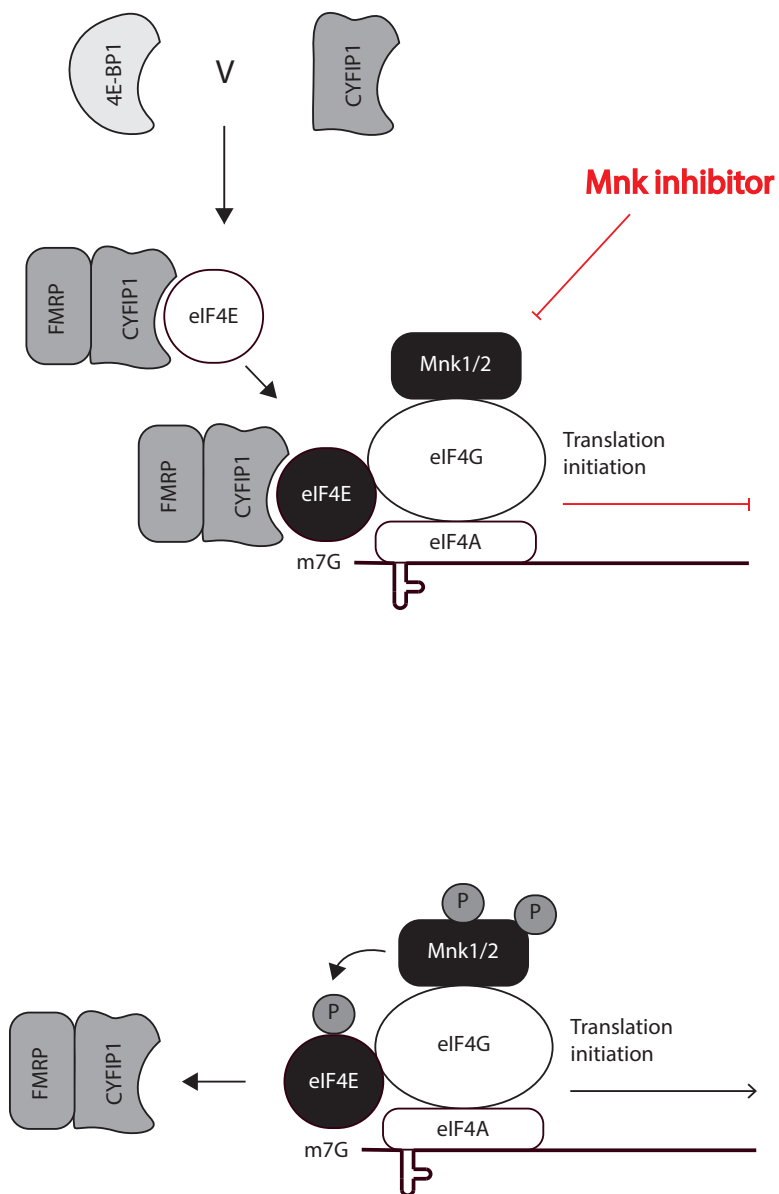


Figure 1.4 The CYFIP1-FMRP complex.

A diagram illustrating how Mnk kinase activity and its inhibition affects the association of the CYFIP1-FMRP complex with the m<sup>7</sup>G 5'cap of mRNAs. Binding of the CYFIP1-FMRP complex to eIF4E is, generally, believed to repress the translation initiation of particular mRNAs. Inhibiting Mnk kinase activity, and therefore phosphorylation of eIF4E, has been shown to increase the association of CYFIP1 with eIF4E. This suggests that phosphorylation of eIF4E normally reduces CYFIP1 binding.



#### 1.3.4.4 YB1

YB1 is another protein, which appears to have a dual role in regulating protein translation. YB-1 competes with eIF4E for the 5' m<sup>7</sup>GTP cap of mRNA. As a result, YB-1 biases translation towards that of cap-independent mRNAs. YB-1 competes with eIF4E for the mRNA cap structures resulting in a repressive effect on the translation of cap-dependent mRNAs, encoding proteins involved in proliferation; in favor, of the translation of cap-independent mRNAs encoding proteins involved in EMT, pro-survival and angiogenesis (Evdokimova et al., 2009b).

#### 1.3.4.5 miRNA

Mechanisms of translational control that act via translation initiation factors tend to have a broader effect on overall cap-dependent translation. miRNAs control the translation of specific mRNAs. miRNAs repress the translation of particular mRNA by binding to a specific sequence in the 3'UTR. miRNAs can either cause direct repression of mRNA translation or stimulate mRNA degradation via deadenylation. The number of miRNAs that bind to the 3'UTR correlates with the degree of repression. miRNAs interact with other sequence-specific RNA-binding proteins or act as adaptors for Argonaute proteins: such as, the Human Argonaute2 (Ago2) protein which, via its endonuclease activity, cleaves the mRNA it is tethered to (Meister et al., 2004).

### 1.3.5 Translational control in cancer

Many of the proteins involved in translation initiation and the pathways that control translation are established oncogenes. Mutations affecting any one of the upstream kinases or phosphatases can, in theory, tip the balance in favour of cell proliferation; to give an idea of the different signalling pathways or parts of the pathways that can cause this effect on translational control a few common mutations are considered here.

Starting at the cell membrane, common sites of tumorigenic mutations are the growth receptors or receptor tyrosine kinases. HER2, a target of the widely publicised Herceptin (Trastuzumab) drug, is a growth factor receptor tyrosine kinase, which is overexpressed in aggressive forms of breast cancer. HER2 activates RAS and PI3K, it is through activation of RAS and the consequent RAS-RAF-MEK-ERK pathway that HER2 overexpression causes activates Mnk1/2 activation (Chrestensen et al., 2007). It has recently been reported that the overexpression of Mnk1 and Mnk2, mediated by the transcription factor YB-1, plays an important role in acquired resistance to Trastuzumab (Astanehe et al., 2012).

PTEN - a phosphatase responsible for de-phosphorylation of PIP<sub>3</sub> (Phosphatidylinositol (3, 4, 5)-triphosphate) and, consequently, inhibiting Akt/PKB activation, - plays a key role in restraining the activity of the PI3K-AKT-mTOR pathway and ultimately has a reductive effect on eIF4E availability. PTEN inactivating mutations, which – via increased mTORC1 activity - cause an increase in eIF4E availability, have been attributed to a wide range of cancers: hormonal based, such as prostate and breast, neurological cancers, skin and those affecting the immune system - lymphoma - and haematological - leukaemia. A recent study, highlighting PTEN as a marker for patients' response to radiotherapy, typifies the

importance of this protein in tumourigenesis (Snietura et al., 2012). The role Mnks could be playing in this PTEN directed tumourigenesis has been highlighted in two studies. In lymphoma cells, which had PTEN knocked out, knocking out Mnk1/2 appeared to inhibit tumourigenesis (Ueda et al., 2010). Similarly, inhibition of Mnks using CGP57380, in prostate cancer cells that had low PTEN levels, reduced the translation of proteins that encourage cell proliferation (Bianchini et al., 2008b).

RAS proteins comprise a family of GTPases: H-RAS, K-RAS and N-RAS. Mutations in RAS proteins can be found in up to 30% of all human cancers (Cox et al., 2014). Two important effectors of the RAS GTPases are PI3K and RAF. RAS proteins are activated by an adaptor protein, Sos, which is associated with SH2/SH3 domains, and activated, via Grb2, by the auto-phosphorylation sites on the intracellular domain of receptor tyrosine kinases. When mutations, commonly in the RAS GTPase, cause RAS to be overactive it causes increased signalling down both the PI3K-Akt-mTOR pathway and the RAF-MEK-ERK pathway, causing a dual effect on increased eIF4E availability and thus uncoupled protein translation (Castellano and Downward, 2011).

eIF4E was established as an oncogene – or, more correctly, a proto-oncogene - over 20 years ago when overexpression of eIF4E transformed fibroblast cells and made them tumourigenic (Lazaris-Karatzas et al., 1990). This was backed up by a converse study, which showed the reversal of RAS-transformed rat fibroblasts in response to knocking down eIF4E expression using antisense RNA (Rinker-Schaeffer et al., 1993). The link between eIF4E expression and cell proliferation was established when eIF4E overexpression was shown to increase cyclin D1 levels (Rosenwald et al., 1993). Since then,

studies – both clinical and in vitro - have repeatedly shown a link between increased eIF4E availability and tumourigenesis (Ruggero et al., 2004, Wendel et al., 2004, Wendel et al., 2007a, Graff et al., 2007).

It is not just at the translational level where p-eIF4E may play a role in promoting the expression of tumour promoting proteins. eIF4E phosphorylation has been shown to increase the expression of proteins involved in regulating cell proliferation, cyclin D1 and Hdm2, by enhancing the nuclear export of their mRNAs ((Topisirovic et al., 2004, Phillips and Blaydes, 2008). By monitoring the nuclear-cytoplasmic distribution of a specific mRNA, each study showed that either inhibiting the phosphorylation of eIF4E or introducing a phospho-defective mutant reduced the cytoplasmic levels of the respective mRNAs, whilst increasing the nuclear level proportionately.

### 1.3.6 Translational control as a drug target

Translational control, like most signalling pathways, is highly complex and adaptable, which makes it difficult to find suitable drug targets. The negative feedback loops and interconnectivity of signalling pathways combined with the ability of other pathways to adapt and compensate for others means that cancer cells can acquire resistance to drugs targeting signalling proteins. Examples of this include the acquired resistance to AZD6244, the MEK inhibitor, and trastuzumab, which targets the HER2 receptor, referred to in previous sections of this introduction. Another problem is that particular domains - catalytic domains and, especially, ATP-binding sites - are well conserved amongst kinase proteins meaning drugs designed to target a particular signalling protein often affect other signalling proteins.

The Mnk inhibitor CGP57380 had been shown to be effective at inhibiting the growth of cancer cells, in particular the breast cancer cell line AU565. AU565 cells overexpress the growth factor receptor HER2, which is frequently mutated in aggressive breast cancers (Chrestensen et al., 2007). These cells also exhibit increased Mnk1 and Mnk2 activity (Chrestensen et al., 2007). However, when CGP57380 was tested against an extensive panel of kinases it was found to be a relatively weak inhibitor of the Mnks and more worryingly that it inhibits other kinases such as MKK1, CK1 and BRSK2 with similar potency (Bain et al. 2007). In addition, CGP57380 was also found to inhibit RSK, which is involved in a negative feedback loop. RSK and Mnk are both activated by ERK, RSK then acts upstream to inhibit MEK – the kinase responsible for activating ERK, which subsequently inhibits the activity of Mnk. By inhibiting RSK and thereby blocking the negative feedback loop, CGP57380 was shown to slightly induce Mnk activity (Zhang et al., 2008). Despite the explicit concern of Bain et al – “CGP 57380 is not a specific inhibitor of MNK isoforms and results obtained from its use in cell-based assays are difficult to interpret” – CGP57380 is still commonly used in published studies (Bain et al. 2007). This means that any findings based on experiments using CGP57380 have to be taken with a degree of caution or be backed up with genetic knockout studies or more selective Mnk inhibitors. Negative feedback loops have undermined attempts to design effective mTOR inhibitors. In theory, inhibiting mTOR should, by increasing the amount of 4E-BP1 that is able to quench eIF4E, inhibit cap-dependent translation. The problem with inhibiting mTORC1 – on its own at least - is that mTORC1 also activates p70S6K, which inhibits AKT upstream of mTOR: therefore, inhibiting mTORC1 relieves the negative feedback pathway (Shi et al., 2005, Um et al., 2004).

Rapamycin, an allosteric mTORC1 inhibitor, has provided the basis for a lot of functional studies of TOR proteins. Although, rapamycin is effective at inhibiting mTORC1, mTORC2 is insensitive to rapamycin and 4E-BP1 is often insensitive to rapamycin (Choo et al., 2008). 4E-BP1 can still be phosphorylated in the presence of rapamycin and cap-dependent translation can still occur (Choo et al., 2008). Consequently, cells continue to proliferate in the presence of rapamycin (Dowling et al., 2010). To find out more about how mTOR regulates cell proliferation, ATP-competitive mTOR inhibitors (TORKinibs), such as PP242, have been developed to inhibit mTORC1. Interestingly, although ATP-competitive inhibitors are more effective than rapamycin at inhibiting cell proliferation the effect is not due to the additive inhibition of mTORC2 (Feldman et al., 2009). Instead, the stronger anti-proliferative effect of TORKinibs is due to the improved inhibition of mTORC1 (Feldman et al., 2009).

The adaptability and interconnectivity of signalling pathways and the problem this poses for inhibiting the pathways that regulate translation is illustrated by the example of AZD8055: a dual mTORC1 and mTORC2 inhibitor. A study by Cope et al. 2014 showed that cells grown in the presence of AZD8055 could acquire resistance to AZD8055 by overexpressing eIF4E (Cope et al., 2014).

The interconnectivity of the RAS/MEK/Mnk and PI3K/Akt/mTOR means that inhibiting one pathway on its own, PI3K/Akt/mTOR, is not sufficiently potent. Some of the crossover between the two pathways is only just being uncovered. Over the last few years there have been several papers linking MNK2 and mTOR. MNK2 was seen to inhibit mTOR dependent phosphorylation of a ribosomal protein S6 kinase (p70S6K), but also

rapamycin, an inhibitor of mTORC1, appears to increase phosphorylation of eIF4E by phosphorylating a site (Ser437) on Mnk2a (Hu et al., 2012, Stead and Proud, 2013, Wang et al., 2007). A more recent study has suggested that this rapamycin dependent increase in p-eIF4E might not be solely dependent on Mnk2 (Teo et al., 2015b). The study showed how a dual Mnk inhibitor appeared to have an additive effect on the inhibition of rapamycin dependent eIF4E phosphorylation caused by a selective Mnk2 inhibitor (Teo et al., 2015b).

One way of overcoming the problem of acquired resistance in cancer cells is to use drugs in combination. Combination therapies involving the combined inhibition of the RAS-MEK-Mnk and PI3K-Akt-mTOR pathways have yielded some promising results. The combined use of AZD6244 – an inhibitor of MEK (two kinases upstream of Mnk) – and rapamycin – a natural mTOR inhibitor - in two prostate cancer models (BxPC-3 and MIA PaCa-2) increased the amount of cell cycle arrest and apoptosis. This caused a marked improvement in growth inhibition compared to that caused by each inhibitor on its own. A common caveat to combination therapies is increased toxicity. Crucially, the combined use of AZD6244 and rapamycin did not appear to have any toxicity (Chang et al., 2009). This lack of apparent toxicity is somewhat surprising; an explanation could be that both drugs act on proteins relatively far down their respective signalling pathways, which limits the number of additional pathways they might affect. Another reason is that these pathways have a common output: eIF4E availability.

The phosphorylation status of 4E-BP1 and how it affects eIF4E availability is emerging as a key consideration in the design of combination therapies that target both the PI3K-mTOR and MAPK pathways. A study has recently shown

that a lower p-4E-BP1<sup>T70</sup>:4E-BP1 ratio correlates with increased sensitivity of leukaemia cell lines to rapamycin following treatment with an Mnk inhibitor (Teo et al., 2015b). The combination of an Mnk inhibitor and rapamycin resulted in a bigger reduction in levels of p-4E-BP1<sup>T70</sup> than when either an Mnk inhibitor or rapamycin was used alone. Coincident with this reduction in p-4E-BP1<sup>T70</sup>, was a reduction in eIF4E-eIF4G binding and cell proliferation (Teo et al., 2015).

Combined inhibition of the Ras-Raf-MEK-ERK and PI3K-AKT-mTOR pathways might not be enough to inhibit cap-dependent translation. A study by Cope et al., already mentioned earlier on in this section, showed that cells could develop resistance to a combined treatment of a MEK inhibitor, AZD6244, and dual mTORC1 and mTORC2 inhibitor, AZD8055, by overexpressing eIF4E (Cope et al., 2014). This study, together with an earlier study on acquired resistance to MEK inhibitors, illustrates just how resilient signalling pathways can be to targeted small molecule inhibition (Little et al., 2011).



## 1.4 Cell migration

Cell migration plays a critical role throughout human development and physiology: from the spatial orientation of cells within an embryo through to the ability to heal a wound (Yamaguchi and Condeelis, 2007). Not all cell migration is beneficial. Cell migration is what allows cancer cells to spread to other parts of the body. This process, which is called metastasis, is responsible for 90% of all cancer deaths (Chaffer and Weinberg, 2011). An understanding of the cellular processes involved in cell migration, therefore, not only improves our understanding of some key physiological processes, but is also helping to guide the development of anti-cancer drugs (Chaffer and Weinberg, 2011, Yamaguchi and Condeelis, 2007).

Cell migration relies on the driving force of a particular type of cell membrane protrusion called a lamellipodia (Yamaguchi and Condeelis, 2007). Lamellipodia are sheet-like protrusions, which attach to a substrate and pull the cell forward (Yamaguchi and Condeelis, 2007). A second membrane protrusion, called the filopodia, is also believed to be important in directing cell movement in response to external cues; however, the precise role of filopodia is not known (Yamaguchi and Condeelis, 2007).

### 1.4.1 Rearranging the cytoskeleton

The formation of cellular protrusions is an active process driven by rearrangements of the cytoskeleton. These cytoskeletal rearrangements are initiated by signaling pathways activated in response to the sensing of external chemoattractants. Lamellipodia, the protrusions that produce the driving force for cell migration, are formed as a result of localized actin polymerization. Actin polymerization requires free barbed ends. Free barbed ends can either be produced by forming new actin filaments, a process

initiated by the Arp2/3 complex; causing breaks in existing actin filaments, for instance by cofilin; or uncapping barbed ends on existing actin filaments (Zigmond, 2004, Condeelis, 2001).

#### 1.4.1.1 WASP and WAVE

The activity of the Arp2/3 complex is regulated by the WASP protein family (Millard et al., 2004). In mammalian cells, the WASP protein family consists of 5 proteins: WASP, N-WASP, WAVE1, WAVE2 and WAVE3 (Yamaguchi and Condeelis, 2007). Several upstream signaling pathways converge on, and activate, the WASP proteins, which by activating Arp2/3 complex drives the actin nucleation necessary for cell migration (Millard et al., 2004).

There are several signaling pathways believed to link receptors responding to chemoattractants at the cell surface to the WASP proteins, which initiate the formation of cellular protrusions. In a resting state, the WASP and N-WASP proteins are auto-inhibited: its own amino-terminal domain binds the VCA domain, which is responsible for binding to and activating the ARP2/3 complex. It is only when a Rho GTPase – such as Cdc42 or Rac – or a member of the Src homology (SH) domain-containing SH2-SH3 adaptor protein family – such as NCK – binds to WASP or N-WASP that its auto-inhibition is removed and ARP2/3 can be activated (Eden et al., 2002). WAVE1 is regulated differently. WAVE1 exists in a complex which inhibits – by trans-inhibition - the activity of WAVE1 (Eden et al., 2002). This complex contains PIR21, CYFIP2 (also known as NCKAP1) and HSPC300, but it is not clear which proteins are directly responsible for the trans-inhibition of WAVE1 (Eden et al., 2002). What is clear is that Rac1 and NCK activate WAVE1 by causing WAVE1 to dissociate from the inhibitory complex (Eden et al., 2002).

PIP3, a phosphoinositide, a product of PI3K activity has also been shown to be important for recruiting WAVE2 to polarized membranes in order to induce lamellipodia formation(Oikawa et al., 2004). As well as regulating the WAVE proteins phosphoinositides have also been reported to regulate WASP, N-WASP(Yamaguchi and Condeelis, 2007).

WASP is only expressed in hematopoietic cells, such as macrophages and dendritic cells. N-WASP is expressed ubiquitously, but is particularly abundant in the brain. Several reports link WASP and N-WASP to a role in particular membrane protrusions called invadopodia, which are involved in cell invasion. It is thought that WASP and N-WASP are involved in the endocytosis of matrix components that have been degraded as a result of cell invasion. Cell invasion is an important part of metastasis, as it allows cancer cells to break out of their tissues. N-WASP has been shown to play an important role in invadopodia formation in carcinoma cells – cancer of the epithelial cells. The role of WASP in driving invadopodia formation in macrophages and dendritic cells is a vital part of the immune system. In patients with Wiskott–Aldrich syndrome, characterized by immune deficiency, their macrophages are unable to form invadopodia due to a mutation in WASP. (Yamaguchi and Condeelis, 2007)

WAVE 2, like N-WASP, is ubiquitously expressed. Particularly high levels of WAVE2 are found in hematopoietic cells. WAVE 1 and WAVE 3 are expressed at low levels in a range of tissues, but are expressed at high levels in the brain. WAVE 2 is believed to be an important driver of metastasis in cancer. Analysis of patient samples, by immunohistochemistry, showed that WAVE 2 was expressed in metastatic human lung cancer cells (Semba et al., 2006). An association was also drawn between WAVE 2 expression and

patient prognosis (Semba et al., 2006). An experiment in a mouse melanoma cell line showed that WAVE 1 and WAVE 2 expression correlates with the progression towards metastasis: WAVE1 was later found to have little influence on this progression, suggesting that it was primarily down to the role of WAVE2 (Kurusu et al., 2005). Suppressing WAVE2 expression has also been shown to inhibit lamellipodia and, consequently, metastasis in a human sarcoma cell line (Huang et al., 2006). Interestingly, WAVE2 does not appear to be important for invadopodia formation in cancer. The role of WAVE2 seems to be restricted to lamellipodia formation and cell migration (Yamaguchi and Condeelis, 2007). WAVE3 has been linked with cancer cell migration, but it is not clear what the exact role of WAVE3 is (Sossey-Alaoui et al., 2005a). Evidence from knockdown studies in adenocarcinoma cell lines suggests that WAVE3 could be important for regulating the expression of matrix metalloproteinases, which are important for cell invasion (Sossey-Alaoui et al., 2005b).

#### 1.4.1.2 Cofilin

The direction a cell moves in is dictated by the activity of cofilin (Ghosh et al., 2004). Cofilin activity has been shown to be important for directional cell migration of carcinoma cells in response to EGF stimulation (Mouneimne et al., 2004). Cofilin is a protein capable of binding to both actin monomers and filaments, in which it can induce a break. At rest, cofilin exists in a trans-inhibitory complex with PIP2. Activation of PLC, for example in response to EGF binding to an EGF receptor, hydrolyses PIP2 releasing cofilin (Mouneimne et al., 2004). As well as initiating the release of cofilin, EGF simultaneously activates LIM kinase. LIM kinase then activates the free cofilin in localized bursts (Song et al., 2006). The localized bursts of cofilin activity cause localized actin polymerization, which results in the formation of

lamellipodia to pull the cell forward in a particular direction (Song et al., 2006, Yamaguchi and Condeelis, 2007). The balance between LIM kinase activity and cofilin activity is also important for cell motility: too much activity of either LIM or cofilin inhibits migration (Zebda et al., 2000, Yamaguchi and Condeelis, 2007).

#### 1.4.1.3 Cortactin

Cortactin, like cofilin, is an actin binding protein that is important for cell migration. For instance, knocking out cortactin has been shown to impair the ability of cancer cells to migrate and invade (Bryce et al., 2005). The role cortactin plays in cell migration is, however, very different to that of cofilin. The cortactin knockout cells could still produce lamellipodia, but they did not persist as long as in wild type cells (Bryce et al., 2005). This observation suggests that cortactin plays an important role in the formation of cell adhesions, which are what allow a lamellipodia to persist. As with lamellipodia formation, ARP2/3 dependent actin polymerization is an important part of cell adhesion (DeMali et al., 2002). It is perhaps surprising that cortactin plays such an important role in actin reorganization when cortactin activity alone has a relatively small effect on Arp2/3-dependent actin nucleation (Urano et al., 2001). Instead of acting directly on actin nucleation, cortactin supplements the activities of Arp2/3 complex. For instance, it is believed that cortactin stabilizes the actin filaments produced by Arp2/3 complex (Weaver et al., 2001). There is also evidence that cortactin activates N-WASP, which then activates Arp2/3 (Martinez-Quiles et al., 2004).

Cortactin is believed to be phosphorylated in response to cell adhesion and growth factor stimulation (Lua and Low, 2005). How cortactin activity is

regulated during lamellipodia and invadopodia formation is not clear. Src has been shown to phosphorylate cortactin during invadopodia formation, but another study showed that knocking out cortactin had no effect on invadopodia formation (Lua and Low, 2005). These findings do not rule out a role for cortactin in stabilizing the invadopodia, as it does in lamellipodia. There is also some evidence to suggest ERK could regulate cortactin activity (Lua and Low, 2005).

#### 1.4.1 Translation

The localized translation of mRNAs encoding WAVE1, ArpC2,  $\beta$ -actin and Rac1 at the leading edge of migrating cells has been shown, using immunofluorescence, to be important in lamellipodia formation (Willett et al., 2013). The same group also published an earlier paper showing how proteins involved in translation initiation colocalise with the leading edge of migrating fibroblasts (Willett et al., 2011). One of these proteins was eIF4E which is central to cap-dependent translation and to the ability of breast cancer cells to metastasise, a process which relies the ability of cancer cells to migrate (Nasr et al., 2013). They also showed that phosphorylated eIF4E, which is indicative of Mnk kinase activity, co-localised with the leading edge. Several papers since – including data presented in the results section of this thesis - have shown that Mnks, which are believed to control the translation of a particular subset of mRNAs, play an important role in cell migration (Ramalingam et al., 2014, Beggs et al., 2015, Robichaud et al., 2014).

#### 1.4.2 Cell polarity

The regulation of cell adhesion is important for converting the individual transient cellular protrusions into a concerted movement. For a cell protrusion to last, it must form new cell adhesions with the extracellular

rmatrix and other cells (Ridley et al., 2003). Once several protrusions have formed and adhered at the leading edge of a cell, signals are transmitted to the rear of the cell causing the removal of cell adhesions. The cell is now polarized.

In a polarized cell the molecular processes going on at the front of the cell are distinct from those at the rear of the cell. The distinct molecular processes between the front and rear of a cell can be established within a relatively narrow extracellular concentration gradient of chemoattractant. The signaling proteins responsible for converting a small extracellular concentration gradient into cell polarity are the phosphoinositides: PIP<sub>3</sub> and PIP<sub>2</sub> (Ridley et al., 2003). PI3K, which generates the phosphoinositides, accumulates at the leading edge; whilst PTEN, which removes the phosphoinositides, becomes localized to the rear and sides of the cell (Ridley et al., 2003). It has been shown that cells that have defective PI3K or PTEN can still migrate but do not respond to a chemo-attractant gradient (Ridley et al., 2003).

The high levels of PIP<sub>3</sub> at the leading edge of migrating cells appears to be important for activating Cdc42, which can initiate actin nucleation (Ridley et al., 2003). Cdc42 is important for guiding the directionality of cell migration: if Cdc42 is activated all over the cell, the cells are unable to migrate, which highlights the importance of localized activation of Cdc42 at the leading edge of cells (Ridley et al., 2003). This also helps restrict lamellipodia formation to the leading edge of cells. As well as initiating actin nucleation, Cdc42 is also important in directing changes in the microtubule structure of a polarized cell (Ridley et al., 2003). Cdc42 is believed to be responsible for moving the microtubule-organising centre (MTOC) and golgi apparatus

towards the leading edge of the cell; or, in front of the nucleus so as to re-orientate the organelles inside a polarized cell (Ridley et al., 2003). Cdc42 then initiates the growth of microtubules from the MTOC into the leading edge of the cell (Ridley et al., 2003). The microtubules then facilitate the transport of vesicles, containing proteins required for lamellipodia formation, from the golgi to the leading edge (Ridley et al., 2003).

The protein complexes involved in maintaining apical-basal cell polarity in epithelial cells are well conserved throughout multicellular organisms and the various tissues types within each organism (Royer and Lu, 2011). The complexes involved in maintaining apical-basal polarity help resist tumour formation by: establishing orientation within the cell during asymmetric cell division – important in cancer stem cells; and, by linking cell polarity to the apical junction complex (AJC) (Royer and Lu, 2011). There are three main complexes Par, Crumbs and Scribble which together help establish and maintain apical-basal cell polarity (Royer and Lu, 2011). Par and Crumbs establish and maintain the apical membrane phenotype, whereas Scribble is central to the basolateral membrane phenotype (Royer and Lu, 2011).

The AJC consists of tight and adherens junction complexes (Royer and Lu, 2011). The complexes responsible for maintaining apical-basal cell polarity help stabilize the tight and adherens junctions (Royer and Lu, 2011). A central component of the adherens junction is E-cadherin. The loss of apical cell polarity during the late stages of EMT is coincident with a loss of E-cadherin and the AJCs, which allow the cells to become motile (Royer and Lu, 2011). The maintenance of apical-basal cell polarity protects against epithelial cells becoming mesenchymal and migrating away from the tissues of which they are a part (Royer and Lu, 2011).



### 1.4.3 Single and collective cell migration

Cell migration is the result of a dynamic interaction between a cell and the surface or substrate to which it is attached. The transient nature of cell migration and the ability of cells to switch between different types of cell migration make it a difficult process to study (Huttenlocher and Horwitz, 2011). At either end of a range of different types of cell migration are single cell migration and collective cell migration. Single cell migration is often categorized into either mesenchymal-like or amoeboid-like migration; however, rather confusingly, non-mesenchymal cells and amoeba-like cells such as *Dictyostellium*, can also undergo mesenchymal cells (Huttenlocher and Horwitz, 2011). Mesenchymal single cell migration involves a cycle of protrusion formation, adhesion formation and stabilization at the leading edge of a cell followed by re-orientation of the cell body and release of cell adhesion at the rear of the cell (Huttenlocher and Horwitz, 2011). This cycle involves integrins, which help generate the tractable forces to drag a cell forward (Huttenlocher and Horwitz, 2011). Conversely, weak integrin interactions and even integrin-independent migration is central to Amoeboid-like single cell migration (Huttenlocher and Horwitz, 2011). The weak integrin-dependent adhesions in amoeboid-like single cell migration allow for a fast gliding motion in migratory amoeboid cells (Huttenlocher and Horwitz, 2011). This is characterized by blebbing, caused by cortical actin tension and is used by dendritic cells, neutrophils and lymphocytes (Huttenlocher and Horwitz, 2011). Cancer cells have been observed to switch between integrin-dependent mesenchymal-like cell migration and integrin-independent amoeboid-like cell migration, making it difficult to identify integrins that would be suitable drug targets (Huttenlocher and Horwitz, 2011).

#### 1.4.4 Integrins

Integrins are what link the dynamic interactions between the extracellular matrix and changes in intracellular actin polymerization that drive cell migration (Hynes, 2002). Integrins are heterodimeric receptors, made up of  $\alpha$  and  $\beta$  chains, with large extracellular domains and short cytoplasmic domains (Takada et al., 2007). In humans there are 24 different integrin heterodimers, made up of different combinations of 18  $\alpha$  and 8  $\beta$  chains (Takada et al., 2007). The different combinations determine which extracellular ligands the integrins can bind to. Integrin ligands include fibronectin, collagen and laminin; and cell surface receptor proteins ICAM-1 and V-CAM1 (Takada et al., 2007). Each integrin heterodimer has different adhesive properties. This means that the strength and persistence with which a cell adheres to a particular matrix, and, consequently, how it migrates, is determined by the particular assortment of integrin heterodimers that are involved (Takada et al., 2007). Altering the integrin profile of a cell can affect how well a cell migrates, for instance:  $\alpha v \beta 3$  expression on melanoma cells has been shown to correlate with tumour invasion, whilst  $\alpha 2 \beta 1$  integrin is associated with rhabdomyosarcoma (Seftor et al., 1992, Chan et al., 1991).

If cells just formed strong permanent adhesions they would not be able to migrate. The turnover of adhesions is as important to cell migration as the formation of cell adhesions. The rate of cell migration is at its optimum when the rate of formation and turn over of cell adhesions is balanced (Zamir and Geiger, 2001). Implicit in this is the need for intermediate levels of particular integrins,  $\alpha 5 \beta 1$  or  $\alpha 2 \beta 1$ , and intermediate concentrations of ligand, such as fibronectin or collagen (Huttenlocher and Horwitz, 2011). The rate of cell adhesion formation is coupled to the rate of actin polymerization

(Huttenlocher and Horwitz, 2011). The precise mechanism behind the formation of adhesions is not known (Huttenlocher and Horwitz, 2011). It is thought to involve clustering of activated integrins in response to binding to integrin ligand such as fibronectin (Huttenlocher and Horwitz, 2011).

Integrins coordinate the generation of tractable forces in a cell with intracellular signaling pathways which as well as feeding back into effects on cell migration also govern cell proliferation, gene expression and cell survival (Huttenlocher and Horwitz, 2011). What links the rate of protrusion to downstream signaling pathways is the amount of tension generated across the integrin-actin linkages (Huttenlocher and Horwitz, 2011). Integrin-actin linkages were first observed in the 1970s and whilst several molecules are known to be involved it is still unclear how exactly these linkages form (Huttenlocher and Horwitz, 2011). Proteins that have been implicated in linking integrins to the actin cytoskeleton at focal adhesions include talin, vinculin, and  $\alpha$ -actinin (Zamir and Geiger, 2001). The rate of cellular protrusion formation is determined by the difference between the rate of actin polymerisation and depolymerisation at the leading edge of a cell (Huttenlocher and Horwitz, 2011). When cell adhesions form, they inhibit actin depolymerisation, which means the rate of protrusion increases (Huttenlocher and Horwitz, 2011). This is thought to generate a shunting force on the extracellular matrix component (Huttenlocher and Horwitz, 2011). Some of this force is absolved by the molecular slippages that are believed to occur within the linkages (Huttenlocher and Horwitz, 2011). It is thought that on some more pliable extracellular matrix components the forces generated by this shunting force could translate into movements within the extracellular matrix (Huttenlocher and Horwitz, 2011).

The binding of ligands to the extracellular domains of integrins causes them to cluster (Huttenlocher and Horwitz, 2011). Inside the cell, integrin clustering results in the formation of multi-protein complexes that include signaling proteins and adaptor proteins, which connect to the actin cytoskeleton (Huttenlocher and Horwitz, 2011). Integrin clusters generate the localised signaling activity that causes cells to polarize (Huttenlocher and Horwitz, 2011). Localized activation of PKA is believed to be important in directing cell migration (Huttenlocher and Horwitz, 2011). Several signalling pathways activated by integrin clusters are thought to converge on the Rho GTPases, which regulate actin polymerization and also a feedback onto cell adhesion (Huttenlocher and Horwitz, 2011). Integrin-based adhesion can be formed from different combinations of over 150 molecules, which means they are hugely varied (Huttenlocher and Horwitz, 2011). Integrin-based adhesions vary from cell to cell and depend on the particular extracellular ligand it is interacting with. Although the size of the focal adhesions varies widely, there is generally an inverse correlation between the size of a focal adhesion and rate of cell migration. The focal adhesions are the best characterized of the integrin-based adhesion complexes (Huttenlocher and Horwitz, 2011).

#### 1.4.5 Focal adhesions

Focal adhesions, or focal contacts, are aggregates of transmembrane proteins, mainly composed of integrins, which link the extracellular matrix to the actin cytoskeleton and a large intracellular signaling hub (Zamir and Geiger, 2001). The intracellular signaling hub of focal adhesions is highly complex, with over 50 different proteins known to associate with it (Zamir and Geiger, 2001). Together, the various anchor proteins and signaling proteins coordinate intracellular responses to changes in cell adhesion.

FAK is an interesting example of one of the many kinases associated with focal adhesion complexes. As well as interacting with the intracellular domain of integrins, FAK also responds to signals from receptor tyrosine kinases (Turner, 2000). FAK phosphorylates another protein paxillin, which has two important roles in coordinating responses to changes in cell adhesion (Turner, 2000). One role of paxillin is to link integrins with the actin cytoskeleton, via another protein called vinculin which provides a physical link between paxillin and the actin cytoskeleton (Turner, 2000). Paxillin, following phosphorylation by FAK, also activates the MAPK signaling pathway can initiate changes in gene expression (Turner, 2000).

Vinculin, Arp2/3 and FAK - another protein known to bind to Arp2/3 - all enter the site of an adhesion at the same time so it is thought they are part of a concerted mechanism involved in initiating adhesion formation (Huttenlocher and Horwitz, 2011, Mitra et al., 2005) Vinculin is known to bind to Arp2/3 and it is thought that this interaction could be a crucial part in adhesion formation (Huttenlocher and Horwitz, 2011). Arp2/3 dependent actin nucleation regulates adhesion. Arp2/3 activity is controlled by Rho GTPase activity and integrin signaling following cell adhesion (Huttenlocher and Horwitz, 2011). Rho GTPase activity - and, therefore, indirectly, Arp2/3 activity - is regulated by paxillin and FAK (Brown and Turner, 2004).

## 1.5 Epithelial-Mesenchymal Transition (EMT)

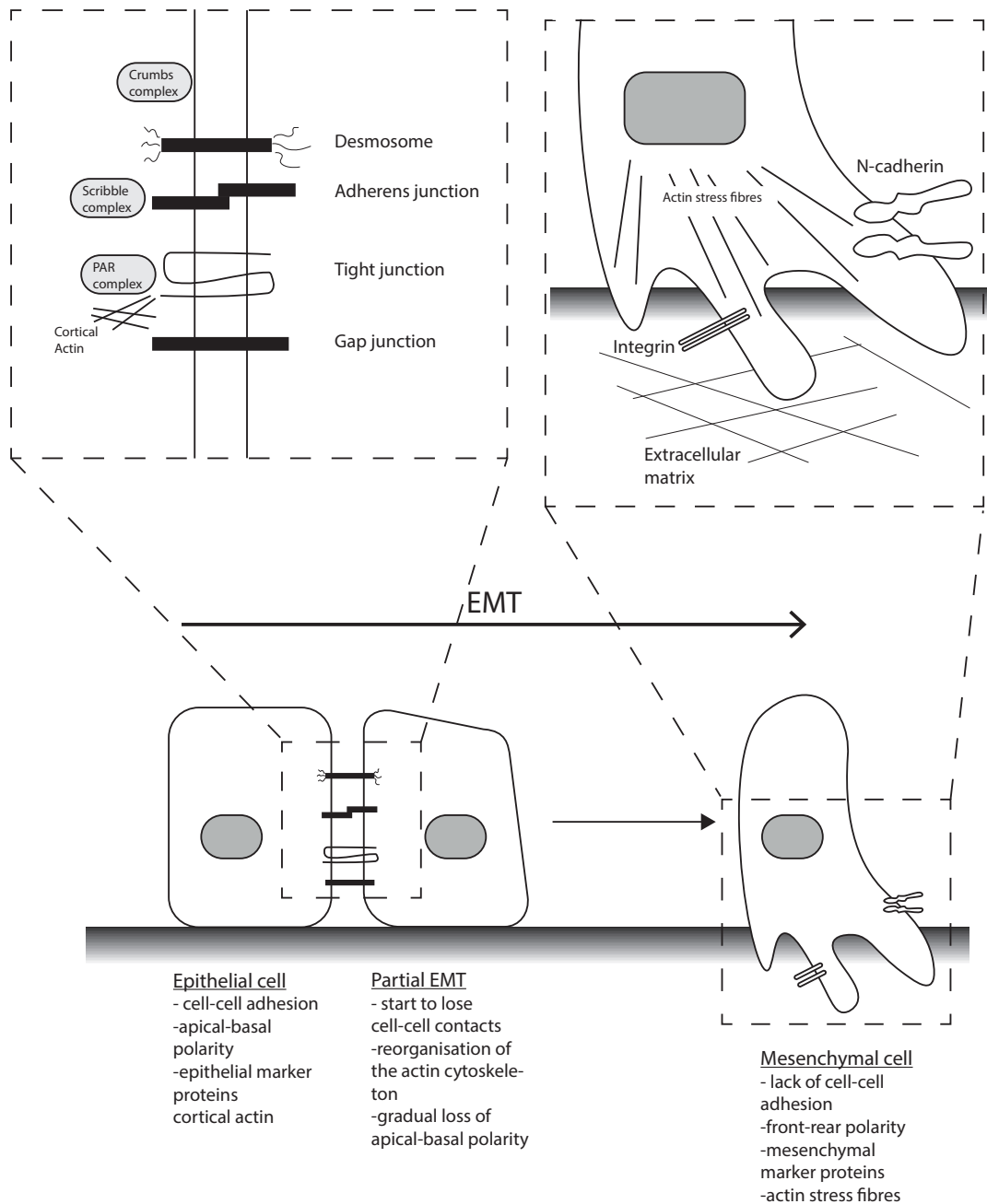


Figure 1.5 Epithelial-Mesenchymal Transition (EMT)

\*Based on diagram featured in (Samy et al., 2014)

Cell migration is an important part of Epithelial-Mesenchymal Transition (EMT). EMT is a transient process by which epithelial cells lose their epithelial phenotype and gain a mesenchymal phenotype. The process is an integral part of development, but is also reactivated during wound repair, fibrosis and in cancer cells. EMT was first observed in chick embryos in the early 1980s (Hay, 1995). Originally, the process was called Epithelial-Mesenchymal Transformation, but it was changed to Epithelial-Mesenchymal Transition (EMT) when it was discovered that it was in fact a transient process: the reverse is called Mesenchymal-Epithelial Transition (MET) (Samy et al., 2014). Epithelial cells can transition between EMT and MET multiple times (Samy et al., 2014).

Epithelial cells usually exist in single layers or in multilayer tissues. They form the permeable boundaries between different organs and tissues (Huang et al., 2012). The function of particular epithelial cells is defined by the basement membranes that they adhere to (Samy et al., 2014). The adhesion to the basement membrane helps establish an apical-basal polarity (Samy et al., 2014). Epithelial cells also form tight-junctions between adjacent epithelial cells, which allow them to communicate with each other (Samy et al., 2014). The loss of these properties, which define epithelial cells, are the key steps in EMT (Samy et al., 2014).

The exact process of EMT can vary between different tissues, but there are a few key processes common to all forms of EMT. These include: the loss of the cell-cell junctions; the loss of apical-basal polarity, to be replaced with a front-rear polarity; reorganization of the cytoskeleton; the replacement of

epithelial gene expression with mesenchymal gene expression; increased cell motility; and, in many cases, the acquisition of the ability to invade other tissues and degrade the extracellular matrix (Samy et al., 2014). Following EMT, cells also become more resistant to apoptosis and senescence (Thiery et al., 2009).

During EMT, cells transition from co-operative cells working together as tissue to cells that act independently of the tissue from which they have derived. Once EMT is initiated, the cell-cell contacts - tight junctions, adherens junctions, desmosomes, gap junctions – are degraded. It is the degradation of adherens junctions which leads to the degradation of an established marker of epithelial cells: epithelial-cadherin (E-Cadherin) (Yilmaz and Christofori, 2009). E-cadherin forms part of the adherens junction in epithelial cells. When the adherens junctions are degraded, E-cadherin is cleaved at the plasma membrane and degraded (Yilmaz and Christofori, 2009). Reduced E-cadherin levels cause a reduction in cell adhesion and increased cell motility (Samy et al., 2014).

Epithelial cells have a cortical actin cytoskeleton, which is important for the cell-cell contacts and interactions with the basement membrane (Samy et al., 2014). During EMT, the actin cytoskeleton changes so that it becomes more focused on driving the formation of cellular protrusions, such as lamellipodia or invadopodia, which allow cells to migrate or invade (Yilmaz and Christofori, 2010). Cells that have undergone EMT also redirect their actin towards the formation of actin stress fibres which cause the cells to contract (Yilmaz and Christofori, 2010).



Rho GTPases are thought to play a central role in the reorganisation of the actin cytoskeleton during EMT (Nelson, 2009). RHOA promotes actin stress fibre formation during EMT. RAC1 and CDC42 promote actin nucleation to initiate the formation of lamellipodia. Rho-associated kinase (ROCK) promotes actin polymerization and stabilizes actin filaments by activating LIM kinase, which inactivates cofilin: a protein responsible for creating breaks in actin filaments (Narumiya et al., 2009). ROCK is also responsible for increasing contractility in cells undergoing EMT, by inducing myosin light chain phosphorylation (Narumiya et al., 2009). RHO-GTPases also play an important role in the transition from apical-basal polarity to front-rear polarity (Nelson, 2009). RHOA localizes at the rear of the cell where it promotes the removal of adhesion complexes, which causes the cell to retract (Nelson, 2009).

A lot of the changes that occur during EMT are driven by changes in gene expression. The 'cadherin switch', from expressing E-cadherin in epithelial cells to expressing N-cadherin in mesenchymal cells, is often used as an indication as to whether cells have undergone EMT (Yilmaz and Christofori, 2010). The expression of other proteins involved in cell-cell contacts is also repressed; such as, occludin and claudin – proteins involved in apical tight junctions – and desmoplakin and plakophilin – proteins involved in desmosomes (Huang et al., 2012). The increased expression of NCAM, which interacts with N-Cadherin, in mesenchymal cells leads to increased focal adhesion assembly through its activation of a SRC family kinase FYN (Lehembre et al., 2008). Increased focal adhesion formation contributes to the increased motility and invasiveness observed in mesenchymal cells (Samy et al., 2014). The expression of the intermediate filament vimentin, another established marker of mesenchymal cells, is switched on during EMT (Huang

et al., 2012). Vimentin is known to increase cell motility, it is thought, by interacting with motor proteins (Mendez et al., 2010). Whilst the expression of vimentin gets switched on during EMT, the expression of keratin – an intermediate filament – becomes switched off (Huang et al., 2012). Keratin is important in directing E-cadherin to the plasma membrane where, in epithelial cells, it forms adherens junctions (Toivola et al., 2005).

The way cells interact with their ECM also changes during EMT. Epithelial cells express integrins such as  $\alpha 6 \beta 4$  which interact with the basement membrane, but during EMT these integrins get replaced with other integrins, such as  $\alpha 5 \beta 1$ , which binds to fibronectin and  $\alpha 1 \beta 1$  and  $\alpha 2 \beta 1$ , which bind to collagen (type 1 ) (Koenig et al., 2006, Samy et al., 2014). In addition,  $\alpha 1 \beta 1$  and  $\alpha 2 \beta 1$  integrins reinforce the removal of E-cadherin by disrupting the adherens junctions (Samy et al., 2014). The increased expression of integrins  $\alpha v \beta 6$  and  $\alpha v \beta 3$  during EMT is thought to contribute to increased invasiveness by increasing the expression of MMPs and their association with invadopodia (Shah et al., 2012, Samy et al., 2014). The release of MMPs from the invadopodia allows invasive cells to break down the extracellular matrix (Nisticò et al., 2012). It is believed that some MMPs also target the extracellular domain of E-cadherin contributing to its removal and the loss of adherens junctions (Nisticò et al., 2012). MMP secretion is also believed to feed back into the EMT progression by unlocking growth factors from the extracellular matrix, such as TGF $\beta$ , which activate signaling pathways that promote EMT (Sheppard, 2005). Active TGF $\beta$  stimulates the release of collagen and fibronectin helping to remodel the extracellular matrix into one which integrins expressed by mesenchymal cells can interact with (Samy et al., 2014).

The change in cell phenotype during EMT is due to a reprogramming of gene expression. This reprogramming of gene expression is driven by the transcription factors SNAIL, TWIST, ZEB1 and bHLH (basic helix-loop-helix) transcription factors (Samy et al., 2014). The expression of these transcription factors is activated early in EMT to coordinate the repression of genes that define an epithelial phenotype and activate the expression of mesenchymal genes. Some of these transcription factors can both repress epithelial genes and also induce the expression of mesenchymal genes.

SNAIL transcription factors bind to E-Box DNA sequences within specific epithelial genes and then initiate histone modifications, which repress the expression of the particular epithelial gene. The E-cadherin gene is one example of an epithelial gene, which SNAIL1 represses by this mechanism. The carboxy-terminal zinc-finger binding domain of SNAIL1 allows it to bind to the E-Box sequence within the promoter region of the E-cadherin gene (Samy et al., 2014). Once bound, SNAIL1 can then recruit the Polycomb repressive complex 2 (PRC2) (Samy et al., 2014). The PRC2 complex contains methyltransferases, which methylate histones H3K9 and H3K27 to form repressive chromatin (Samy et al., 2014). Interestingly, the methyltransferases and acetyltransferases, which are part of the PRC2 complex, also make histone modifications normally associated with active chromatin, such as H3K4 methylation and H3K9 acetylation (Samy et al., 2014). These 'bivalent domains', within the promoter of the E-cadherin gene, are essential for the transience of EMT: allowing for a quick transition between an epithelial and mesenchymal state (Samy et al., 2014). Bivalent domains are also found in some of the mesenchymal genes, which SNAIL1 helps activate (Samy et al., 2014).

SNAIL1 is activated by TGF $\beta$ , Wnt signaling, Notch and RTKs (Samy et al., 2014). By cooperating with ETS1, SNAIL1 activates matrix-metalloproteinase (MMP) expression in response to MAPK activation (Jordà et al., 2005). SNAIL1 also cooperates with SMAD3-SMAD4 to repress E-cadherin and occludin expression in response to TGF $\beta$  (Theresa et al., 2009).

The basic loop-helix-loop transcription factor TWIST1 plays an important role in the 'cadherin switch' during EMT (Samy et al., 2014). TWIST1 binds to both the promoters in the E-cadherin and N-cadherin genes and recruits the methyltransferase SET8 (Yang et al., 2012). SET8 methylates histone H4K20, which in the E-cadherin promoter leads to repression, but in the N-cadherin promoter, induces expression (Yang et al., 2012). It is this dual response to the histone H4K20 that defines the switch from E-cadherin to N-cadherin expression in mesenchymal cells (Yang et al., 2012). A diverse array of signaling pathways has been shown to activate TWIST1. In hypoxic conditions, HIF1 $\alpha$  has been shown to induce TWIST1 expression leading to EMT, which eventually allows cancer cells to escape (Muh-Hwa et al., 2008). Similar to SNAIL1, TGF $\beta$  activates TWIST1, indirectly, by repressing the expression of another bHLH transcription factor, which binds to and inhibits TWIST activity (Kang et al., 2003). Activity along the MAPK signaling pathway helps prolong TWIST1 activity, by protecting it from degradation (Hong et al., 2011).

ZEB1 and ZEB2, the two vertebrate ZEB transcription factors, like TWIST and SNAIL, bind to E-box domains in the promoter regions of genes (Héctor et al., 2007). Similarly, ZEB transcription factors can also act as both repressors of epithelial genes, such as E-cadherin, and activators of mesenchymal gene expression (Samy et al., 2014). ZEB can either repress E-cadherin by bringing

in a C-terminal Binding Protein or a SWI/SNF chromatin remodeling protein (Sánchez-Tilló et al., 2010). The expression of ZEB1 is thought to be promoted by SNAIL and TWIST (Dave et al., 2011). In terms of signaling, TGF $\beta$ , Wnt signaling and RAS-MAPK signalling, in response to growth factors, have been shown to induce ZEB expression (Shirakihara et al., 2007).

The expression of proteins involved in EMT is not just regulated by transcription. Alternative mRNA splicing and the translation of particular mRNAs into protein are also known to contribute to EMT.

One essential splicing event in EMT involves the mRNA that codes for the CD44 protein, which is involved in cell adhesion. There are two isoforms of CD44: the variant isoforms (CD44v) and the standard isoforms (CD44s) (Brown et al., 2011). The variant isoform is expressed in epithelial cells. During EMT, CD44v is replaced by CD44s (Brown et al., 2011). The switch is governed by the mRNA levels and corresponding expression of epithelial regulatory splicing protein 1 (ESRP1) (Brown et al., 2011). ESRP1 causes a splicing event in the CD44 mRNA, which produces CD44v (Brown et al., 2011). A drop in the level of ESRP1 mRNA, and consequently ESRP1 protein levels, during EMT means that the CD44s isoform is produced in favor of the CD44v isoform. The pivotal nature of ESRP1 in EMT is highlighted by a study showing that overexpressing ESRP1 prevents EMT (Brown et al., 2011). The study showed that as well as preventing the expression of CD44s, ESRP1 overexpression also prevented the expression of other mesenchymal markers such as vimentin and N-cadherin, whilst preserving the expression of E-cadherin at cell-cell junctions (Brown et al., 2011).

### 1.5.1 Translation

The expression of proteins involved in EMT is not only controlled at the transcriptional level. Changes in the way particular mRNAs are translated into proteins are also important in driving EMT. As mentioned in the section on translational control, the CYFIP1-FMRP complex has been shown to play an important role in regulating the translation of both E-cadherin and vimentin (Lucá et al., 2013). CYFIP1 binds to eIF4E, FMRP then binds to CYFIP1 where it is thought to act mainly as a repressor of mRNA translation (Napoli et al., 2008). It has been shown that FMRP-dependent repression of E-cadherin expression is important in driving EMT and metastasis of breast tumours in mice (Lucá et al., 2013). The same publication also suggests that FMRP mRNA levels correlate with, and indeed stimulates, expression of vimentin: however, it shows that knocking out FMRP reduces vimentin mRNA levels, so it could be acting via transcription rather than translation (Lucá et al., 2013). The study also shows that knocking down FMRP using siRNA reduces cell protrusion number (Lucá et al., 2013). The data presented in Chapter 5 and in another publication suggests, indirectly, that CYFIP1-FMRP binding to eIF4E correlates with a reduction in the proportion of vimentin mRNAs associated with polysomes (Beggs et al., 2015). The study shows that inhibiting eIF4E phosphorylation by the Mnks increases CYFIP1 binding whilst, in the same cell type, reducing vimentin expression (Beggs et al., 2015).

miRNAs can alter the expression of proteins involved in EMT by binding to particular mRNAs and either inhibiting their translation or causing their degradation (Lamouille et al., 2013). The miR-106b-25 cluster suppresses SMAD7 expression, which causes increased TGF $\beta$  signaling and an induction of EMT (Lamouille et al., 2013). miR-200 and miR-205 on the other hand

prevent EMT, by repressing ZEB1 and ZEB2 expression (Lamouille et al., 2013). miR-200 and miR-205 are involved in a double negative feedback mechanism whereby miR-200 repress the expression of the ZEB proteins and the ZEB proteins suppress the expression of miR-200 (Lamouille et al., 2013). The expression of miR-200 is decreased during EMT, allowing ZEB1 and ZEB2 to be expressed (Lamouille et al., 2013). Activation of SNAIL1 and TWIST – two other transcription factors important in driving EMT – is also suppressed by miRNA (Samy et al., 2014). miRNA's also act directly on the markers of EMT. The expression of E-cadherin – a epithelial marker - is repressed by miR-9 whilst N-cadherin – a mesenchymal marker – is repressed by miR-194 (Furic et al., 2010, Meng et al., 2010). During EMT, expression of miR-9 increases, whilst miR-194 expression is reduced: bringing about the characteristic switch in cadherin expression (Samy et al., 2014).

### 1.5.2 Regulation of EMT

#### 1.5.2.1 TGF $\beta$

TGF $\beta$  signaling regulates EMT at the transcriptional, post-transcriptional, translational and post-translational levels (Samy et al., 2014). TGF $\beta$  binds to and activates TGF $\beta$  'type II' family receptors, which then activates and forms a complex with 'type I' family transmembrane kinases (Samy et al., 2014). The TGF $\beta$  receptor complex can then activate signaling via the SMAD complexes or other signaling pathways such as RHOA, CDC42-RAC, PI3K and MAPK (Derynck and Zhang, 2003).

SMAD protein complexes translocate to the nucleus where they combine with DNA-binding transcription factors to activate or repress transcription (Feng and Derynck, 2005). SMAD3 activates transcription of proteins such as SNAIL2 that drive EMT, whilst SMAD2 prevents EMT (Samy et al., 2014).

SMAD3-SMAD4 cooperates with SNAIL1 to inhibit the transcription of genes encoding E-cadherin and occludin, proteins which are central to the cell-cell contacts of epithelial cells (Theresa et al., 2009). SMAD3-SMAD4 also interacts with ZEB1 and ZEB2 to drive transcriptional changes during EMT (Samy et al., 2014). It has also been shown that some SMAD proteins can activate the expression of mesenchymal proteins – such as vimentin, fibronectin and collagen  $\alpha 1$  – directly (Nawshad et al., 2007, Kaimori et al., 2007). SMAD proteins also control the expression and repression of particular miRNAs to bring about EMT (Samy et al., 2014).

Non-SMAD dependent signaling can bring about further changes in cells undergoing EMT (Samy et al., 2014). The activation of the PI3K-mTOR pathway increases translation, cell size and invasive behavior – via the expression of MMP9; whilst activation of RHOA and RAC-CDC42 brings about the changes to the cytoskeleton, which drive the increased motility of mesenchymal cells (Lamouille and Derynck, 2007). The activation of AKT, downstream of PI3K, leads to the phosphorylation of the splicing factor hnRNPE1 causing it to dissociate from the 3'UTRs of disabled 2 (DAB2) and interleukin(II)-like EMT inducer (ILE1), proteins which enable EMT (Arindam et al., 2010). TGF $\beta$  can also induce a low level of RAS-RAF-MEK-ERK signaling, which is thought to contribute to the switch in cadherin expression during EMT (Grände et al., 2002). RAS-RAF-MEK-ERK signaling is also implicated in a recent study showing that TGF $\beta$  can promote the translation of SNAIL and MMP-3 via Mnk-dependent phosphorylation of eIF4E (Robichaud et al., 2014).



### 1.5.3 MAPK signalling

The binding of growth factors to receptor tyrosine kinases (RTKs) is another way of activating signaling pathways, which induce EMT. The PI3K-AKT, RAS-RAF-MEK-ERK, p38MAPK, JNK and SRC signaling pathways have all been shown to contribute to EMT (Samy et al., 2014).

RAS and RAF signaling has been shown to contribute to EMT in two ways: one, by activating the expression of SNAIL1 and/or SNAIL2, to drive the transcriptional changes; and, secondly, by activating RHO-GTPases to increase cell motility (Makrodouli et al., 2011). FGFs stimulate SNAIL2 expression which induces  $\alpha 2\beta 1$  integrin and MMP3 expression coincident with a destabilization of desmosomes: cell-cell contacts which are important for anchoring cells to other neighboring cells in a tissue (Vallés et al., 1996, Savagner et al., 1997, Billottet et al., 2008, Samy et al., 2014). HGF similarly acts via ERK to activate transcription of the SNAIL proteins (Grotegut et al., 2006). Like FGF, HGF also destabilizes desmosomes (Savagner et al., 1997). IGF1 can act, via the ERK MAPK pathway, to induce ZEB1 expression and, via the PI3K-AKT pathway and NF- $\kappa$ B, to increase SNAIL1 expression (Graham et al., 2008). Together, IGF1 has been shown to activate the cadherin switch – from E to N-cadherin – and the expression of mesenchymal proteins vimentin and fibronectin (Kim et al., 2007). IGF1 can also bind directly to E-cadherin and  $\alpha v$  integrin to disrupt these cell adhesions and allow cells to move (Canonici et al., 2008).

#### 1.5.3.1 EGF

EGF acts via the ERK MAPK pathway to induce internalization of E-cadherin whilst at the same time inducing SNAIL1 and TWIST expression, which

reinforce the reduction in E-cadherin based cell adhesion by reducing E-cadherin expression (Lu et al., 2003). EGF has been shown to stimulate the release of MMP2 and MMP9, which allows the cells to invade their way through the extracellular matrix (Ahmed et al., 2006).

VEGF, which plays an important role in stimulating angiogenesis, also induces EMT, via an induction in SNAIL expression in breast cancer cells (Yang et al., 2006). It is also believed to induce SNAIL1, SNAIL2 and Twist expression (Yang et al., 2006). The role of VEGF in driving the transcriptional changes during EMT is amplified by a feedback mechanism where SNAIL1 increases VEGF expression (Samy et al., 2014).

#### 1.5.4 Wnt

Wnt signaling, although primarily involved in driving EMT during development, is also believed to have a role in driving EMT in cancer cells (Samy et al., 2014). By inhibiting GSK3, Wnt signaling prevents  $\beta$ -catenin degradation so that it is free to stimulate expression of EMT proteins (Christof, 2012). An increase in  $\beta$ -catenin mediated gene expression is localized to the invasive front of tumours undergoing EMT (Brabletz et al., 2001).

#### 1.5.5 Hypoxia

The hypoxic environment of tumours stimulates EMT, via expression of the transcription factor HIF1 $\alpha$  (Samy et al., 2014). HIF1 $\alpha$  activates expression of TWIST and SNAIL1, which then activate the transcription of proteins, or loss of proteins such as E-cadherin, that bring about EMT (Muh-Hwa et al., 2008). EMT allows cancer cells to escape these restrictive hypoxic conditions and continue their growth at a secondary site.

### 1.5.6 Stroma

EMT is a self-fulfilling process. By interacting with and recruiting other cells, such as fibroblasts and other immune cells, mesenchymal cells create an environment, which encourages other cells to undergo EMT (Samy et al., 2014). This environment is called the stroma.

## 1.6 Metastasis

Metastasis refers to the process by which cancer cells are able to move from the site of a primary tumour to a second site in another tissue or part of the body. It is this ability to spread that is responsible for 90% of all cancer deaths. Despite its severity, the process is, compared to other processes involved in tumourigenesis, poorly understood.

Existing knowledge of metastasis is sufficient to be able to break it down into discrete steps. Metastasis usually proceeds as follows: loss of cell adhesion, increase in cell motility and ability to invade, entry and survival in the blood circulation, invasion into a new tissue, adaptation to and proliferation in a new tissue (Gupta and Massagué, 2006).

The loss of adhesion to the extracellular matrix normally triggers anoikis, a type of programmed cell death. Metastatic tumour cells are able to survive this loss of adhesion by increasing the expression of anti-apoptotic proteins, such as BCL2 and XIAP Mehlen, P. and A. Puisieux (2006). These anti-apoptotic proteins also allow the metastatic cells to survive the nutrient deprivation and hypoxic conditions of the hostile microenvironment. One study showed that as cells become metastatic the loss of integrin attachments to the extracellular matrix, which is responsible for cell adhesion, reduces the transcription of the gene encoding the pro-apoptotic protein caspase 8 (Stupack et al., 2006).

For tumour cells to escape the initial tumour site, they have to be able to break through the basement membrane. The basement membrane is formed of glycoproteins, such as fibronectin, and proteoglycans, such as collagen.

Together, the constituents of the basement membrane form an integral part of epithelial tissues: forming a barrier to invading cells; and, also helping to orientate the individual epithelial cells that make up a tissue. The proteins that allow cells to break down the basement membrane, matrixmetalloproteases (MMPs), are usually held under tight control (Liotta and Kohn, 2001). Metastatic cancer cells disrupt the mechanisms controlling MMP secretion, releasing sufficient amounts of MMPs to break down both the extracellular matrix and basement membrane.

The bloodstream is a very hostile environment for metastatic cancer cells, but they have several mechanisms to deal with these environmental stresses. One mechanism metastatic cells have been shown to rely on, in order to shield them from the forces generated by the blood flow, is to interact with platelets in the blood (Nash et al., 2002). Without interacting with the platelets the cancer cells would be more susceptible to the shearing forces of the blood flow and more exposed to circulating immune cells (Nash et al., 2002).

Another way cancer cells are believed to cope with environmental stresses during metastasis is to enter a state of dormancy. This involves reversing the hyperproliferation, which lead to the formation of the initial tumour. One protein that is thought to be central to this switch is YB-1. YB-1, as already alluded to in section 1.3.4.4, binds to the 5' m<sup>7</sup>GTP cap found in some mRNAs. By binding to this cap, YB-1 prevents cap-dependent translation initiation in favour of cap-independent translation. The mRNAs of many proliferative proteins, such as cyclin D1, require cap-dependent translation. YB-1, by preventing cap-dependent translation, prevents the expression of these proliferative proteins. The mRNAs for the transcription factors SNAIL,

ZEB and TWIST, which are known to co-ordinate the process of EMT, are cap-independent so they are still translated (Evdokimova et al., 2009a, Evdokimova et al., 2009b). By entering a state of dormancy, cancer cells reduce their energy demands whilst reducing the chances of further genomic instability. It is thought that this process explains why cancer can often reemerge in patients years after a seemingly successful treatment (Evdokimova et al., 2009b).

## **1.7 Cell proliferation**

### **1.7.1 Cell cycle progression**

Sustained proliferative signalling is, arguably, the most fundamental hallmark of cancer (Hanahan and Weinberg, 2011). Regulating these signals is crucial for maintaining order within tissues. In normal cells the production of growth factors, which stimulate proliferative signaling, is tightly controlled (Hanahan and Weinberg, 2011). Cancer cells often acquire mutations that free the proliferative signaling from the constraints of growth factor stimulation (Hanahan and Weinberg, 2011). Mutations in the growth factor receptors or in the downstream signaling proteins - such as those affecting the constituents of the RAS-RAF-MEK-ERK pathway, referred to in section 1.1 - can result in these proteins remaining active even in the absence of growth factors (Hanahan and Weinberg, 2011). Alternatively, some cancer cells may increase the expression of the growth factor receptors, such as HER2 in advanced breast cancer, so that they become hyper-responsive and respond to lower levels of growth factor that are insufficient to stimulate normal cells (Hanahan and Weinberg, 2011). It has also been shown, as mentioned in section 1.5 - on EMT, that the digestion of the extracellular matrix (ECM) by MMPs secreted by invasive cancer cells and unlock growth factors sequestered in the ECM (Hanahan and Weinberg, 2011). Cancer cells can also secrete growth factors themselves or communicate with other normal cells in the stroma – see section 1.5.6 - to stimulate them to release growth factors (Hanahan and Weinberg, 2011). This local increase in growth factor levels accentuates any increases in responsiveness the cancer cells may have over normal cells (Hanahan and Weinberg, 2011).

As well as acquiring mutations that increase the activity of a protein, usually termed oncogenic, cancer cells can gain a proliferative advantage as the result of mutations, which reduce or remove the activity of a protein, called a tumour suppressor (Hanahan and Weinberg, 2011). Two tumour suppressors important in regulating proliferation are the retinoblastoma protein (RB) and p53 (Hanahan and Weinberg, 2011). Phosphorylation of RB is the trigger for entry into the S phase of the cell cycle (Sherr and McCormick, 2002). RB is phosphorylated by cyclin-D dependent kinases (Sherr and McCormick, 2002). Cyclin D kinase complexes form following RAS-RAF-MEK-ERK signaling, which by activating transcription factors, such as AP1, increase the transcription of cyclin D1: the central component of cyclin-D1 dependent kinases (Sherr and McCormick, 2002). The import of cyclin D1 complexes into the nucleus and its persistence is determined by Ras-PI3K signaling which inhibits GSK-3 $\beta$  downstream (Sherr and McCormick, 2002). GSK-3 $\beta$  is responsible for phosphorylating cyclin-D1 an event which leads to its export from the nucleus and eventual degradation (Sherr and McCormick, 2002). Without the cyclin-D dependent kinase activity RB remains in its inactive state, where it sequesters the transcription factor E2F (Sherr and McCormick, 2002). E2F initiates the transcription of the *cyclin E* gene which drives progression into the S phase of the cell cycle (Sherr and McCormick, 2002). E2F can only do this when it is free from the RB protein (Sherr and McCormick, 2002).

p53 is responsible for synchronizing cell cycle progression with cellular stresses. Normally, if there isn't sufficient oxygen, nutrients, factors necessary for growth or the genome has suffered too much damage, p53 will stall any further progression through the cell cycle (Hanahan and Weinberg, 2011). If



the damage to a cell is too extensive p53 can direct cells into apoptosis (Hanahan and Weinberg, 2011).

There is thought to be extensive overlap between the functions of RB and p53. Experiments involving knocking out either RB or p53, in mice, have shown that the mice lacking one of these proteins appear to develop normally, with some tumour development later in life (Lipinski and Jacks, 1999, Nader and Lawrence, 1999). This suggests that normal cell cycle progression is not solely dependent on either RB or p53.

### 1.7.2 Cell viability/apoptosis

Apoptosis, programmed cell death, is a co-ordinated process that removes damaged cells (Portt et al., 2011). This programmed removal is characterized by DNA cleavage, a reduction in cell size and eventually the breaking up of the cell into small vesicles, which are then consumed by other cells (Elmore, 2007)(Adams and Cory, 2007). It is vital process in embryo development, maintaining healthy tissues and removing pathogens (Adams and Cory, 2007). It is also important for removing tumour cells (Adams and Cory, 2007). By reducing the level of apoptosis tumour cells exploit this control mechanism, to promote their survival (Fernald and Kurokawa, 2013). Disruptions in the regulation of apoptosis can also lead to autoimmune diseases and neurodegenerative diseases (Elmore, 2007). Thus, it is hoped that an increased understanding of the processes governing apoptosis will yield new treatments for a range of diseases.

The two canonical pathways regulating apoptosis are the stress pathway, also referred to as the intrinsic pathway, and the extrinsic pathway (Fernald and Kurokawa, 2013). The intrinsic pathway is activated in response to

intracellular stresses – such as DNA damage - and developmental cues (Adams and Cory, 2007) (Portt et al., 2011). The intrinsic pathway acts primarily via the Bcl-2 family of proteins (Adams and Cory, 2007). The interactions between the 17 different members of the Bcl-2 family act as a 'life/death switch' (Adams and Cory, 2007). Of the three subfamilies, one family is made up of pro-survival proteins – such as: Bcl-2, Bcl-x<sub>L</sub> and Mcl-1 - whilst the other two subfamilies – Bax-like apoptotic subfamily and the 'BH3-only proteins' - consist of pro-apoptotic proteins (Adams and Cory, 2007). The Bax-like apoptotic family consists of Bax, Bak and Bok, which all closely resemble the Bcl-2 pro-survival protein (Adams and Cory, 2007). The 'BH3-only proteins' consist of 8 members: Bim, Bad, Bik, Bid, Bmf, Puma, Noxa, Hrk (Adams and Cory, 2007). The BH3-only proteins are structurally distinct from Bcl-2 protein except for the BH3 domain – 'Bcl-2 Homology' region (Adams and Cory, 2007). The BH3 proteins are what sense the intracellular damage (Adams and Cory, 2007). Once activated the BH3 proteins bind and inhibit the pro-survival, Bcl-2, proteins (Adams and Cory, 2007). This inhibition of the Bcl-2 proteins removes their inhibition of the other pro-apoptotic subfamily, the Bax-like proteins (Adams and Cory, 2007). Consequently, the Bax-like proteins are activated leading to the permeabilisation of the outer mitochondrial membrane, resulting in the release of cytochrome c (Adams and Cory, 2007). The release of cytochrome c leads to the activation of the initiator caspase, caspase 9 (Adams and Cory, 2007). Caspase 9 then activates, by cleavage, several caspases, caspase 3, 6, and 7 (Adams and Cory, 2007). Caspase 3 is responsible for the DNA cleavage and blebbing, which is characteristic of cells going through apoptosis (Portt et al., 2011)(Elmore, 2007). The extrinsic pathway also leads to the activation of caspase 3, but through a different route (Portt et al., 2011). The extrinsic pathway is activated by TNF receptors - TNFR, Fas and

TRAIL – which leads to the activation of caspase 8 and caspase 10, via activation of the Fas-Associated-Death-Domain (FADD) complex and Death Inducing Signalling Complex (DISC) (Elmore, 2007, Adams and Cory, 2007, Portt et al., 2011).

Cancer cells are under constant stress: from the inherent genomic instability and low oxygen levels (Fernald and Kurokawa, 2013). Normally, these stresses would activate the intrinsic pathway. Apoptotic pathways are often disabled in cancer cells due to the overexpression of members of the pro-survival Bcl2 protein sub-family, such as Mcl-1 or the repression of pro-apoptotic proteins (Fernald and Kurokawa, 2013). Studies have shown that disabling apoptosis is not in itself sufficient to cause tumourigenesis, but is often a contributing factor exacerbating any proliferative advantages the cancer cells may have gained (Fernald and Kurokawa, 2013). p53, which is involved in the expression of BAX and other pro-apoptotic proteins is inactivated in 50% of cancers; the most commonly inactivated tumour suppressor gene (Fernald and Kurokawa, 2013). The loss of p53, reduces BAX expression, which means cancer cells cannot respond to DNA damage and metabolic stresses (Fernald and Kurokawa, 2013). In cancer cells with high ERK activity, such as RAS-mutant cancers, the transcription of *BIM* is suppressed due to inhibition of one of its transcription factors: FOXO (Fernald and Kurokawa, 2013). Without BIM, cancer cells are unable to respond to growth factor withdrawal: something that would normally send cells into apoptosis (Fernald and Kurokawa, 2013). A number of tyrosine kinase inhibitors, which inhibit kinases upstream of BIM, such as ERK, AKT and HER2, have been designed to try and restore BIM levels (Fernald and Kurokawa, 2013). The effectiveness of a TKI is determined by how well it restores BIM RNA levels (Fernald and Kurokawa, 2013). BIM RNA levels are

also used as a marker for patient prognosis and as an indicator as to how well a patient might respond to TKIs (Fernald and Kurokawa, 2013). Studies have shown that in order to fully activate apoptosis it may require inhibition of both the ERK pathway and the PI3K/AKT pathway (Fernald and Kurokawa, 2013). This is because PUMA, another pro-apoptotic factor transcribed in response to FOXO, was shown to increase in cells treated with PI3K/AKT inhibitors (Fernald and Kurokawa, 2013). It is thought that the ERK pathway is responsible for activating BIM transcription via FOXO whilst the PI3K/AKT pathway is responsible for activating PUMA transcription, again, via FOXO (Fernald and Kurokawa, 2013). It is not clear exactly how the two are independently regulated. The reduced expression of BIM and PUMA in cancer cells, by reducing activation of BAX and BAK, inhibit the cytochrome c release from the mitochondria (Fernald and Kurokawa, 2013). The processes linking cytochrome c release to the eventual death of a cell have also been shown to become disabled in cancer cells (Fernald and Kurokawa, 2013). Studies have shown that loss of p53 can also lead to reduced expression of some caspases (2,7,8 and 9), the enzymes which by cleaving various cellular substrates bring about cell death (Fernald and Kurokawa, 2013).

## 1.8 Project aims

To elucidate the role of the Mnks in tumourigenesis and help establish whether they are suitable targets to develop treatments for cancer. Knockout studies in mice have shown that the Mnks do not appear to be essential for normal cell growth and development; however, knockout of the Mnks did inhibit tumourigenesis (Ueda et al., 2010). Using novel Mnk inhibitors the aim of this project is to see whether inhibiting Mnk kinase activity has any effect on cancer cells. If any effects are observed, MEF Mnk KO cells are available for use in supporting experiments. Ultimately, the aim is to uncover possible mechanisms through which the Mnks might have a role in tumourigenesis.

## 2. Materials and Methods

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### 2.1 Cell culture

MDA-MB-231 adenocarcinoma cells were maintained in Dulbecco's modified Eagle medium (DMEM, Invitrogen), supplemented with 10% (v/v) fetal bovine serum (FBS).

SCC25 (squamous cell carcinoma) cells were maintained in a 1:1 ratio of DMEM, containing high glucose, (Sigma) to Ham F-12 medium (Life Technologies).

COLO205 cells were maintained in RPMI (Invitrogen), supplemented with 10% (v/v) fetal bovine serum (FBS) and 2mM glutamine. COLO205-AZD6244 resistant (C6244R) cells were maintained in supplemented RPMI media, which contained 1 $\mu$ M AZD6244.

HT29 cells were maintained in McCoy's medium (Invitrogen), supplemented with 10% (v/v) fetal bovine serum (FBS) and 2mM glutamine. HT29-AZD6244 resistant (HT29-6244R) cells were maintained in supplemented McCoy's media, which contained 1 $\mu$ M AZD6244.

HCT116 cells were maintained in Dulbecco's modified Eagle medium (DMEM, Invitrogen), supplemented with 10% (v/v) fetal bovine serum (FBS) and 2mM glutamine. HCT116-AZD6244 resistant (H6244R) cells were maintained in supplemented DMEM media, which contained 2 $\mu$ M AZD6244.

LoVo cells were maintained in Dulbecco's modified Eagle medium (DMEM, Invitrogen), supplemented with 10% (v/v) fetal bovine serum (FBS) and 2mM

glutamine. LoVo-AZD6244 resistant (L6244R) cells were maintained in supplemented DMEM media, which contained 4 $\mu$ M AZD6244.

A549 cells were maintained in Dulbecco's modified Eagle medium (DMEM, Invitrogen), supplemented with 10% (v/v) fetal bovine serum (FBS) and 2mM glutamine.

SW620 cells were maintained in Leibovitz's media supplemented with 10% (v/v) fetal bovine serum (FBS), 2mM glutamine and 0.075% sodium bicarbonate.

Primary mouse embryonic fibroblasts (MEF) cells were prepared from 13.5 day-old embryos and grown in DMEM plus 10% (v/v) FBS; all experiments using them employed cells passaged <4 times. Cells were maintained at 37°C in humidified air with 5% CO<sub>2</sub>.

Immortalised MEF cells were produced by continuous passage of primary MEFs until they immortalised. They were maintained in Dulbecco's modified Eagle medium (DMEM, Invitrogen), supplemented with 10% (v/v) fetal bovine serum (FBS).

#### Freezing down medium

For most cell types: complete media with 5% DMSO.

For MEFs:

70% DMEM

20% FBS

10% DMSO

1ml vials of cells were stored in a Mr. Frosty™ overnight at -80°C before being transferred to a liquid nitrogen cryostore.

### Western blots

Lysates (typically 30µg of protein) were prepared in 1x sample buffer prior to loading. \*For ZEB1, double the amount of protein lysate was loaded, using a 1.5mm 10 well gel.

Lysates were pre-heated for 5 minutes at 95°C in the sample buffer. To detect high molecular weight proteins the samples would be loaded on a 12.5% acrylamide gel; for low molecular weight proteins (such as 4E-BP1) the samples would be loaded on a 13.5% acrylamide gel. \*For ZEB1 (220 kDa), an 8% acrylamide gel was used.

Acrylamide gels were run at a constant voltage of 200V for 50 min using a Bio-Rad electrophoresis system. \*The 8% gel was run at 180V for 1h 30min. The samples were then transferred to nitrocellulose/PVDF (polyvinylidene difluoride) membrane - pre-soaked in methanol to reduce hydrophobicity - using a Bio-Rad electrotransfer system for 1h 30min at 80V. The electrotransfer system was placed in a box and surrounded with ice. \* The 8% gel was transferred at 100V for 2 hours in a cold room on ice using pre-chilled transfer buffer.

Following transfer of the samples to the PVDF membrane, the membranes were blocked in blocking agent (see buffers) for 1h at room temperature to prevent non-specific binding upon subsequent addition of the antibodies. Primary (1°) antibody solutions (in PBST + 2% BSA) were added to the membranes and left overnight at 4°C. The membranes were then washed 3x



in PBST to remove excess antibody solution. Fluorescently tagged secondary antibody was then added to the membrane and left – in a black box – at room temperature for 1h.

For chapters 3-5 membranes were developed using a LI-COR Odyssey® Quantitative Imaging System. For chapter 6, the immunoblots were developed using the ECL method and an X-ray machine.

Loading Gel	<u>12.50%</u>				<u>13.50%</u>			
	x2		x4		x2		x4	
Gel thickness	1mm	1.5mm	1mm	1.5mm	1mm	1.5mm	1mm	1.5mm
Total volume	10ml	16ml	20ml	32ml	10ml	16ml	20ml	32ml
Distilled water (ml)	3.7	5.92	7.4	11.84	2.18	3.488	4.36	6.976
Tris pH8.8 (ml)	2.5	4	5	8	2.5	4	5	8
40% acrylamide (ml)	3.15	5.04	6.3	10.08	3.375	5.4	6.75	10.8
2% Bis-acrylamide (ml)	0.5	0.8	1	1.6	1.8	2.88	3.6	5.76
10% SDS (μl)	100	160	200	320	100	160	200	320
TEMED (μl)	5	8	10	16	5	8	10	16
10% APS (μl)	50	80	100	160	100	160	200	320
Stacking Gel	x2		x4					
	1mm	1.5mm	1mm	1.5mm				
Tris pH6.8	0.62	1.24	1.24	2.48				
40% Acrylamide	0.62	1.24	1.24	2.48				
Bis-acrylamide	0.322	0.644	0.644	1.288				
SDS 10%	62.4	124.8	124.8	249.6				
Make up to this final volume with water:	5	10	10	20				
TEMED	5	10	10	20				
APS 10%	25	50	50	100				

## 2.2 Antibodies

Antibodies were prepared in PBST + 2% BSA.

### 1° Antibodies:

eIF4E (Cell Signaling Technology) 1:1000

p-eIF4E (Cell Signaling Technology) 1:500

4E-BP1 (Homemade) 1:1000

Mnk1 (Cell Signaling Technology) 1:1000

Cyclin D1 (Santa Cruz) 1:200  
YB1 (Cell Signaling Technology) 1:1000  
CD44 (Cell Signaling Technology) 1:1000;  
ERK (Cell Signaling Technology) 1:1000;  
P-ERK1/2(T202/204) (Cell Signaling Technology) 1:1000  
Paxillin (Cell Signaling Technology) 1:1000  
P-Paxillin (Tyr-31) Santa Cruz 1:1000  
FAK (Cell Signaling Technology) 1:1000  
P-FAK (Y397) (Abcam) 1:1000  
CYFIP1/Sra-1 (Upstate Cell Signaling Solutions) 1:1000  
NCKAP1 (Novus Biologicals) 1:1000  
Mnk2 (Sigma) 1:1000  
S6 (Santa Cruz) 1:1000  
P-S6 (S240/244) (Cell Signaling Technology) 1:1000  
PKB (Cell Signaling Technology) 1:1000  
P-PKB (S473) (Cell Signaling Technology) 1:1000  
GAPDH (Cell Signaling Technology) 1:2000  
Vimentin (Cell Signaling Technology) 1:1000  
ZEB1 (Cell Signaling Technology) 1:1000  
PARP (Cell Signaling Technology) 1:1000  
E-cadherin (Cell Signaling Technology) 1:1000  
SNAIL (Cell Signaling Technology) 1:1000  
eEF2 (Cell Signaling Technology) 1:1000  
β-actin (Cell Signaling Technology) 1:2000

2° Antibodies:

Anti-rabbit (Fisher Scientific) 1:20,000  
Anti-mouse (Fisher Scientific) 1:20,000

Anti-goat (Fisher Scientific) 1:20,000

### **2.3 Inhibitors**

The Mnk inhibitors used were Mnk-I1, documented in patent WO 2011/104340 A1, three unpatented compounds Mnk-I2, Mnk-I3, Mnk-I4 and the commercially available compound CGP57380 [21]. The MEK inhibitor used was AZD6244 (Selumetinib). A RAF inhibitor AZ'9304 was used for some experiments. Agents were added to the medium in DMSO vehicle at the appropriate concentration (always <1% v/v DMSO). The amount of vehicle added was normalised across the different treatment concentrations within each experiment.

### **2.4 Buffers and solutions**

#### Blocking agent

1g milk in 20ml PBST

#### Bradford reagent

1 part Bio-rad Bradford reagent: 4 part distilled water

#### Lysis buffer

25mM Tris

50mM  $\beta$ -glycerol phosphate

50mM KCl

1% Triton x100

For lysing cells: DTT (1:1000)  $\text{Na}_3\text{VO}_4$  (1:1000) and Roche: Complete Protease Inhibitor Cocktail - EDTA free (1:25) was added to the lysis buffer.

### PBST

1x PBS

0.1% Tween

### Running buffer

(1L): 100ml 10xrunning buffer (Tris/Glycine/SDS buffer from Bio-Rad)

MiliQ or distilled water was added up to 1L

### Sample buffer (5x)

62mM Tris pH6.8

7% SDS

20% sucrose

0.01% bromophenol blue

\* For a 1ml aliquot of 5x sample buffer 950µl of 5x sample buffer would be combined with 50µl DTT. This would then be diluted in MiliQ water to make 2x or 1x stocks.

### Transfer buffer

(1L): 100ml 10x transfer buffer (Tris/Glycine buffer from Bio-Rad)

200ml methanol

1ml 20% SDS

MiliQ or distilled water was added up to 1L.

## **2.5 m<sup>7</sup>-GTP pull-down**

For each sample 7.5µl of m<sup>7</sup>-GTP Sepharose 4B beads (GE Healthcare) is added to 7.5µl Sepharose CL-4B beads. 300-400µg of protein lysate is added to the bead mixture and put on a tumbler for 1.5 hours at 4°C.

Following the pull-down, 20µl of 2x sample buffer is added to each bead mixture and heated at 90°C for 5 minutes in preparation for loading.

For eIF4E, 5µl of the sample buffer/bead supernatant is loaded. For phospho-eIF4E, 15µl is loaded.

\*\*During the project, GE Healthcare stopped producing the m<sup>7</sup>-GTP Sepharose 4B beads. For most of the data presented here total p-eIF4E levels were used. The Immobilized -Aminoethyl-m<sup>7</sup>-GTP (bulk material) from Jenabiosciences was found to be a suitable replacement and was used by Shuye Tian to produce the data for Figure 5.5.

## **2.6 Proliferation Assays**

### **2.6.1 Cell counting**

500 µl of growth medium, containing about 20,000 cells, was added to each well of a 24-well plate, along with indicated concentrations of Mnk-11, CGP57380 and/or DMSO. Cells were then incubated at 37°C for a further 24h.

After 24h, medium was aspirated off and wells were washed once with 0.5 ml PBS; 0.5 ml of 0.05% trypsin-EDTA (1X) solution was then added and left for 30 min. The number of cells in each well was counted on a CASY 1 counter (Scharfe System GmbH, Germany); 0.5 ml of trypsinised cells were added to 9.5 ml of CASYton medium, inverted 3 times and inserted into the CASY 1 counter.

### 2.6.2 Thymidine Incorporation

#### For a 24 hour treatment

On day 1, HCT116 and H6244R cells,  $7.5 \times 10^4$  cells/ml were seeded 1ml per well into a 24 well plate. For COLO205 and C6244R cells,  $1 \times 10^5$  cells/ml were seeded 1ml per well into a 24 well plate. The cells were left to adhere overnight.

At 4pm on day 2, the medium is removed from the wells of the 24 well plate where the cells have been seeded the day before. The media is replaced with the media containing the respective concentrations of inhibitor(s).

On day 3, 18 hours after treatment, 25 $\mu$ l/per well of [ $^3$ H]thymidine/medium mix is added per well. For x2 24 well plates, 100ul cold 5mM thymidine is added to 2.4ml medium along with 50ul 1 $\mu$ Ci/ $\mu$ l [ $^3$ H] thymidine. After adding the 25 $\mu$ l of the thymidine mix to each well the plates are incubated for 6 hours. At 6 hours, the media is aspirated from each well and 0.5ml ice-cold 5% TCA is added to each well. This is repeated once. The plates are then washed in water to remove the TCA and 0.5ml 0.1M NaOH was added to each well to lyse the cells. The lysed cells are added to a 5ml scintillation vial along with 4ml of scintillation fluid. The counts are then measured on a Tri-carb 2100 liquid scintillation analyzer.

### 2.7 Cell cycle analysis

Treatments of the cells with the indicated concentrations for compounds were conducted such that they were all harvested at 50-70% confluence. Following treatment, the media were removed and kept. The cells were then incubated in  $\times 1$  trypsin/EDTA (0.5%) for 4 min at 37°C. DMEM

was added and the cells gently dispersed by pipetting, combined with the saved media and centrifuged at 200g for 5 min at room temperature. The media were discarded and the cell pellets gently resuspended in DMEM and equilibrated by incubation at 37°C for 30 min. The cells were pelleted by centrifugation at 200 g for 10 min at room temperature and the media removed. Cells were stained using 25 µg/ml of propidium iodide, before analysing approximately 20,000 cells on a FACS Calibur Instrument (Becton Dickinson). The data were then analysed on Cell Quest software.

## **2.8 Caspase assays**

200 µl of a suspension containing about 10,000 cells were added to each well of a 96-well plate. Cells were allowed to settle overnight. At time zero, medium was replaced with 100 µl of growth medium, or serum-free media in the case of the MEF experiments, containing the appropriate concentration of compound. After 24 h, 50 µl of Caspase-Glo® 3/7 Assay reagent (Promega) was added to each well and left for 2 h with constant shaking at room-temperature. Luminescence was measured using a BMG Labtech FLUOstar Optima Filter-based multi-mode microplate reader.

## **2.9 Migration assays**

### **2.9.1 Scratch-wound healing assays**

A 30 mm diameter culture dish, containing a monolayer of cells, immersed in 2 ml growth medium, was scratched using a P-20 pipette tip forming a 'wound' across its diameter. At time zero, a Zeiss Axiovert 200 microscope, (10x objective lens), was used to take images. Plates were then incubated at 37°C and images taken at the indicated time-points. (Beggs et al., 2015)

### 2.9.2 Transwell cell migration assays

Transwell migration assays were performed using polycarbonate inserts (8  $\mu\text{m}$  pore size, Transwell®, Beckton Dickinson) placed into a standard 24-well plate. Wells were coated with the respective chemo-attractants, 10  $\mu\text{g}/\text{ml}$  fibronectin (Sigma) for SCC25 cells and 10 $\mu\text{g}/\text{ml}$  collagen (Millipore) for MDA-MB-231 cells. For MEF migration assays, wells were left uncoated, but, instead, 500  $\mu\text{l}$  of serum-containing medium were added to the wells. Cells were pre-treated with compounds 30 min before seeding, at  $5 \times 10^4$  cells in 200  $\mu\text{l}$ , into the Transwell inserts. Cells that had migrated into the bottom well were trypsinised and counted on a CASY counter. (Beggs et al., 2015)



## Transwell Migration Assay

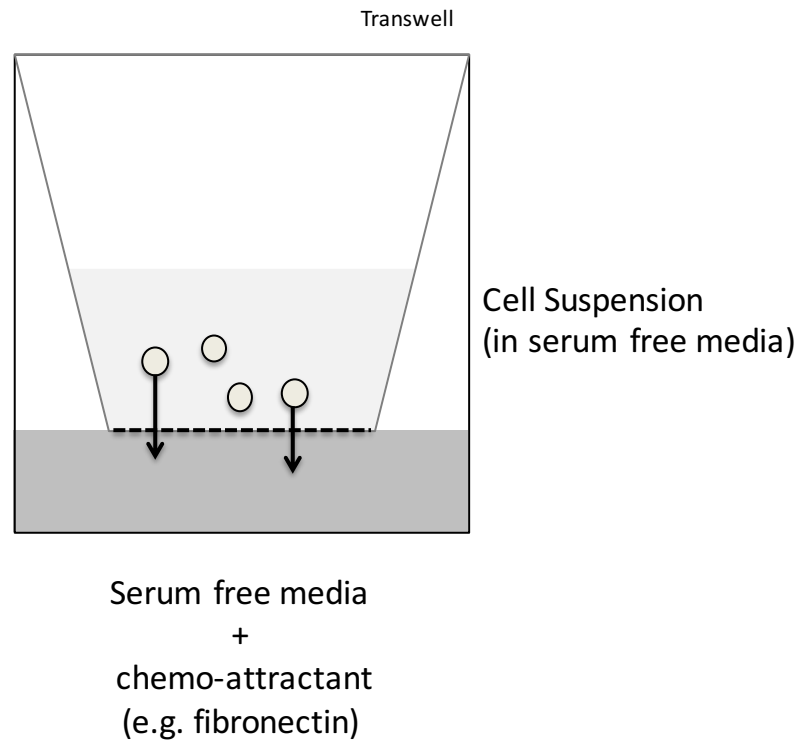


Figure 2.1 Transwell migration assay

Diagram illustrating how the transwell migration assay is set up. Cells migrate through a  $0.8\mu\text{M}$  porous membrane at the bottom of the transwell insert and into the well of a 24 well plate. The cells are added in serum free media and migrate towards a chemoattractant, such as fibronectin, or serum.

### 2.10 RT-qPCR on total RNA samples

For MDA-MB-231 cells, total RNA was extracted from 10cm plates using the GeneJET RNA purification kit (Thermo Scientific) according to the supplier's instructions. For MEF cells, total RNA was extracted from 10cm plates using Trizol (Invitrogen) extraction. RT-Real time PCR amplification was carried out using the ImProm-Itm Reverse Transcription System (A3800 Promega) with oligo(dT)15 and random primers following the manufacturer's protocol.

Subsequently, real-time quantitative (q) PCR was performed using primers for human vimentin (5'-TTCTCTGCCTCTTCCAACTTT-3')/(5'-CGTTGATAACCTGTCCATCTCTA-3'); mouse vimentin (5'-CTGCTGGAAGGCGAGGAG-3')/ 5'-ACCGTCTTAATCAGGAGTG TTC-3'); or 18S rRNA (PrimerDesign). Samples were analysed in triplicate with SYBR Green dye (Primer Design mix) on an ABI StepOnePlus quantitative PCR instrument (Applied Biosystems). The comparative Ct method was used to measure amplification of vimentin mRNA levels compared to 18S rRNA.

## 2.11 Polysome profiles

### 2.11.1 Sample preparation

MDA-MB-231 cells were lysed using 300  $\mu$ L of lysis buffer (268  $\mu$ L TNM lysis buffer, 1% Triton X-100, 10 mM DTT, 40 U RNase inhibitor). The lysates were centrifuged at 17,000g at 4°C for 5 min. The supernatant was transferred to an new eppendorf tube, on ice, containing 3  $\mu$ L heparin (10  $\mu$ g/ $\mu$ L). The samples were snap frozen in liquid nitrogen before storing the samples at -80°C.

### 2.11.2 Preparing the density gradients

Before starting, polyallomer centrifuge tubes were placed in dry ice. 50% sucrose solution was carefully added in the bottom of the tube. Once the 50% layer had frozen, the 44% sucrose solution was added on top of the frozen layer. This process of sequentially adding layers of increasingly more dilute sucrose solutions was repeated until a gradient consisting of 6 layers of increasingly dilute sucrose solution was formed. The frozen sucrose gradients were then stored at -80°C until the day before they were required, when they would be transferred to 4°C and left to thaw overnight.

### 2.11.3 Collecting sample fractions

The lysates were carefully added to the top of the thawed sucrose gradients, before inserting the gradients into individual pre-cooled centrifuge buckets. The buckets were then carefully, to avoid disturbing the fractions, attached to the pre-cooled centrifuge rotor. The rotor was then, very carefully, lowered into the centrifuge. The samples were centrifuged at 160000 g for 110 min at 4°C. Immediately after the centrifugation had finished, the sucrose gradients were passed through a fraction collector (Gilson FC 203B/FC 204) which continuously monitored the absorbance at 254 nm. The RNA fractions were collected in 9 microcentrifuge tubes, per sample.

### 2.11.4 RT-qPCR on polysome fractions

For the RT-qPCR on the polysome fractions, an equal amount (20 pg) of kanamycin RNA was added to each fraction and the total RNA in each fraction was extracted using Trizol (Invitrogen) extraction. Half of the re-suspended RNA pellet from each of the 9 fractions was then pooled for fractions 1-3; 4-6; and, 7-9 before carrying out reverse transcription on the pooled fractions, using the ImProm-II<sup>tm</sup> Reverse Transcription System (A3800 Promega) as described above. The cDNA product was then used for real-time quantitative (RT-q) PCR, which was carried out using primers for human vimentin (as above), actin or kanamycin (PrimerDesign). Samples were analysed in triplicate with SYBR Green dye (Primer Design mix) on an ABI StepOnePlus quantitative PCR instrument (Applied Biosystems). The comparative Ct method was used to measure amplification of vimentin and actin cDNA, compared to kanamycin external standard cDNA. To normalise the Ct values, the average (from three replicates)  $\Delta$ Ct value for kanamycin

was subtracted from the  $\Delta\text{Ct}$  value for the cDNA of interest (vimentin or actin) before calculating the relative level of the test RNA. (Beggs et al., 2015)

## **2.12 Statistical analysis**

Data were analysed by performing a one-way ANOVA with Dunnett's multiple comparisons test for significance and, for the cell cycle (FACS) analyses, a two-way ANOVA with Tukey's multiple comparisons test, using GraphPAD Prism 6 software. For the MEF proliferation assay, where there were only two columns, a Mann-Whitney test was used to test for significance. (Beggs et al., 2015)

## 3. Genetic knockout of the Mnks impairs cell migration

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### 3.1 Introduction

The first indication that Mnk activity might be involved in cell migration was a study published in 2011 by Willett, M. et al. Using immunofluorescence, they were able to show how p-eIF4E associates with the leading edge of migrating cell(Willett et al., 2011). Mnks are the only known kinases to phosphorylate eIF4E. From this, it is possible to infer that Mnk activity is associated with the leading edge of migrating cells and may, therefore, play an important role in cell migration. The results presented in this chapter show this to be the case.

## 3.2 Results

### 3.2.1 Mnk knockout MEFs show impaired migration

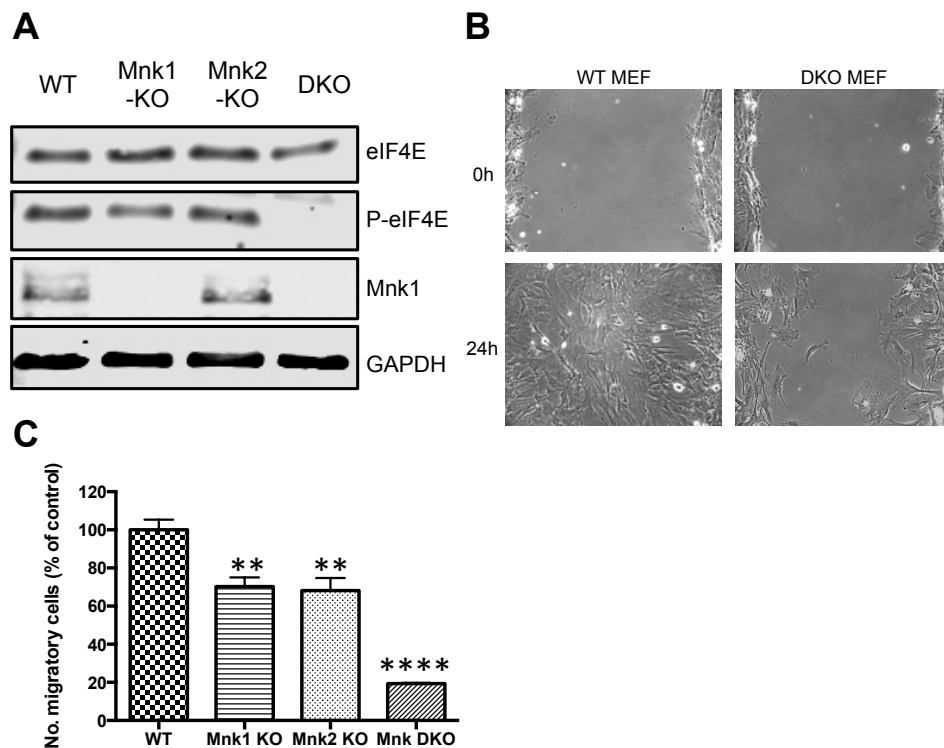


Figure 3.1 Genetically knocking out both Mnk1 and Mnk2, in Mouse Embryonic Fibroblast (MEF) cells, impairs 2D and 3D cell migration.

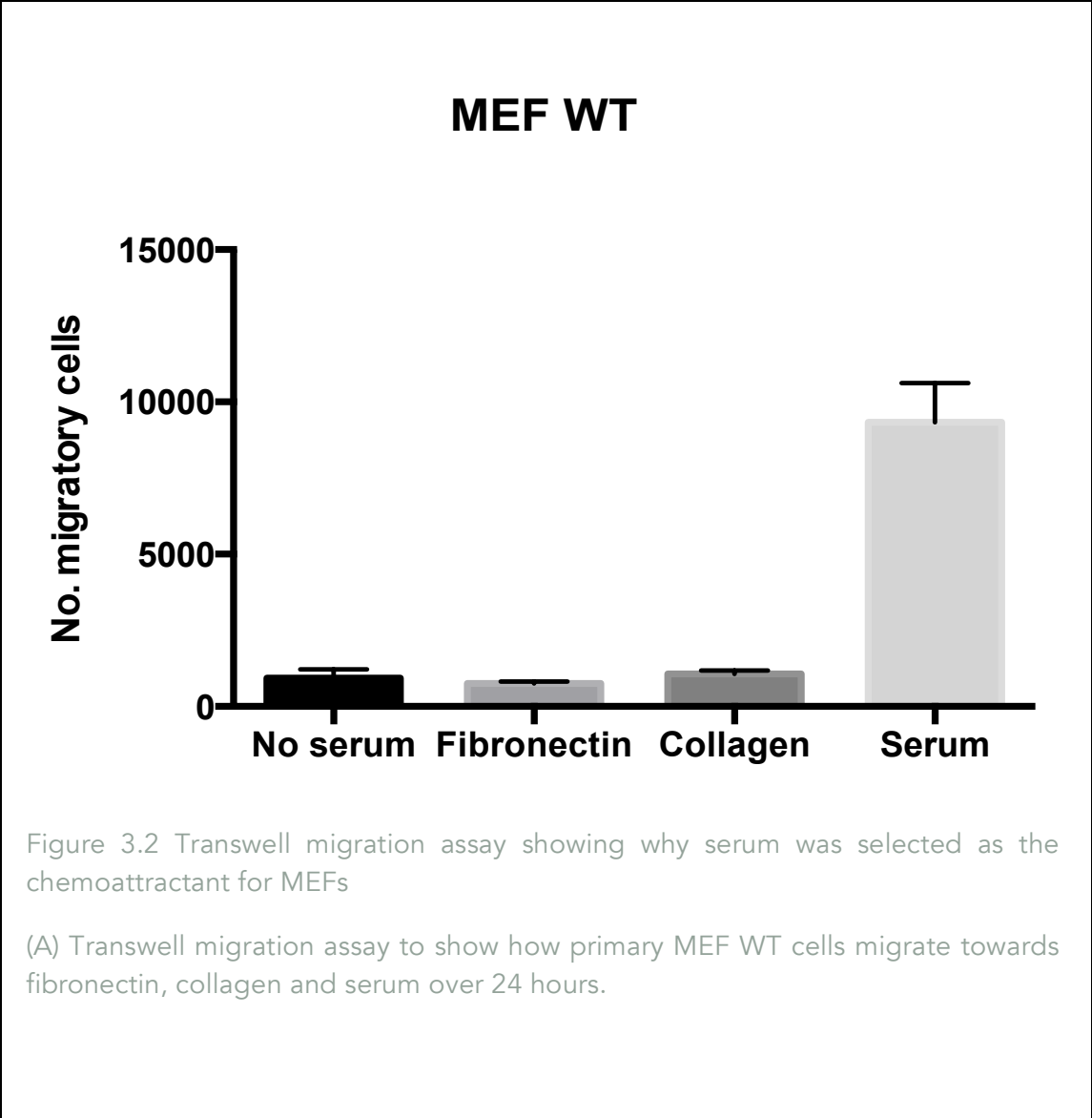
(A) Western blot confirming the effect of knocking out Mnk1 and Mnk2\* on eIF4E phosphorylation in primary MEF cells. GAPDH is used as a loading control. \*The commercial Mnk2 antibodies tested were not specific for Mnk2 (B) Scratch-wound healing assay showing the effect of knocking out both the Mnks (DKO) on wound closure, over 24 h. (C) Transwell migration assay to show the effect of knocking out Mnk1 and Mnk2 on the ability of MEF cells to migrate towards serum, over 24 h. Data are shown as mean percentages, of MEF WT migration, + – S.E.M. from three replicates. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001; \*\*\*\*P < 0.0001

The scratch-wound-healing assay was used for the initial experiments. This involves growing the experimental cell line to 100% confluence to form a monolayer. Then, using a small pipette tip, a scratch is formed across the diameter of the monolayer. Once satisfied that the scratches are the same size across the different experimental conditions, the plates of cells were then left for 24 hours before comparing the degree to which the wound has healed.

Although it is a rather crude experiment, it was possible to see a clear difference between the MEF WT cells and MEF Mnk DKO cells (**Figure 3.1B**). After 24 hours the scratch-wound left in a monolayer of primary WT MEF cells is almost completely healed; however, the MEF DKO cells have made little impact on the wound after 24 hours.

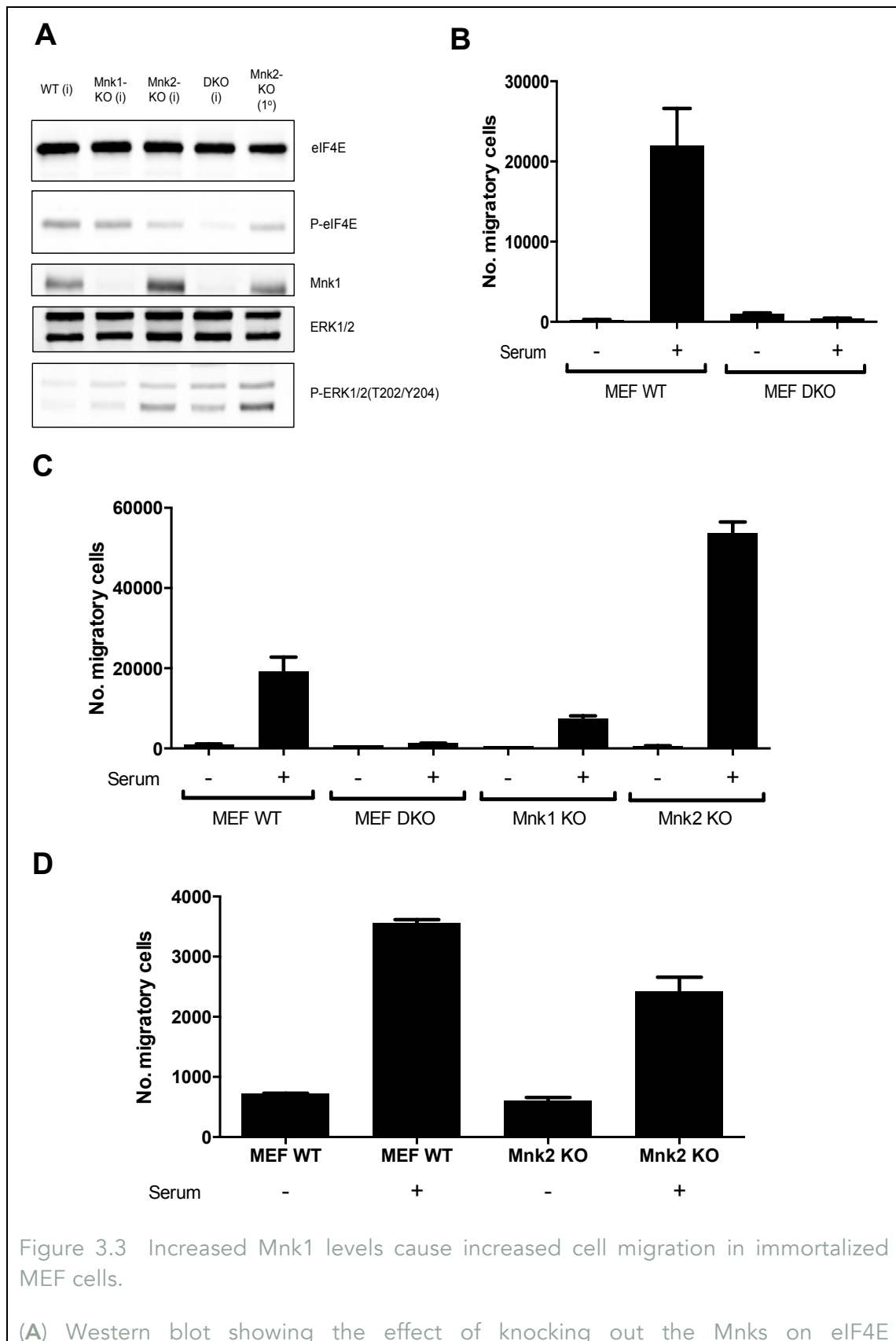
**Figure 3.1C** is the result of a transwell migration assay comparing MEF WT, single Mnk1 and Mnk2 KO cells and MEF Mnk DKO cells, over 24 hours, are shown. The result shows that knocking out both of the Mnks in primary MEFs (MEF DKO) causes an ~80% reduction – when compared with MEF WT cells – in the number of cells that manage to migrate through the transwell over 24 hours. This result corroborates with the effect of knocking out both the Mnks in the scratch-wound healing assay. Knocking out either Mnk1 or Mnk2 causes a ~25% reduction in the number of migratory cells. This result is shown alongside a western comparing the levels of p-eIF4E – to give an indication of how much Mnk activity is left in the knockout cells – and Mnk1 (**Figure 3.1A**). Unfortunately, there isn't a specific Mnk2 antibody available. The absence of any p-eIF4E in the MEF DKO lane confirms that all Mnk activity has been removed.

A control transwell migration assay experiment was carried out prior to these experiments to identify a suitable chemoattractant to use. Figure 3.2 shows why serum was used as the chemoattractant; the MEFs failed to migrate towards two commonly used chemoattractants: fibronectin and collagen.





### 3.2.2 Increased Mnk1 levels increase cell migration



phosphorylation, Mnk1 levels and ERK1/2 phosphorylation in immortalised MEF cells (and primary MEF Mnk2 KO cells for comparison). Total eIF4E and ERK blots show that the differences in phosphorylation of these proteins are not due to changes in the total levels of these proteins. \*The commercial Mnk2 antibodies tested were not specific for Mnk2.

(B) Transwell migration assay showing the effect of knocking out both the Mnk1 and Mnk2 (DKO) on the rate of immortalised MEF cell migration.

(C) Transwell migration assay showing the effect of knocking out Mnk1 and Mnk2 on the rate of immortalised MEF cell migration.

(D) Transwell migration assay showing the effect of knocking out Mnk2 on the rate of *primary* MEF cell migration.

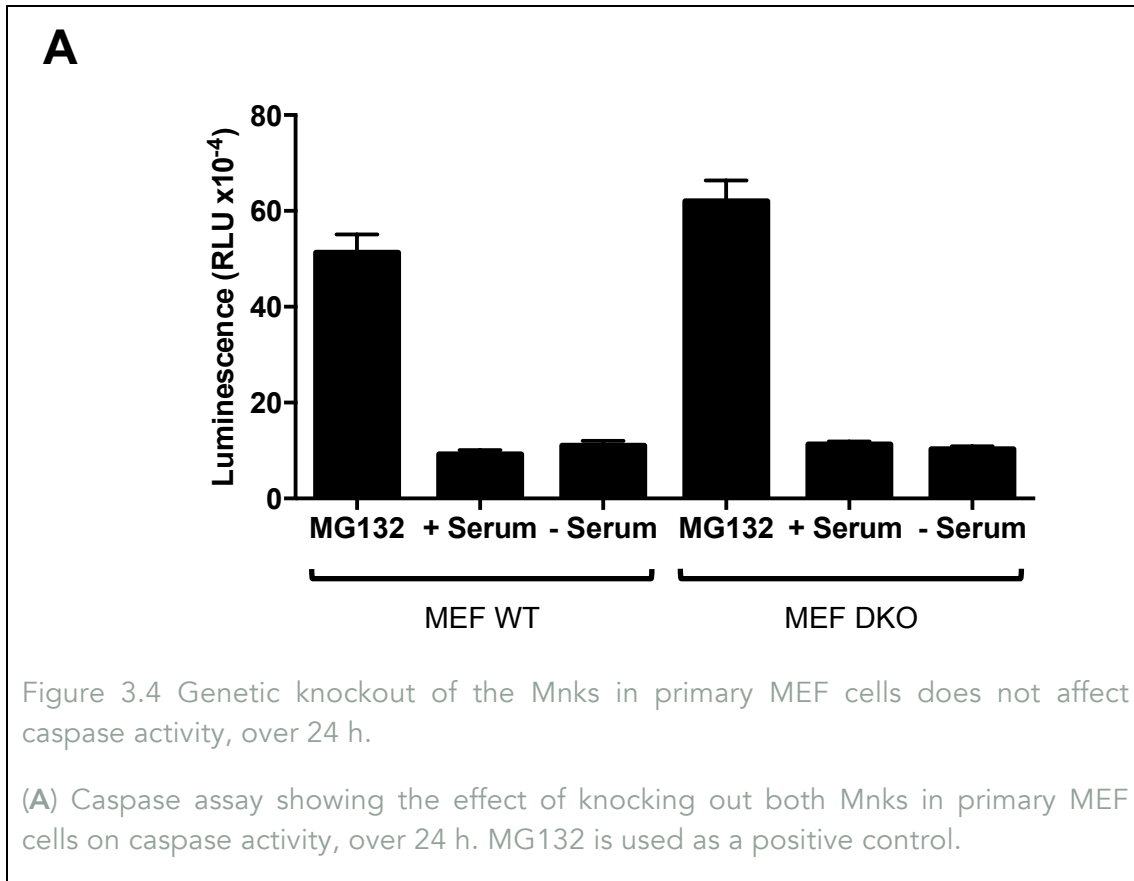
All of the transwell migration assay results in this figure are based on the number of cells that have migrated through the transwell towards serum over 24 h.

In immortalised MEFs an interesting effect was observed whereby knocking out Mnk2 caused an increase in the rate of migration through a transwell over 24 h. A western blot, shown in **Figure 3.3A**, helps explain this result. The western blot compares the levels of Mnk1 across immortalised MEF WT, Mnk1 KO, Mnk2KO, DKO cells and primary MEF Mnk2 KO cells. Mnk1 levels are reduced in both the single Mnk1 KO cells and DKO cells, as you would expect. In immortalised Mnk2 KO cells Mnk1 levels actually increase above that of immortalised MEF WT cells. Knocking out Mnk2 only appears to increase Mnk1 levels in the immortalised cells; in the primary Mnk2 KO cells, the Mnk1 levels are similar to that of the MEF WT cells (as was the case in **Figure 3.1A**). It must, therefore, be an artifact of the immortalisation process. Given that knocking out Mnk1 reduces cell migration, the observed increase in cell migration in immortalised Mnk2 KO cells (**Figure 3.3C**) must, in some way, be linked to the increase in Mnk1 levels. Based on the reduced cell migration over 24 h in primary Mnk2 KO MEF cells, Mnk2 must also play an

important role in cell migration. The effect of knocking out Mnk2 on cell migration in the immortalised MEFs must be interpreted in the light of the observed increase in Mnk1 levels. In contrast, knocking out both of the Mnks in immortalised MEF cells (**Figures 3.3B and 3.3C**) prevents any active migration towards the serum.

Interestingly, phosphorylation of ERK1/2 (P-ERK1/2) appears to increase in response to genetically knocking out Mnk2 in MEFs (**Figure 3.3A**). This effect was observed in both the immortalized and primary Mnk2 KO MEFs. An explanation for this effect could be that Mnk2 inhibits the phosphorylation of ERK1/2 via a feedback-loop, either directly on ERK1/2 or further upstream. This would explain why removing Mnk2, which would remove this inhibition, would result in increased P-ERK1/2. The total ERK blot shows that this effect is not due to an affect on the expression of ERK1/2.

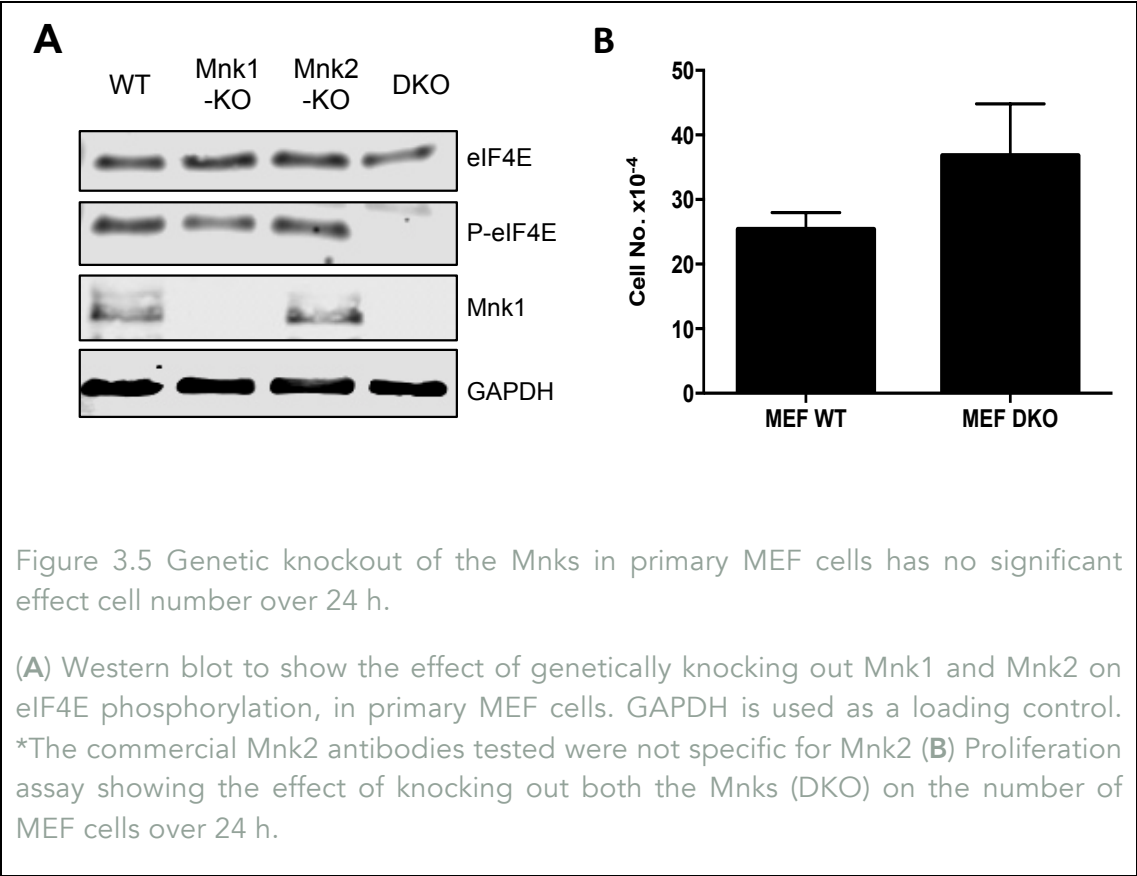
### 3.2.3 Mnk knockout does not have an effect on cell viability over 24 h



To make sure that the effects of knocking out the Mnks on cell migration are not simply a consequence of an adverse effect on cell viability, a caspase assay was carried out. This assay measures the activity of the caspase enzymes, which are involved in apoptosis (Köhler et al., 2002). Increased caspase activity represents an increase in apoptosis or programmed cell death. MG-132 was used as a positive control in these experiments because it is known to induce apoptosis, by inhibiting the proteasome (Goldberg, 2012). The caspase assay was carried out on WT and Mnk-DKO MEF cells both in the presence and absence of serum. The reason for testing caspase activity both in the presence and absence of serum was because in the migration assays the cells are exposed to both conditions: the cells are seeded into the transwell, the top well, in the absence of serum and then left

to migrate towards serum in the bottom well. **Figure 3.4A** shows that genetically knocking out both the Mnks does not affect caspase activity in the presence or absence of serum.

3.2.4 Mnk knockout does not affect cell proliferation over 24 h



The migration assays were carried out over a relatively short period of 24 h to reduce the influence of proliferation on the number of cells in the bottom well: it is believed that cells stop proliferating during active cell migration – mimicking the harsh environment metastatic cells would experience when breaking off into the blood (Evdokimova et al., 2009b). Despite this provision, it was important to confirm that knocking out the Mnks did not significantly affect cell number over the same 24-h period as the migration assay. **Figure 3.5B** (with **Figure 3.5A** verifying the knockdown), confirms that

genetically knocking out the Mnks does not significantly affect cell number over 24 h.

### **3.3 Discussion**

The data presented in this chapter is the first and only data to show the effects of genetically knocking out the Mnks on cell migration. The striking inhibitory effect of knocking out both the Mnks on MEF cell migration suggests the Mnks play a central part in cell migration. A suggestion that is further supported by the observation that an increase in Mnk1 levels causes an increase in cell migration.

Given that these experiments were performed using non-immortalised primary MEF cells it is possible that the Mnks could play a role in the migration of a wide array of cell types. What is surprisingly, given that cell migration is important in embryogenesis, is that the DKO embryos were able to form at all. All there is not data to present, there did tend to be fewer and sometimes no embryos in the plugged female DKO mice compared with the WT mice. The experimental set up is also very different to the conditions during early embryo development. For a start the results presented in this chapter are based on 24 hours of migration. Embryo development occurs over a much longer time frame so although there is very little migration over 24 hours there might be enough residual migration over the 13.5 days that it took for these embryos to develop. Another caveat is that in the transwell migration assays the rate of migration was measured towards serum, which although was useful as a proof of principal is rather simplistic compared to the migration which occurs during embryogenesis. The three-dimensional tissues and matrices which cells migrate through during embryogenesis add several layers of complexity (Satoshi and Anna, 2008). Each of these tissues

and matrices have their own distinct physical and chemical properties which influence the rate of migration (Satoshi and Anna, 2008). The type of cell migration during embryogenesis could be different to the type measured in a transwell migration assay. A transwell migration assay measures the migration of single cells that have actively migrated through the pores in the bottom of the transwell insert. During embryogenesis cells tend to migrate collectively in a spatial and temporal pattern (Satoshi and Anna, 2008). The scratch wound healing assay measures collective sheet-like cell migration but has several limitations in that it is carried out in 2D, there are no chemoattractants stimulating the migration, it occurs on a hard surface and it lacks the additional layers of complexity involved in embryonic migration, which have already been alluded to (Satoshi and Anna, 2008).

## 4. Mnk kinase activity is important for cancer cell migration

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### 4.1 Introduction

The previous chapter showed how the Mnks appear to be important in MEF cell migration. Given that the aim of this project was to establish the value of the Mnks as anti-cancer targets, the next step was to look at whether this effect translates into an effect on cancer cell migration. Cell migration is an important process in metastasis, giving rise to secondary tumours, which are responsible for about 90% of all cancer deaths. It had already been shown that eIF4E is important in the translation of a subset of proteins involved in the metastasis of a breast cancer cell line in a paper published by Nasr, Z. et al. in 2013. They also showed that knocking down eIF4E reduced the ability of breast cancer cell lines to migrate and invade, central processes in metastasis. Although this finding doesn't necessarily implicate the Mnks, eIF4E is a substrate of the Mnks and the Mnks are the only kinases known to phosphorylate eIF4E. To establish whether the kinase activity of the Mnks, in phosphorylating eIF4E, is important for cancer cell migration it required the use of an effective Mnk inhibitor.

To date, published literature on Mnk kinase activity has focused on the use of commercially available Mnk inhibitors, such as CGP57380 or cercosporamide (Knauf et al., 2001) (Chrestensen et al., 2007) (Bianchini et al., 2008b) (Grzmil et al., 2011) (Robichaud et al., 2014). This is despite evidence linking these compounds to off-target effects on other kinases (Bain et al., 2007, Konicek et al., 2011). To be able to form reliable conclusions about the role of Mnk kinase activity it is important to have a potent and selective Mnk inhibitor.



The data presented in this Chapter shows that a novel, and more effective, Mnk inhibitor inhibits cancer cell migration.

## 4.2 Results

### 4.2.1 Mnk-I1 is effective at inhibiting Mnk kinase activity in MEF, SCC25 and MDA-MB-231 cells

A new Mnk inhibitor Mnk-I1 (**Figure 4.1D**) was reported to be a potent inhibitor of the Mnks in patent WO 2011/104340 A1. To see whether Mnk-I1 is more potent than existing Mnk inhibitors a selectivity profile (**Table 1**) was carried out to compare Mnk-I1 with cercosporamide. The selectivity profile showed Mnk-I1 to have a much lower IC<sub>50</sub> compared with cercosporamide. Mnk-I1 also appeared to inhibit fewer kinases than cercosporamide and more importantly no other kinases were inhibited as much as it's intended targets Mnk1 and Mnk2.

To see whether Mnk-I1 is an effective Mnk inhibitor in cells western blots were carried out (**Figure 4.1**) to see the effect of the compound on p-eIF4E levels: an indicator of Mnk activity. Two cancer cells lines, MDA-MB-231 and SCC25, were chosen on the basis that they are commonly used in migration assays; they exhibited high basal levels of p-eIF4E, which has been shown to be indicative of sensitivity to Mnk inhibition; and, because they derive from two different types of cancer: breast and tongue, respectively (Matthew et al., 2010). In the two-cancer cell lines tested, MDA-MB-231 and SCC25 (**Figure 4.1B and Figure 4.1C**), and mouse embryonic fibroblasts (MEFs) (**Figure 4.1A**) Mnk-I1 reduced p-eIF4E levels significantly at concentrations as low as 1 $\mu$ M. Even at 20 $\mu$ M, the effect of CGP57380 on p-eIF4E levels in all 3 cell lines was weaker than that of 1 $\mu$ M Mnk-I1.

To be confident that the reduction in p-eIF4E was due to the effect of Mnk-I1 on its intended target, the Mnks, it was important to check that Mnk-I1 wasn't affecting the activity or expression of some other key proteins, which could indirectly affect Mnk activity. P-S6(240/244) is a readout of mTOR activity, which by affecting 4E-BP – eIF4E binding is a contributing factor in the availability of eIF4E. Mnk-I1 did not affect the expression of 4E-BP1 or levels of P-S6(240/244) in MEFs (**Figure 4.1A**) or two cancer cell lines tested MDA-MB-231 or SCC25(**Figure 4.1B and Figure 4.1C**). Similarly, Mnk-I1 had no effect on the expression of 4E-BP1 or its phosphorylation by mTOR at site T37/46 (**Figure 4.1**). PKB, which is upstream of mTOR, expression and activity appear to be unaffected by Mnk-I1 up to 5 $\mu$ M. The lack of effect of Mnk-I1 on Erk expression and P-Erk shows that it did not appear to affect signalling further upstream the MAPK signaling pathway from Mnk.

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<b>Kinase</b>	<b>Residual activity with 1<math>\mu</math>M cercosporamide [Dundee, 141 kinases]</b>	<b>Residual activity with 1<math>\mu</math>M Mnk-I1 [Dundee, 79 kinases]</b>
Mnk1	5	6
Mnk2	8	3
ERK8	23	-
PRK2	2	50
PKC $\zeta$	21	100
Aurora B	15	76
AMPK	15	41
MARK1	17	-
MARK2	2	-
MARK3	7	77
BRSK1	9	24
BRSK2	6	24
TSSK1	13	-
IKK $\epsilon$	1	83
TBK1	13	88
GCK	15	73
MAP4K3	7	-
ULK2	11	-
DYRK1A	96	25
IRAK1	96	51
NUAK1	57	23
PHK	90	23
RIPK2	87	19
AMPK	15	41

- Indicates not tested.

#### IC50 values

<b>Kinase</b>	<b>Cercosporamide (<math>\mu</math>M)</b>	<b>Mnk-I1 (<math>\mu</math>M)</b>
MNK1	0.043	0.023
MNK2	0.038	0.016

Table 4.1 Selectivity assay comparing cercosporamide and Mnk-I1, a novel Mnk inhibitor

Residual activity of a panel of kinases following treatment with either cercosporamide (on the left) or Mnk-I1 (on the right). IC50 values for cercosporamide and Mnk-I1, with respect to Mnk1 and Mnk2, are included in the table below.

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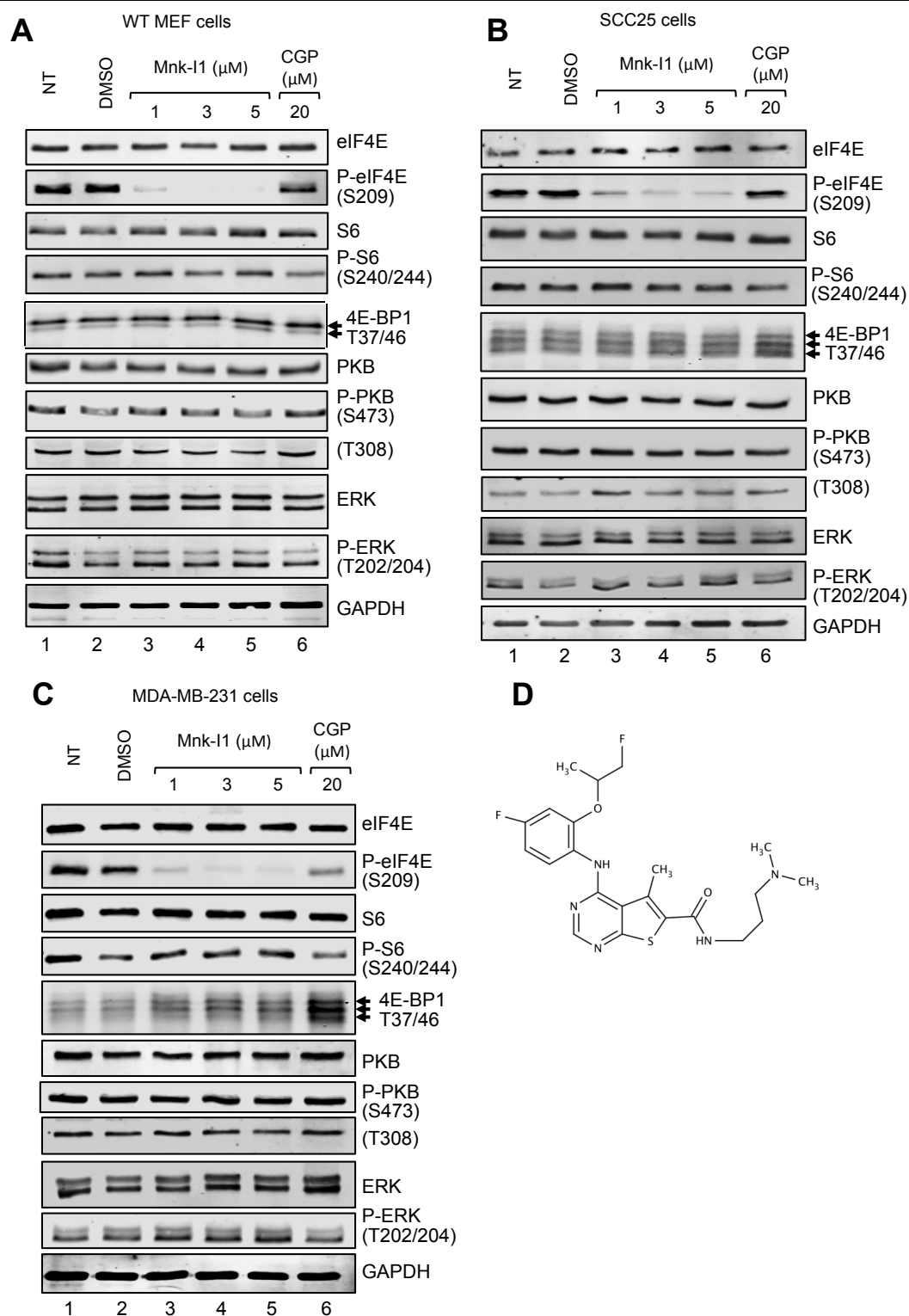
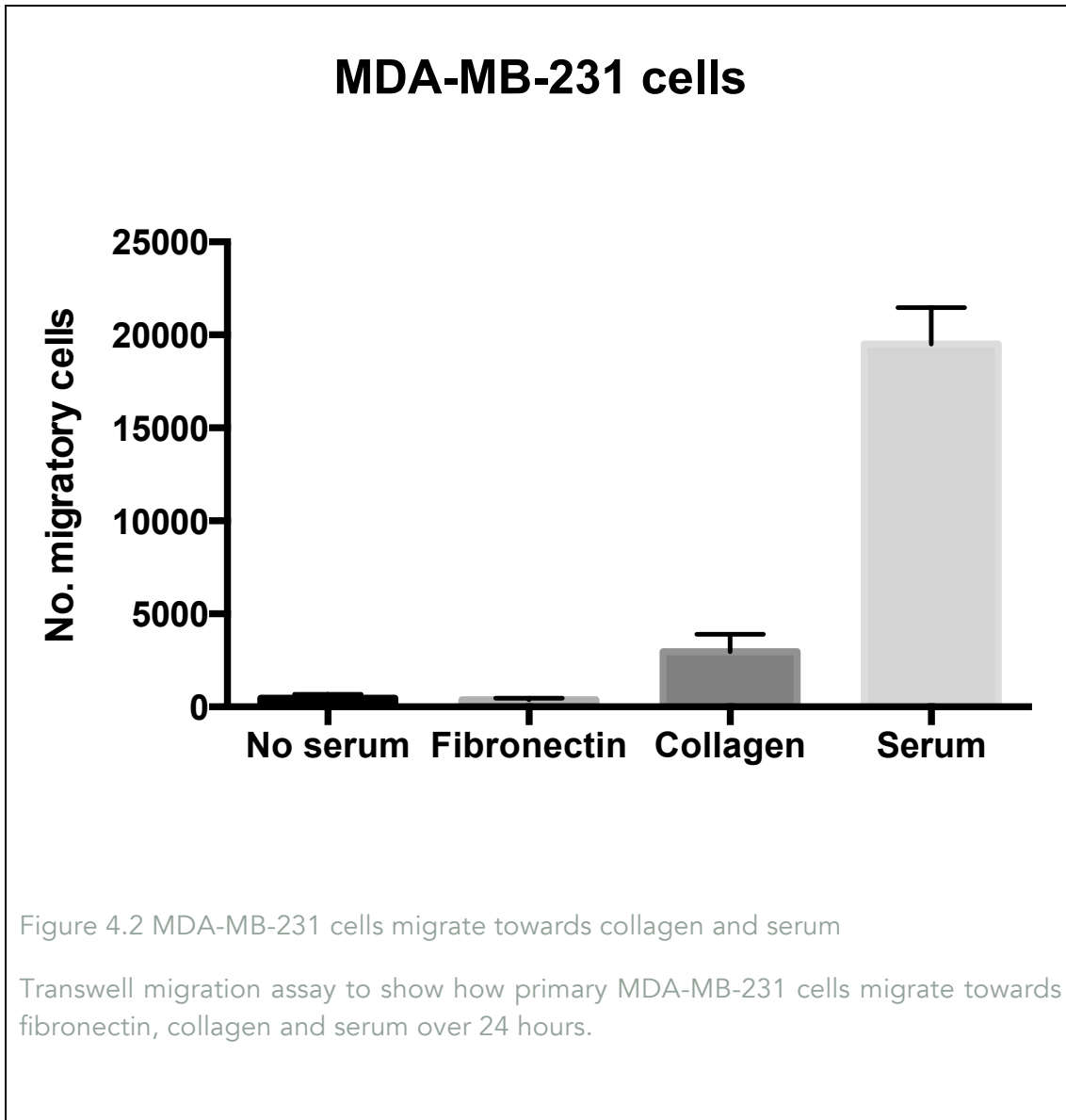


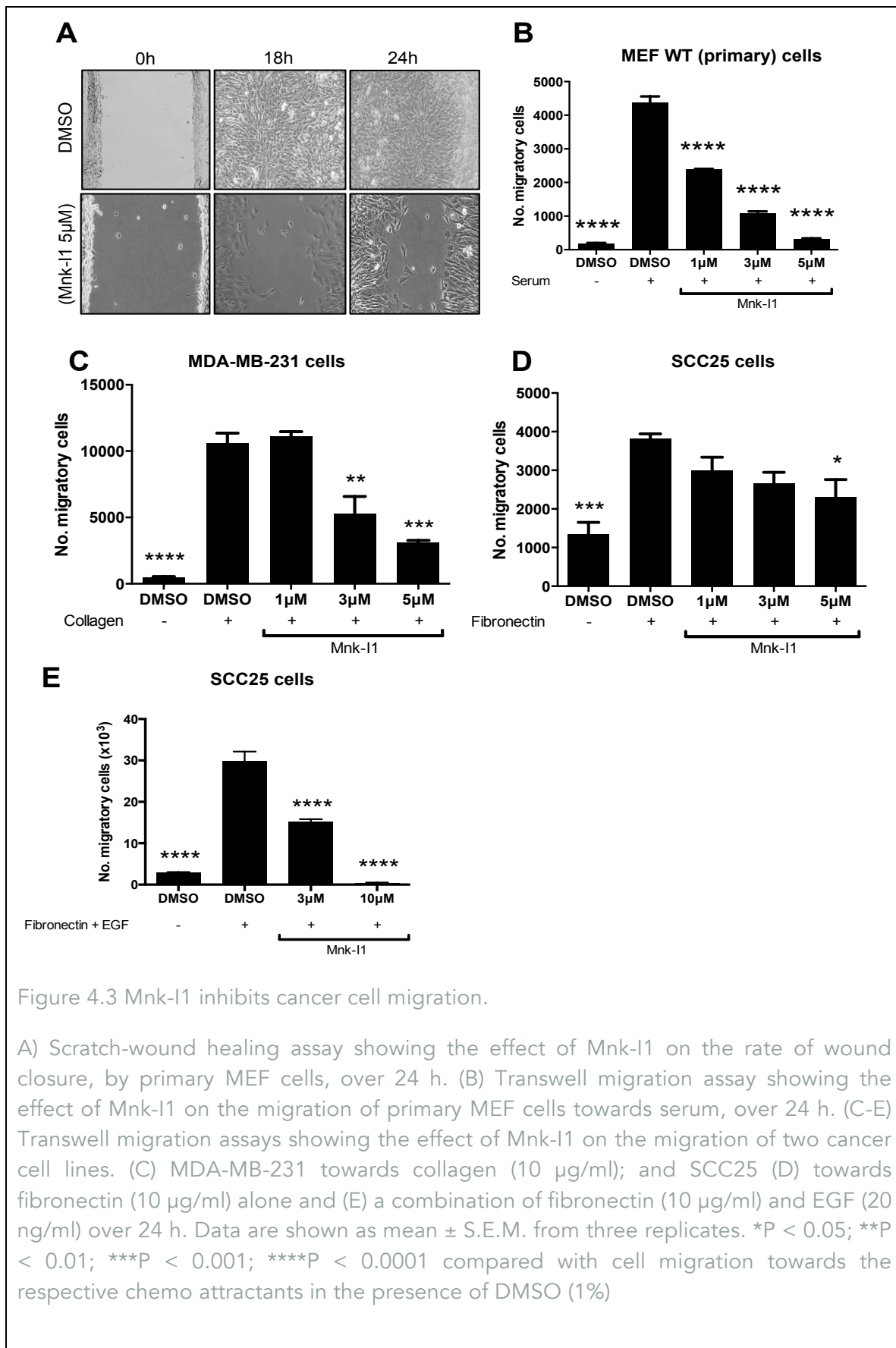
Figure 4.1 Mnk-I1 inhibits Mnk kinase activity at lower concentrations than CGP57380.

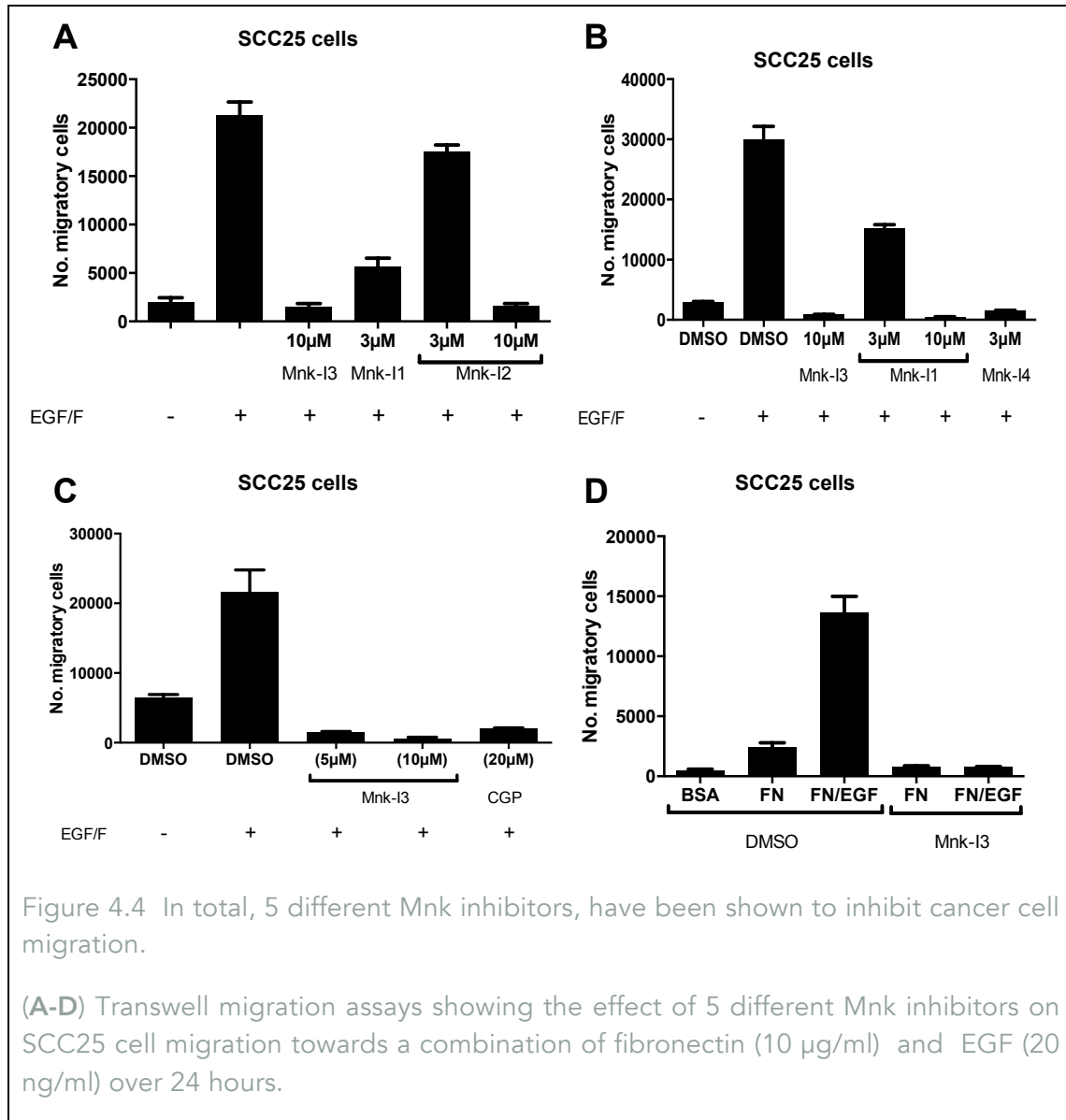
Western blots showing the effect of Mnk-I1 and CGP57380 on the phosphorylation of eIF4E, S6, 4E-BP1, PKB and ERK in (A) MEF WT, (B) SCC25 and (C) MDA-MB-231 cells.

Mnk-I1 is a novel Mnk inhibitor, believed to be a type I or ATP-competitive inhibitor. The results featured in **Figure 4.1** show that Mnk-I1 is effective at inhibiting Mnk activity in two cancer cell lines: SCC25 tongue (squamous cell carcinoma) and MDA-MB-231 (breast). Given that there is no reliable Mnk2 antibody, meaning it would be difficult to confirm the success of siRNA knockdown of the Mnks, it made sense to focus on the effect of inhibiting Mnk kinase activity on cancer cell migration. Both SCC25 and MDA-MB-231 cell lines are known to migrate in transwell migration assays and have relatively high levels of Mnk activity making them suitable model cell lines for these experiments. The SCC25 cell migration assay was carried out using fibronectin and EGF as chemoattractants; a preliminary experiment showed that MDA-MB-231 cells did not migrate very well towards fibronectin, showing a 'preference' for collagen, another chemoattractant (**Figure 4.2**).



#### 4.2.2 Mnk inhibition impairs cancer cell migration





At a concentration range previously shown to be effective in inhibiting Mnk activity (**Figure 4.1**), Mnk-I1 significantly inhibited the rate of MDA-MB-231 cell migration towards collagen (**Figure 4.3C**). A similar effect was seen with respect to inhibiting the migration of SCC25 cells towards fibronectin alone (**Figure 4.3D**) and, with greater statistical significance, with the addition of EGF (**Figure 4.3E**). Mnk-I1 also inhibited the rate at which MEF WT cells migrate towards serum (**Figure 4.3B**) and heal a wound (**Figure 4.3A**), in the scratch-wound healing assay.



In total, 5 different Mnk inhibitors – 4 of which are structurally unrelated – were shown to be effective at inhibiting SCC25 cell migration towards fibronectin and EGF (**Figure 4.4**). Each inhibitor was used at concentrations known to inhibit eIF4E phosphorylation (**Figure 4.5**).

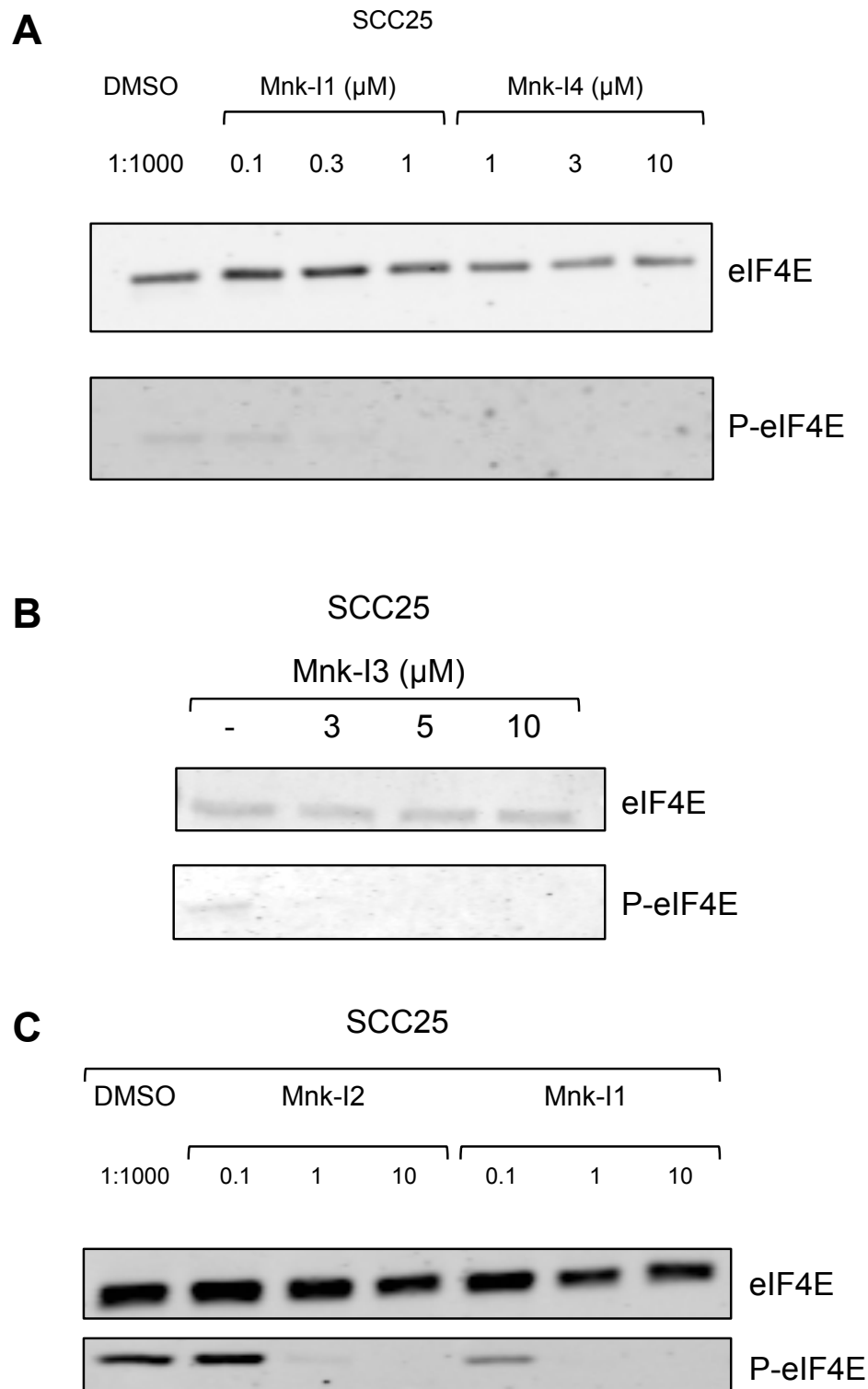
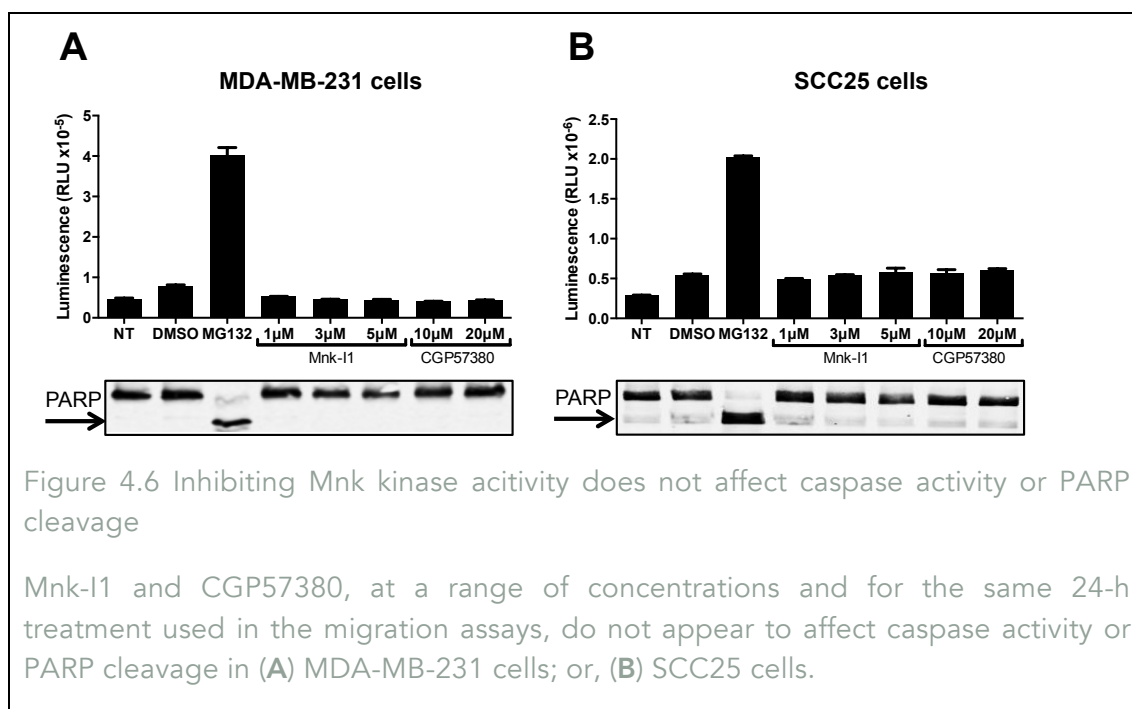


Figure 4.5 Western blots showing that the other Mnk inhibitors used in the transwell migration assays inhibit eIF4E phosphorylation

(A) Western blot to show the effect of Mnk-I1 and Mnk-I4 on eIF4E phosphorylation in SCC25 cells. (B) Western blot to show the effect of Mnk-I3 on eIF4E phosphorylation. (C) Western blot to show the effect of Mnk-I1 and Mnk-I2 on eIF4E phosphorylation in SCC25 cells.

#### 4.2.3 Mnk-I1 does not affect cell viability over 24 h

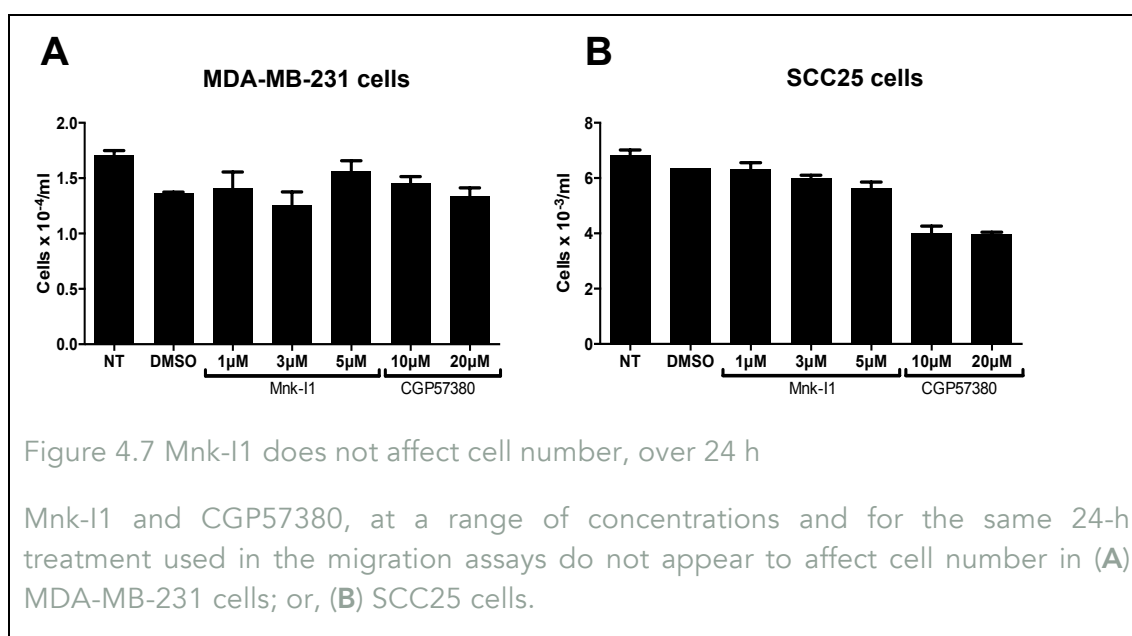


As with the Mnk knockout experiments, it was important – especially when testing a novel inhibitor - to make sure that the effects of Mnk-I1 on cell migration were not due to an effect on cell viability.

Using the same range of concentrations of Mnk-I1 as in the western blots – 1, 3 and 5 μM – and the same 24 h treatment, a caspase assay was carried out in both the MDA-MB-231 cells and SCC25 cells. Compared to the DMSO vehicle control, and using MG132 as a positive control, there didn't appear to be any significant effect of Mnk-I1 on caspase activity in either cell line following a 24 h treatment. Similarly, CGP57380 - a commercially available Mnk inhibitor - also had no significant effect on caspase activity over 24 h in both the MDA-MB-231 cells (**Figure 4.6A**) and the SCC25 cells (**Figure 4.6B**). In parallel with the caspase assay, a PARP cleavage blot was also produced in each cell line tested. PARP cleavage – indicated by a lower molecular weight band on a western blot - is another indicator of apoptosis. Again, neither

Mnk-I1 nor CGP57380 had any noticeable effect on PARP cleavage in MDA-MB-231 and SCC25 cells (**Figures 4.6A and 4.6B**).

#### 4.2.4 Mnk inhibition does not affect cell number over 24 h



The migration assays were carried out over a 24 h period – as opposed to a longer 48 or 72 h period – purposely to reduce the chances of an effect on cell number distorting the results; however, it was still important to confirm this. Inhibiting Mnk kinase activity using the more selective Mnk-I1 Mnk inhibitor in MDA-MB-231, (**Figure 4.7A**), and SCC25 cells, (**Figure 4.7B**), did not significantly affect cell number over 24 h. CGP57380 had an inhibitory effect on the SCC25 cells, but this could be the consequence of an effect of CGP57380 on one of its many reported off-target kinases (Bain et al., 2007).

#### 4.2.5 The effect of Mnk-I1 on cell cycle progression

The effect of Mnk-I1 on cell cycle progression was tested to make sure that the effects on cell migration were not a consequence of an effect on cell proliferation. To simulate the conditions in the migration assay, flow

cytometry was carried out on cells that had been treated with Mnk-I1 (at 5  $\mu$ M – the highest concentration used) for 24 h in the absence of serum (**Figure 4.8B**). As with the lack of an effect on cell number, Mnk1-I1 again had no effect on cell cycle distribution in MDA-MB-231 cells, but did slightly decrease the proportion of SCC25 cells in S-phase (**Figure 4.8A and 4.8B**). In contrast, even at concentrations that did not affect eIF4E phosphorylation, CGP57380 had a significant effect on increasing the number of MDA-MB-231 cells in the G1-phase of the cell cycle and reducing the number in S-phase (**Figure 4.8B**), indicating these observations reflect off-target effects.

Flow cytometry was also used to compare the effect of Mnk-I1 at 5  $\mu$ M – the highest concentration used in the migration assay – on cell cycle progression in MDA-MB-231 cells over 72 h, in the presence of serum (**Figure 4.8A**). Although, the migration assays are carried out over 24 h and in the absence of serum in order for the cells to survive for the duration of the 72-h experiment it was important to maintain them in the presence of serum. Although, there was a significant effect at 24 h on reducing the number of cells in the G1-phase of the cell cycle the effect was not evident after 48 or 72 h.

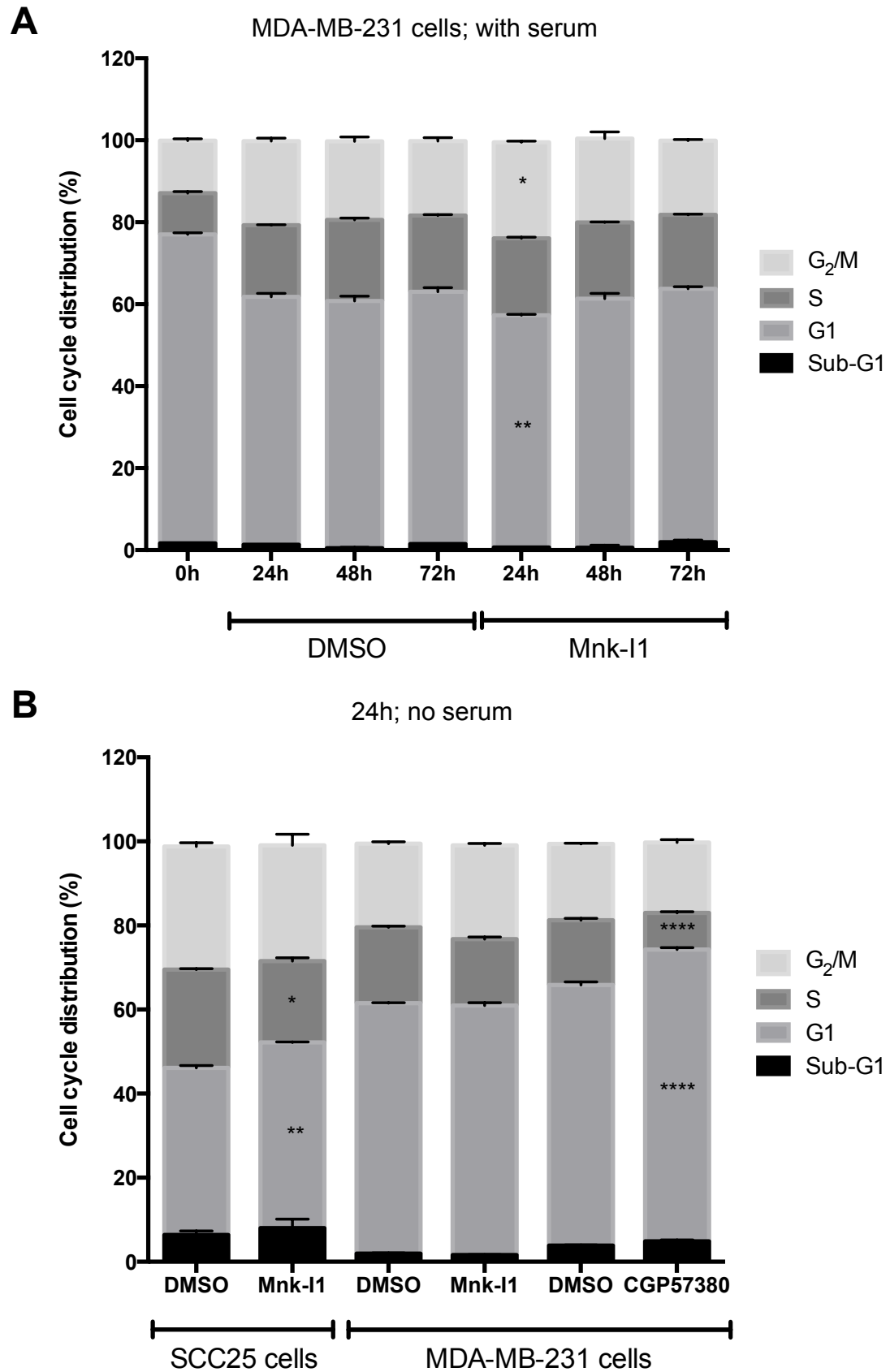


Figure 4.8 Mnk-I1 does not have a consistent effect on cell cycle progression

Flow cytometry analysis of the effect of Mnk-I1 (5 $\mu$ M) on cell cycle progression in **(A)** MDA-MB-231 cells in the presence of serum over 72 h. **(B)** Flow cytometry analysis of the effect of Mnk-I1 (5  $\mu$ M) on SCC25 and MDA-MB-231 cell cycle progression and the effect of CGP57380 on MDA-MB-231 cell cycle progression following 24 h in the absence of serum.

Data are mean +  $\pm$  S.E.M., n = 3. \*P < 0.05; \*\*P < 0.01; \*\*\*\*P < 0.0001.

### 4.3 Discussion

The results featured in this Chapter, by focusing on the Mnks, provide an explicit link between the Mnks and cell migration. By using both cells that have had the Mnks genetically knocked out (Chapter 3) and an effective Mnk inhibitor (this chapter), Mnk-I1, the results are an extension of previous studies linking eIF4E and its phosphorylation to cell migration (Nasr et al., 2013, Willett et al., 2011).

Whilst these data were being prepared for publication, two other groups published studies supporting a role for the Mnks in cancer cell migration. The first paper to suggest a link between the Mnks and cancer cell migration was published by Ramalingam and co-workers (Ramalingam et al., 2014). The studies focused around the use of retinoic acid metabolism-blocking agents (RAMBAs), which were shown to cause the degradation of the Mnks and also to inhibit the migration of MDA-MB-231 cells. A major limitation of this paper is that they give no indication as to how specific the RAMBAs are at degrading the Mnks. The RAMBAs would likely be degrading other proteins as well as the Mnks, which could be responsible for the effects on migration. The issue of selectivity is dealt with in this Chapter by showing that 5 different Mnk inhibitors, 4 of which are structurally unrelated, inhibit cancer cell migration. Even if some of these inhibitors do have small effects on other proteins, the effects on migration must presumably be through a target common to all the inhibitors, which given they all target the Mnks, is most likely to be the Mnks themselves. This assumption is strongly supported by the results showing that knocking out the Mnks has a substantial effect on inhibiting cell migration. Further support comes from the observation that increasing Mnk1 levels actually increases cell migration.



The second paper, showed that introducing a non-phosphorylatable eIF4E<sup>S209A</sup> inhibits random cell migration (Robichaud et al., 2014). They go on to show that preventing Mnk phosphorylation of eIF4E inhibits the translation of proteins involved in metastasis: SNAIL and MMP3. Although the study uses a more directed approach to preventing Mnk phosphorylation of eIF4E, compared with the Ramalingam paper there are also some limitations of the Robichaud paper (Ramalingam et al., 2014, Robichaud et al., 2014). Data published alongside the results of this Chapter, obtained by Shuye Tian, raised question marks over how reliable the eIF4E<sup>S209A</sup> mutant is as a mimic of non-phosphorylated eIF4E (Beggs et al., 2015). If eIF4E<sup>S209A</sup> interacts with other proteins differently, compared to endogenous eIF4E, then this alone could cause some effects. In addition to this, the inhibitor used to support some of the eIF4E<sup>S209A</sup> mutant results was CGP57380, which is actually a relatively weak inhibitor of the Mnks and is known to inhibit other proteins with similar potency\*, including RSK1: a protein which itself has been linked to cell migration (Sulzmaier and Ramos, 2013, Bain et al., 2007). \*1  $\mu$ M CGP57380 caused a greater reduction in RSK1 activity than Mnk2 and only a 16% less reduction compared to Mnk1 (Bain et al., 2007).

The results from the scratch-wound healing assays provided a useful indication as to whether the Mnks might be playing a role in 2D cell migration, before investing in transwell migration assays. The reason it was important to study the effects of Mnk knockout and inhibition on, 3D, migration in transwell migration assays particularly when linking this to cancer metastasis is that 3D migration is more physiologically representative of the migration (Yamaguchi et al., 2005). In scratch-wound healing assays, the cells migrate collectively so that they close the wound. Whilst 2D migration assays still reflect effects on cell polarity and cells' ability to form protrusions, which drag cells forward; the 3D transwell migration assays reflect the ability of

individual cells to actively migrate through the pores of the transwell (Yamaguchi et al., 2005). This is similar to metastasis where single cancer cells migrate and invade their way out of the primary tumour site.

Whilst proliferation is known to be an important driver in the initial formation of a tumour, during metastasis cancer cells are believed to reduce their rate of proliferation: a process believed to be co-ordinated by YB1 and its effects on translation (Evdokimova et al., 2009b). On this basis, over the course of a 24-h migration assay proliferation was unlikely to distort any effects on the number of migratory cells. It was still important to rule out the possibility that knocking out the Mnks or inhibiting Mnk kinase activity reduced cell number or cell viability over the course of the migration assay.

## 5. The Mnks play a role in the translation of the mRNA for vimentin, a marker of the epithelial-mesenchymal transition (EMT)

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### 5.1 Introduction

Based on the data presented in the previous chapters, it is clear the Mnks are playing a role in cell migration, what remained unclear is *how* the Mnks influence cell migration.

A sensible starting point in trying to work out how the Mnks might play a role in cell migration was to look at the established roles of the Mnks. The Mnks are kinases and eIF4E - involved in protein translation initiation - is their best-characterised substrate. So, the first considerations were: could the Mnks have a direct effect, via their kinase activity, on proteins involved in the processes of cell migration; or, could the Mnks affect the translation, and therefore expression, of proteins involved in cell migration?

## 5.2 Results

### 5.2.1 Mnk inhibition does not affect the activity or expression of proteins important in cell adhesion

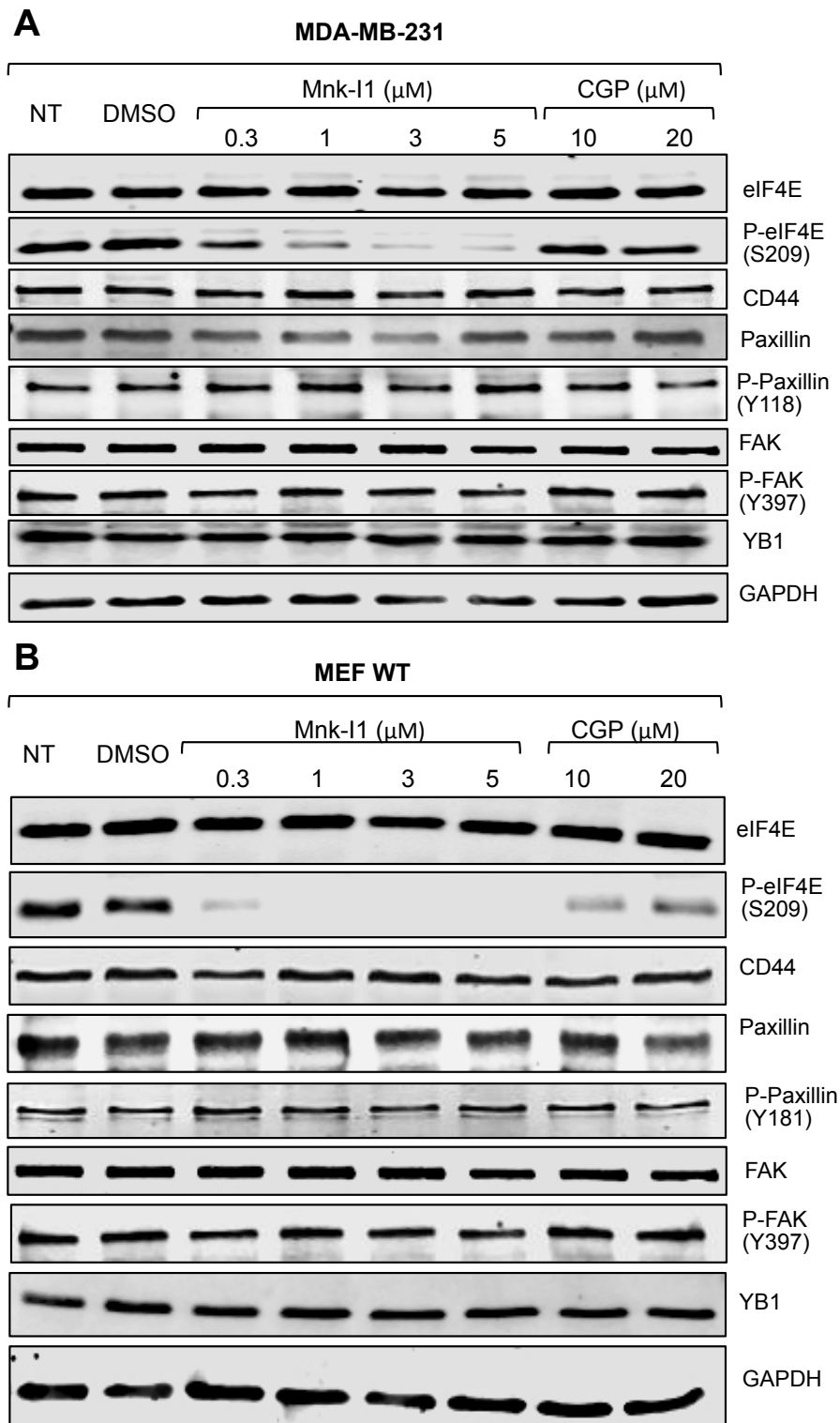


Figure 5.1 Mnk-I1 and CGP'57380 have no effect on the expression levels or phosphorylation of proteins involved in cell adhesion.

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Western blots showing the effect of 24h treatments of Mnk-I1 (0.3  $\mu$ M – 5  $\mu$ M) and CGP57380 (10  $\mu$ M or 20  $\mu$ M) on eIF4E phosphorylation; the expression of CD44, paxillin, FAK and YB1; and the phosphorylation of Paxillin (Y181) and FAK (Y397) in (A) MDA-MB-231 cells and (B) MEF WT cells.

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The rate at which cell adhesions are turned over determines the rate at which cells migrate (Huttenlocher and Horwitz, 2011). Based on previous publications, it is possible to infer a link between cell adhesion and the Mnks: focal adhesion complexes (FACs) - the macromolecular structures that allow a cell to adhere to the ECM – are known to relay signals to the RAS pathway, which is upstream of the Mnks (Fincham et al., 2000b, Ishibe et al., 2003). It has also been shown that integrins, which are a central component of focal adhesion complexes, can signal via the Mnks to influence translation – of VEGF mRNA (Korneeva et al., 2010).

FAK - a tyrosine kinase - and paxillin - an adaptor protein - are two proteins, which are both known to associate with FACs and play a central role in relaying signals onto intracellular signaling pathways (Fincham et al., 2000a). Using the same concentrations and 24-h treatments of Mnk-I1 and CGP57380, as used in the transwell migration assays, there was no effect was observed on the total levels and phosphorylation of FAK and paxillin in MDA-MB-231 (**Figure 5.1A**) or MEF WT cells (**Figure 5.1B**).

CD44, a cell adhesion protein, activates signaling via FAK to the MAP-kinase pathway, following its binding to hyaluronan in the extracellular matrix (ECM) (Fujita et al., 2002). The transcription of the CD44 gene is regulated by Y-box binding protein 1, which is overexpressed in 40% of metastatic cancers (To et al., 2010). Again, using the same concentrations and 24-h treatments of Mnk-I1 and CGP57380, as used in the transwell migration assays, there was no effect was observed on the total levels of CD44 or YB1 (**Figure 5.1**).

### 5.2.1 Mnks regulate vimentin expression

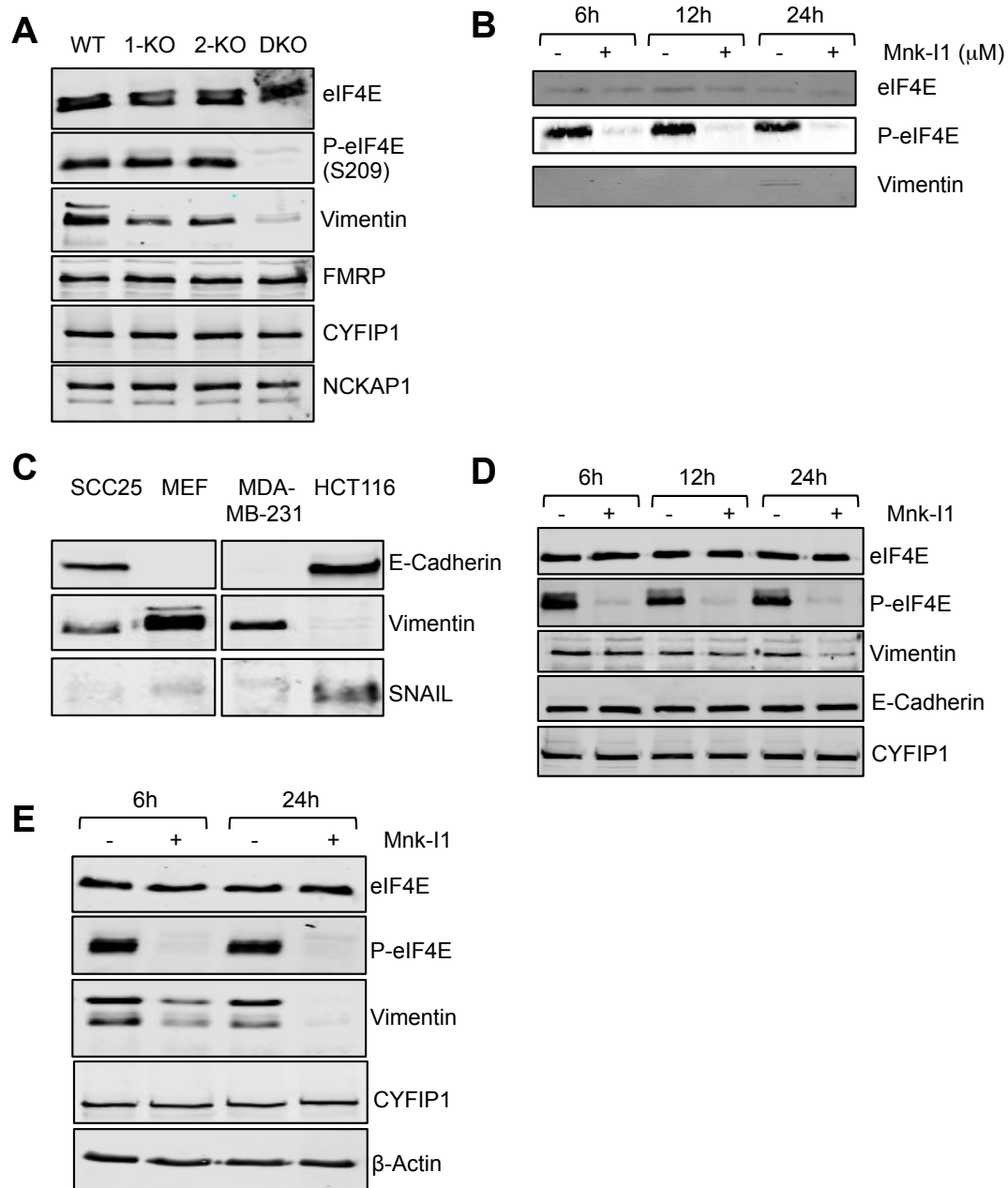


Figure 5.2 Genetic knock out of both Mnks and inhibition of Mnk kinase activity reduces vimentin protein expression, a marker of EMT.

(A) Western blots showing the effect of knocking out Mnk1 and Mnk2 on eIF4E phosphorylation and vimentin, FMRP, CYFIP1 and NCKAP1 expression in primary MEF cells.

(B) Western blots showing the effect of a 6 h, 12 h or 24 h treatment of Mnk-I1 (5 μM) on the expression of vimentin in primary WT MEF cells.

(C) Western blot comparing the expression levels of E-cadherin, vimentin and SNAIL in SCC25, primary MEF WT, MDA-MB-231 and HCT116 cells.

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(D) Western blot showing the effect of a 6 h, 12 h or 24 h treatment of Mnk-I1 (5  $\mu$ M) on the expression of vimentin, E-cadherin and CYFIP1 in SCC25 cells.

(E) Western blot showing the effect of a 6 h or 24 h treatment of Mnk-I1 on vimentin and CYFIP1 expression in MDA-MB-231 cells.

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The epithelial-mesenchymal transition (EMT) is another important process cells undergo in order to become motile and migrate. In cancer, EMT is an important process cancer cells have to undergo in order to be able to metastasize. During EMT cells switch from a polarized cell with many cell-ECM and cell-cell contacts, characteristic of an epithelial cell, to a depolarized, detached and motile mesenchymal cell no longer bound to a particular tissue. As well as a switch in cell morphology during EMT, there is also a switch in protein expression. During the transition, the expression of E-cadherin a protein involved in cell-cell contacts is switched off whilst the expression of vimentin, an intermediate filament protein, is switched on. This switch in protein expression is controlled by transcription factors, such as TWIST and SNAIL (Samy et al., 2014)

A striking observation was made when comparing the protein levels of vimentin in MEF WT and DKO cells by western blot (**Figure 5.2A**). The expression of vimentin in DKO cells appeared to be very weak. The single Mnk1 and Mnk2 KO cells also appeared to express less vimentin compared with the MEF WT cells. Mnk-I1 reduced the expression of vimentin following a 24 h treatment in MEF WT cells (**Figure 5.2B**). In addition, treatment with Mnk-I1 caused a clear decrease in the expression of vimentin in SCC25 (**Figure 5.2D**) and, to slightly lesser extent, MDA-MB-231 cells over 24 h (**Figure 5.2E**). Interestingly, there was no effect of the Mnk inhibitor on E-cadherin levels in SCC25 cells - MDA-MB-231 cells did not express E-cadherin (**Figure 5.2C**).

### 5.2.2 Mnk activity does not affect vimentin mRNA levels

To work out what was causing the decrease in vimentin protein expression it was important to look at whether knocking out the Mnks or inhibiting their kinase activity was having an effect on the vimentin mRNA levels. Interesting

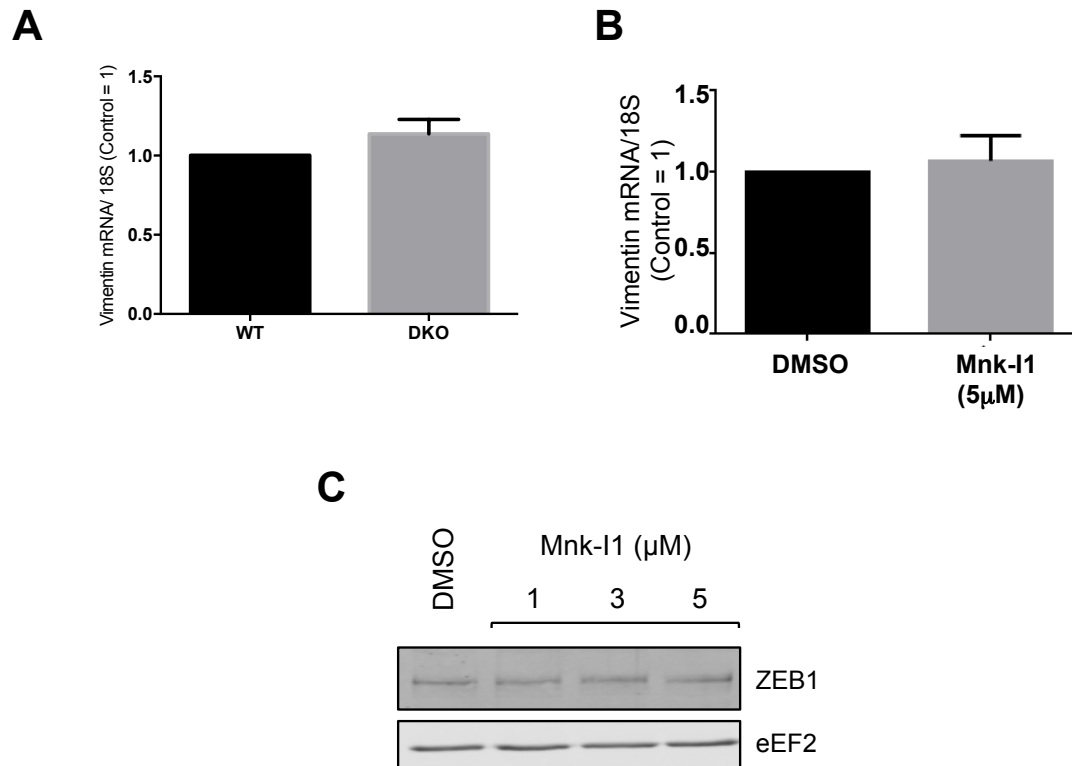


Figure 5.3 Genetic knock-out of both Mnks and inhibition of Mnk kinase activity has no effect on vimentin mRNA levels.

- (A) RT-qPCR to show the effect of knocking out both the Mnks (DKO) on vimentin mRNA levels in primary MEF cells. 18S mRNA levels were used to normalise the levels of mRNA across different samples.
- (B) RT-qPCR to show the effect of treating MDA-MB-231 cells with Mnk-I1 (5 µM) for 24 h on vimentin mRNA levels. 18S mRNA levels are used to normalise the levels of mRNA across different samples.
- (C) Western blot to show the effect of treating MDA-MB-231 cells with Mnk-I1 (5 µM) for 24 h on ZEB1 expression. Total eEF2 is used as a loading control.

RT-qPCR results showed that there was no difference between the levels of vimentin mRNA in the MEF WT and DKO cells (**Figure 5.3A**) or in response to treatment with Mnk-I1 in the MDA-MB-231 cells (**Figure 5.3B**). Similarly, there was no effect of Mnk-I1 on the protein levels of ZEB1 a transcription



factor known to regulate the transcription of vimentin (**Figure 5.3C**). What these results suggest is that the Mnks are not affecting the expression of vimentin protein at the transcriptional level; instead, the results point towards a post-transcriptional effect.

### 5.2.3 Mnk kinase activity is important for vimentin mRNA association with polysome and vimentin protein stability

If the Mnks do not appear to be important for vimentin mRNA levels, but do appear to be important in determining the final vimentin protein levels it suggests they are playing a role somewhere in between. Given that previous studies have suggested the Mnks may regulate the translation of a subset of mRNAs in tumorigenesis, it seemed obvious to look at whether the Mnks might be playing a role in regulating the translation of vimentin. To do this, RT-qPCR was carried out on polysomal fractions: where the lighter fractions (1-3) contain mRNAs associated with monosomes or non-polysomal material (i.e. not being translated) and the heavier fractions (7-9) contain mRNAs associated with polysomes (i.e. actively being translated). If the Mnks are playing a role in vimentin translation then you would expect that inhibiting Mnk kinases activity would reduce the amount of vimentin mRNA associated with ribosomes and therefore cause a shift towards the lighter fractions. This is exactly what was observed.

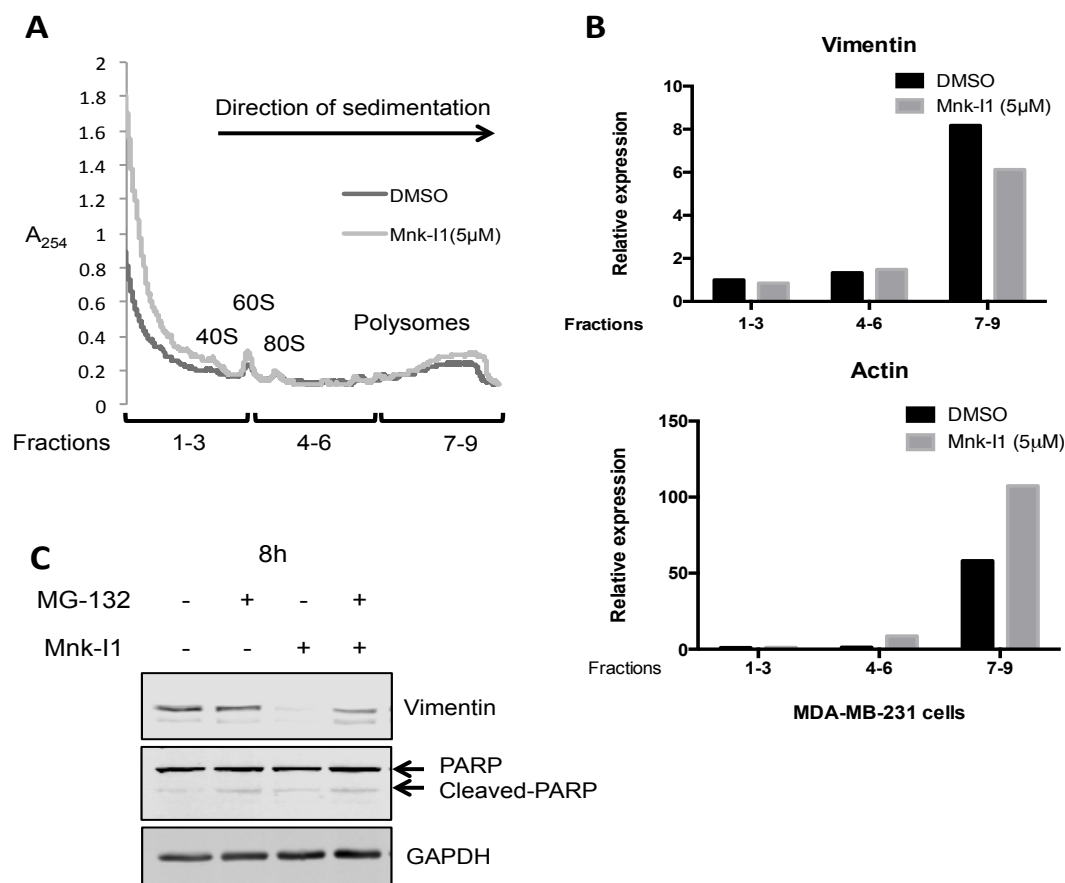


Figure 5.4 Inhibition of Mnk kinase activity inhibits the association of vimentin mRNA with polysomes.

- (A) Polysome gradient showing the effect of treating MDA-MB-231 cells with Mnk-I1 (5 µM) for 24 h on the association of the global mRNA population with polysomes.
- (B) RT-qPCR to show the effect of treating MDA-MB-231 cells with Mnk-I1 (5 µM) on the distribution of vimentin and actin mRNA across the different fractions of the polysome gradient in Figure 5.4A.
- (C) Western blot to show the effect of MG132, which blocks proteasomal degradation, on vimentin expression in MDA-MB-231 cells treated with Mnk-I1 (5 µM) for 8 h.

Compared with the DMSO control, the amount of vimentin mRNAs associated with the heavier fractions in the MDA-MB-231 cells was less (**Figure 5.4B**). This is despite there being a, slight, overall increase in the mRNAs associated with the heavier fractions; indicated both in the polysome

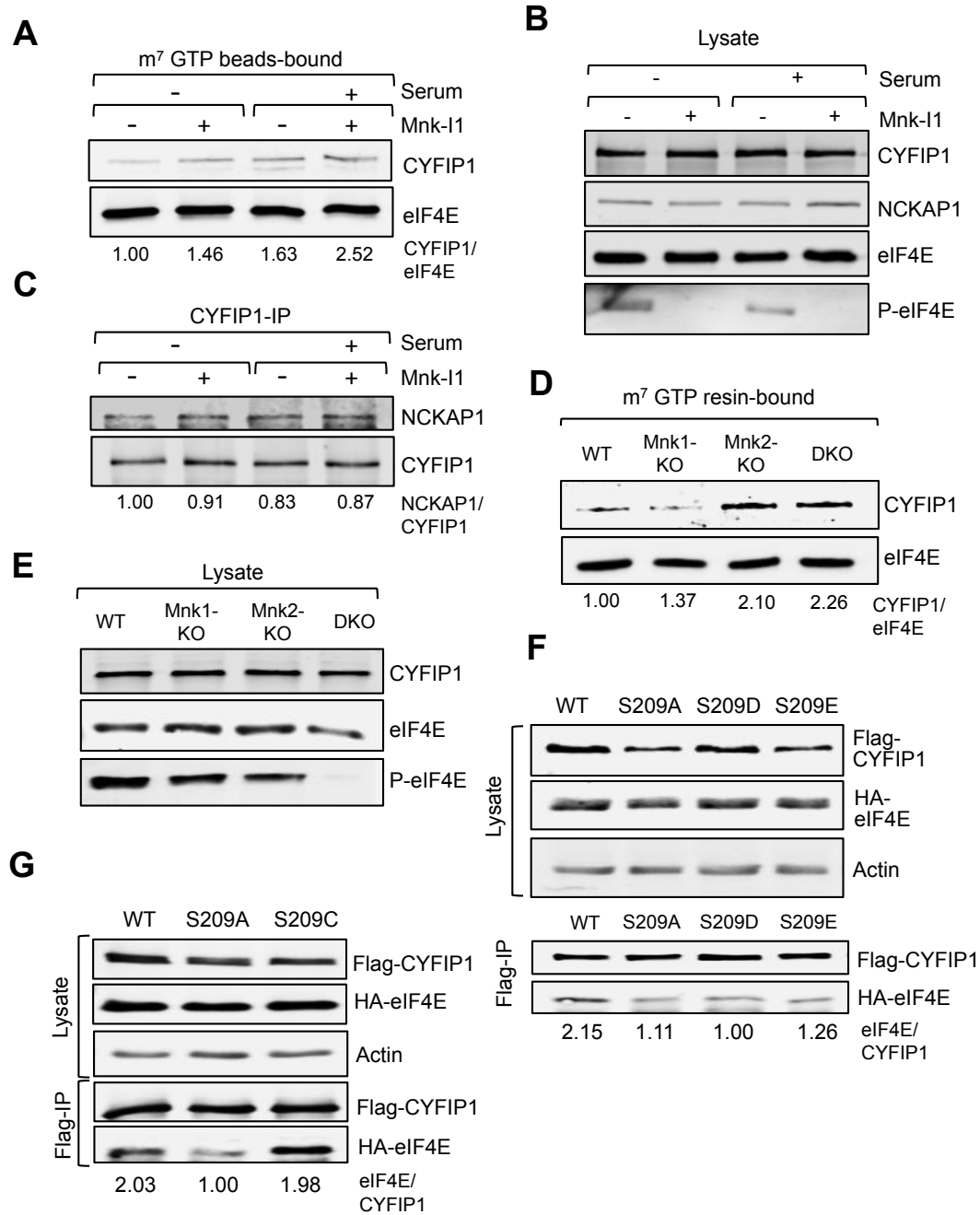
gradient traces and in the shift in actin mRNAs towards the heavier fractions (**Figure 5.4A and 5.4B**).

The differences in vimentin protein expression could also be explained by protein turnover. If a protein is turned over or degraded more rapidly the protein expression level will appear lower. The addition of a proteasome inhibitor, MG-132, appeared to restore vimentin protein levels in the presence of an Mnk inhibitor (**Figure 5.4C**). This suggests that inhibiting the Mnks increases the turnover of vimentin and, conversely, that the Mnks help stabilize vimentin.

### 5.3 Discussion

The data presented in this chapter show that the Mnk1s play a crucial role in the expression of vimentin. Vimentin is an established marker of EMT (see section 1.5 of the introduction), which is an important part of metastasis: a process responsible for 90% of all cancer deaths. Vimentin is emerging as an important target in the design of anti-cancer drugs because of its association with poor patient prognosis (Satelli and Li, 2011). The finding, presented in this chapter, that inhibiting the Mnk1s inhibits vimentin expression, could make the Mnk1s a useful target in prolonging the lives of cancer patients.

Both genetically knocking out the Mnk1s and inhibiting Mnk1 kinase activity caused a defect in vimentin protein expression. Given previous evidence linking the Mnk1s to a role in regulating the translation of a subset of mRNAs, it is perhaps not surprising that this defect was due to an effect on the translation of vimentin (Furic et al., 2010, Wendel et al., 2007a). The notion that the Mnk1s only play a role in regulating a subset of mRNAs, as opposed to a global effect on protein translation, is given further support by the data in Figure 5.4A which shows that inhibiting Mnk1 kinase activity had little effect on the overall distribution of the global mRNA population across a polysome gradient. In fact, there was a slight increase in the number of mRNAs associated with the polysomal fractions of the gradient indicating an overall increase in translation initiation. This slight increase, confirmed by the actin control RT-qPCR, makes the decrease in the proportion of vimentin mRNA associated with the polysomal fractions even more striking, emphasizing that the effect of the Mnk1s is selective for certain mRNAs.



Produced by Shuye Tian

Figure 5.5 Mnk activity inhibits the interaction between CYFIP1 and eIF4E.

Western blot showing the effect of treating MDA-MB-231 cells with Mnk-I1 (5  $\mu$ M), on the association of eIF4E and CYFIP1 with m<sup>7</sup>GTP beads, in the presence and absence of serum. (B) Western blot showing the effect of Mnk-I1 (5  $\mu$ M) and serum on total levels of CYFIP1, NCKAP1 and eIF4E in MDA-MB-231 cells. (C) Western blot

showing the effect of Mnk-11 (5  $\mu$ M) and serum on the association of NCKAP1 with CYFIP1 in MDA-MB-231 cells. **(D)** Western blot showing the effect of knocking out Mnk1 and Mnk2, in primary MEF cells, on the association of eIF4E and CYFIP1 with m<sup>7</sup>GTP beads, in the presence and absence of serum. **(E)** Western blot showing the effect of knocking out Mnk1 and Mnk2 on the total levels of eIF4E and CYFIP1 in primary MEF cells. **(F, G)** Western blot showing the effect of mutating the S209 residue in eIF4E on the total levels of eIF4E and CYFIP1 and the interaction between eIF4E and CYFIP1 in primary MEF WT cells. Actin is used as a loading control.

A mechanism for the regulation of vimentin translation by the Mnks is proposed in the data produced by Shuye Tian for our publication – shown in Figure 5 (Beggs et al., 2015). The data shows that the Mnks, by phosphorylating eIF4E, inhibit the binding of CYFIP1 to eIF4E in cancer cells. It has previously been shown that FMRP, a protein that can both enhance mRNA stability and also repress the translation of particular mRNAs, binds to CYFIP1 (Schenck et al., 2001, Napoli et al., 2008). Mnk activity has been shown to cause the dissociation of the CYFIP1/FMRP complex from the 5'-mRNA cap in neuron cells (Panja et al., 2014). A paper by Luca, R. et al. 2013 showed that FMRP binds to vimentin mRNA in cancer cell lines; however, in the particular cell lines they looked at they showed that FMRP increases vimentin expression (Lucá et al., 2013). This would contradict the link, inferred from the data presented here, which would suggest that increased CYFIP1/FMRP binding, due to inhibition of the Mnks, represses translation of vimentin mRNA (Beggs et al., 2015).

CYFIP1 (also known as Sra-1) has been shown to stabilise the WAVE complex, which drives the actin reorganisation at the leading edge of migrating cells (Kunda et al., 2003). It is thought that Sra-1 might deliver the mRNAs for the proteins that make up or are associated with the WAVE complex to the leading edge of migrating cells (Willett et al., 2013). An example of Sra-1 directing localised mRNA translation has been shown in neuronal cells, where

Sra-1 has represses the translation of FMRP mRNAs until they are delivered to the synapse (Napoli et al., 2008). In our publication, the effect of Mnk-I1 on the binding of CYFIP1 to NCKAP1 - a component of the WAVE complex - was assessed to see whether the Mnks affect the association of CYFIP1 with the WAVE complex (Beggs et al., 2015). The lack of effect, suggests the Mnks do not directly influence the WAVE complex. Further work is needed to clarify this.

Another mechanism by which the Mnks could be having a post-transcriptional effect on vimentin protein levels is by regulating its stability. Data presented in this chapter show that the Mnks play a role in protecting vimentin protein from degradation by the proteasome. Further work is required to ascertain the exact the mechanism by which the Mnks might be playing a role improve vimentin protein stability.

This is not the only set of data to suggest a link between the Mnks and EMT, via an effect on translation. The Robichaud paper, already mentioned in the discussion in the previous chapter, showed that the ability of the Mnks to phosphorylate eIF4E was important in the translation of SNAIL (Robichaud et al., 2014). SNAIL helps trigger the loss of an epithelial phenotype during EMT, by repressing the transcription of E-cadherin – a marker of epithelial cells.

The data presented this chapter, together with published studies, provide strong evidence linking the Mnks to a role in epithelial-mesenchymal-transition in cancer cells. The data suggests that the Mnks play an important role in promoting expression of vimentin, a marker of mesenchymal cells. The mechanism appears to involve a dependence on the Mnks for vimentin expression by translation of vimentin mRNA into protein and also for

protecting vimentin protein from degradation by the proteasome. Published data suggests that the CYFIP1-FMRP complex could be important for regulating the translation of vimentin mRNA into protein; however, there is conflicting evidence as to whether this complex induces or represses vimentin expression (Beggs et al., 2015, Lucá et al., 2013). Further work is required to clarify exactly how the Mnks regulate the translation of vimentin and how they appear to stabilize vimentin protein expression.



## 6. Does inhibition of the Mnk overcome resistance to MEK inhibitors?

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### 6.1 Introduction

The MEK1/2 kinases have emerged as an important target for inhibiting the RAS-RAF-MEK-ERK pathway, which drives the proliferation and survival of a number of human cancers (Little et al., 2011). What makes MEK an attractive target is its unique binding-pocket next to the ATP binding site, which, compared with other kinases, allows the design of relatively specific inhibitors, such as AZD6244 (Ohren et al., 2004). MEK is the only known substrate of RAF and ERK1/2 is the only known substrate of MEK1/2 (Mercer and Pritchard, 2003, Shaul and Seger, 2007). The MEK inhibitor AZD6244 (Selumetinib), which has entered clinical trials, has already proven to be effective at inhibiting proliferation and having a pro-apoptotic effect when tested on various cancer cell lines and xenografts (Little et al., 2011).

Acquired resistance to the MEK inhibitor, AZD6244, has previously been shown to occur due to the amplification of two upstream driving mutations: BRAF<sup>600E</sup> and KRAS<sup>13D</sup>. The consequence of these amplifications is that these resistant cell lines continue to proliferate even in the presence of relatively high concentrations of AZD6244. It has been shown that inhibiting RAF, upstream of MEK, can overcome the resistance in the BRAF<sup>600E</sup> –amplified cell line but not in the KRAS<sup>13D</sup>-amplified cell line. The differing responses can be explained by the fact that the KRAS<sup>13D</sup>-amplified cell line has hyper-activated PKB pathways on top of the hyperactive ERK1/2 signaling pathway found in the BRAF<sup>600E</sup> –amplified cell line. It is likely that additional pathways are hyperactivated in the KRAS<sup>13D</sup>-amplified cell line, because despite a

combination of AZD6244 and a combined PI3K-mTOR inhibitor (AZ12321046) inhibiting ERK1/2 and PKB activity the cells continued to express high levels of cyclin D1 and proliferate (Little et al., 2011).

Mnk inhibitors, which act downstream of MEK and ERK, might provide a useful combination therapy in overcoming some forms of resistance to AZD6244. By working downstream of ERK, the Mnk inhibitors might help to counteract the increased ERK activity caused by the BRAF<sup>600E</sup> and KRAS<sup>13D</sup> amplifications. Given that cells with the BRAF<sup>600E</sup> amplification appear to be more dependent on ERK1/2 activity - compared with cells containing the KRAS<sup>13D</sup> amplification - it is more likely that Mnk inhibition might translate into an effect on cell proliferation in these cells. To overcome the resistance in KRAS<sup>13D</sup>-amplified cell lines it will probably require inhibition of KRAS itself; however, the structure of RAS makes it very difficult to design inhibitors against (Cox et al., 2014).

Mnk-I1 has previously been shown to be effective at inhibiting the Mnks in cancer cell lines (Beggs et al., 2015). To test whether Mnk-I1 is effective in overcoming resistance to AZD6244, several colorectal cancer cell lines with single activating alleles encoding either BRAF<sup>600E</sup> or KRAS<sup>13D</sup> mutations were used. The parental COLO205 and HT29 cell lines contain the BRAF<sup>600E</sup> mutation; whereas, the parental HCT116 cell line contains the KRAS<sup>13D</sup> mutation. The resistant versions of these cell lines - C6244R, HT29-6244R and H6244R, respectively - have amplifications of their corresponding mutation. To maintain these amplifications and their resistance, the resistant version of the cell lines are grown in the presence of AZD6244 - 1 $\mu$ M for C6244R and HT29-6244R and 2 $\mu$ M for H6244R.

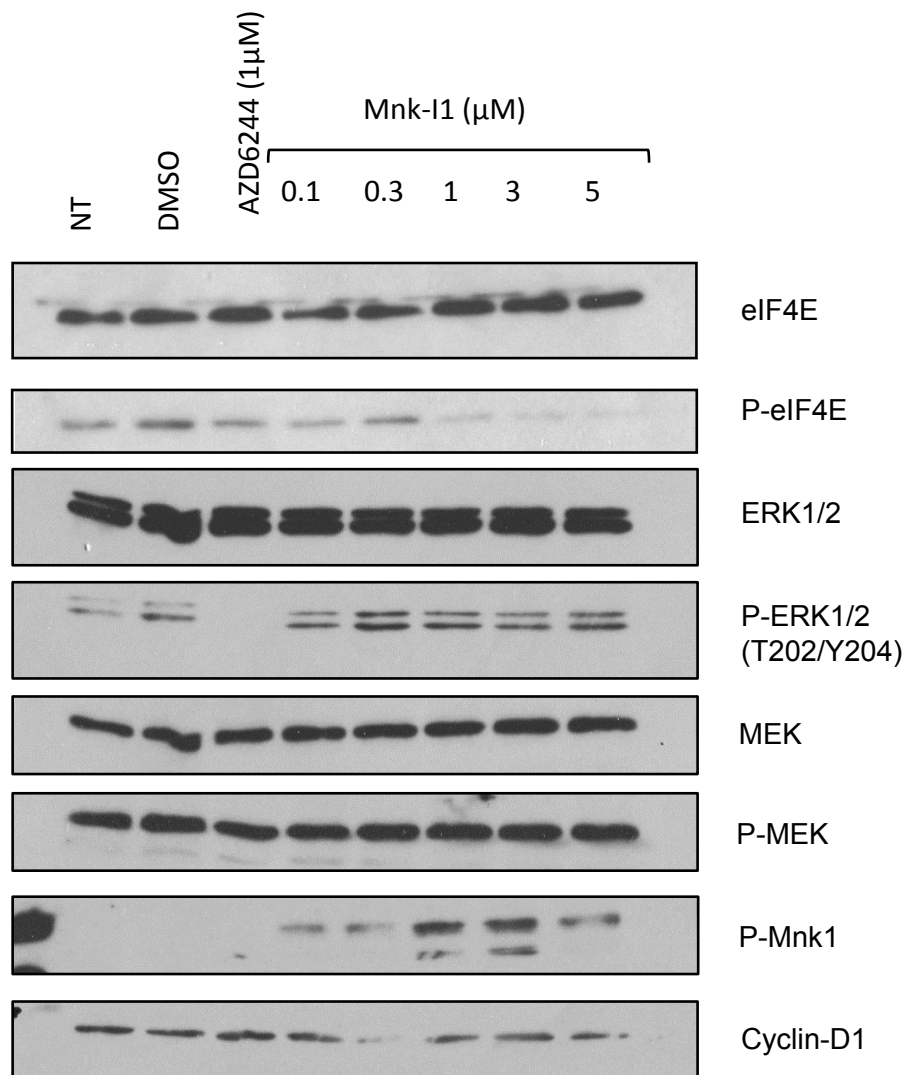
This chapter presents the results of experiments examining the effect of Mnk-I1 on the proliferation of AZD6244 resistant cell lines and parental cell lines. The results also provide some clues as to the mechanism behind an unexpected, possible feedback loop involving the Mnks.

## 6.2 Results

### 6.2.1 Mnk-I1 is effective at inhibiting Mnk kinase activity in COLO205-AZD6244 resistant (C6244R) cells

Mnk-I1 has been shown to be effective in the cancer cell lines MDA-MB-231 and SCC25; however, it was important, especially given the amplifications in the resistant cell lines, to first make sure that Mnk-I1 inhibits the Mnks in the particular cell lines that were due to be used for this series of experiments (Beggs et al., 2015).

A range of Mnk-I1 concentrations from 0.1 $\mu$ M to 5 $\mu$ M were tested on both the parental and resistant COLO205 cells under basal conditions – which includes 1 $\mu$ M AZD6244 for the C6244R cell line. P-eIF4E levels, an established indicator of Mnk activity, were used to check whether the Mnk kinase activity is being inhibited. In parental COLO205 cells, Mnk-I1 seemed to be effective at inhibiting Mnk kinase activity above 1 $\mu$ M (**Figure 6.1A**). This was the same in C6244R cells, where again Mnk-I1 appeared to be effective at inhibiting the Mnks at concentrations as low as 1 $\mu$ M (**Figure 6.1B**). Interestingly, despite having no obvious effect on P-MEK and P-ERK Mnk-I1 appeared to cause an increase in P-Mnk1. This result suggests that Mnk-I1 could be activating a feedback loop or perhaps stabilizing the Mnk structure in such a way that makes it more readily phosphorylated. Importantly, this increase in P-Mnk1 did not appear to affect Mnk-I1's ability to inhibit Mnk kinase activity.

**A****COLO205**

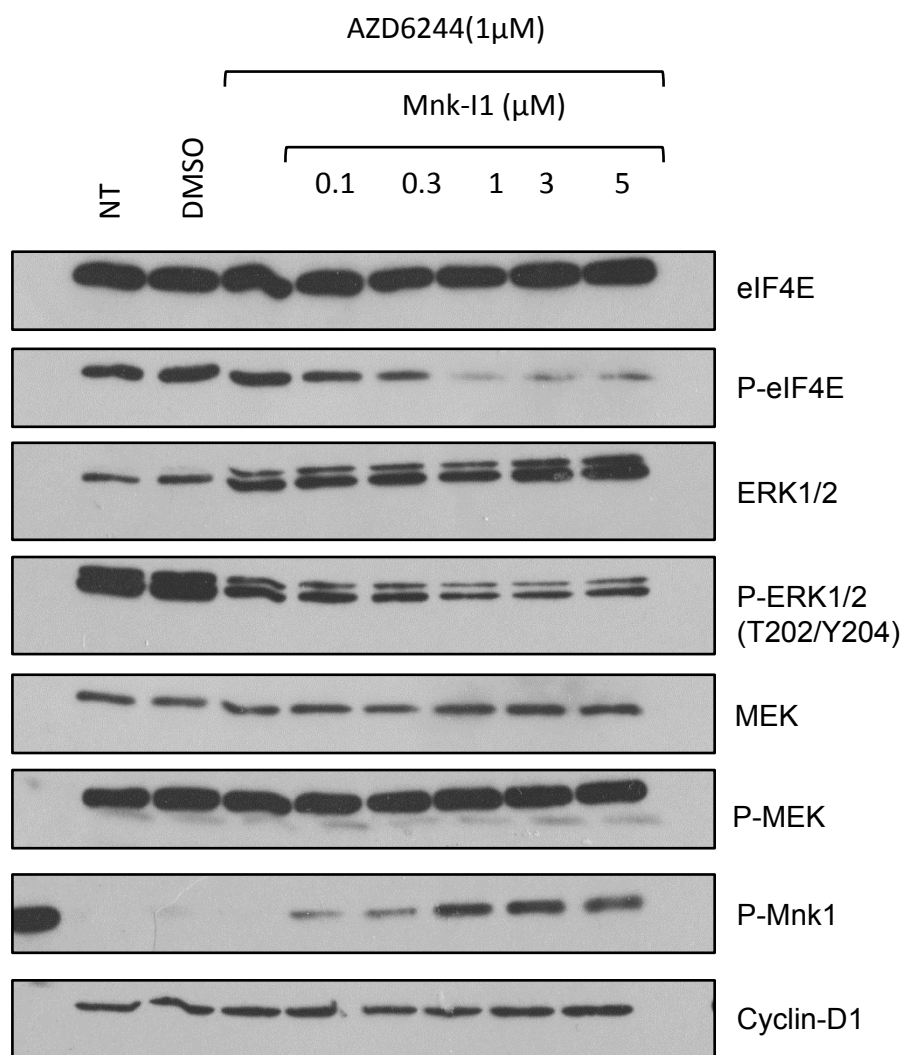
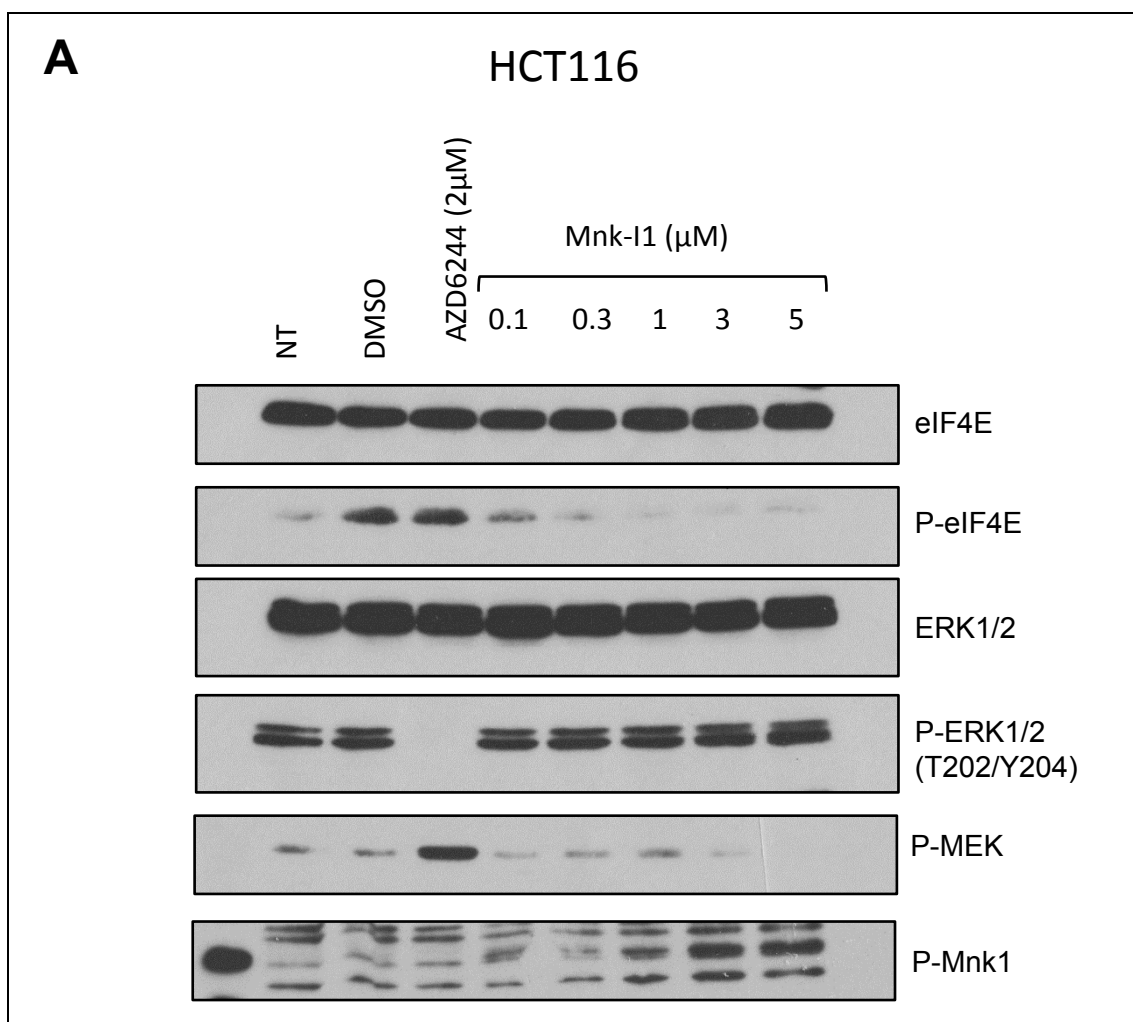
**B****C6244R**

Figure 6.1 Mnk-I1, in combination with AZD6244(1  $\mu$ M), inhibits eIF4E phosphorylation in AZD6244-resistant COLO205 (C6244R) cells

Western blots showing the effect of a 24h treatment with Mnk-I1 (0.1-5  $\mu$ M) on total expression levels and phosphorylation of eIF4E, ERK1/2 and MEK1/2 in (A) COLO205 cells and (B) C6244R cells – in combination with AZD6244(1  $\mu$ M). The effect on Mnk-1 phosphorylation and total cyclin-D1 levels is also shown.

### 6.2.2 Mnk-I1 is effective at inhibiting Mnk kinase activity in HCT116-AZD6244 resistant (H6244R) cells

The same effect- of increasing P-Mnk1 levels - was observed in the parental and AZD6244 resistant HCT116 cell lines (**Figures 6.2A & 6.2B**). In parental HCT116 cells, Mnk-I1 was still effective at inhibiting eIF4E phosphorylation at concentrations above 0.3 $\mu$ M. In HCT116-AZD6244 resistant (H6244R) cells, Mnk-I1 was effective at inhibiting eIF4E phosphorylation at concentrations above 0.1 $\mu$ M. In both the parental and AZD-6244 resistant HCT116 cell lines, Mnk-I1 did not appear to affect P-MEK1/2 or P-ERK1/2 levels.



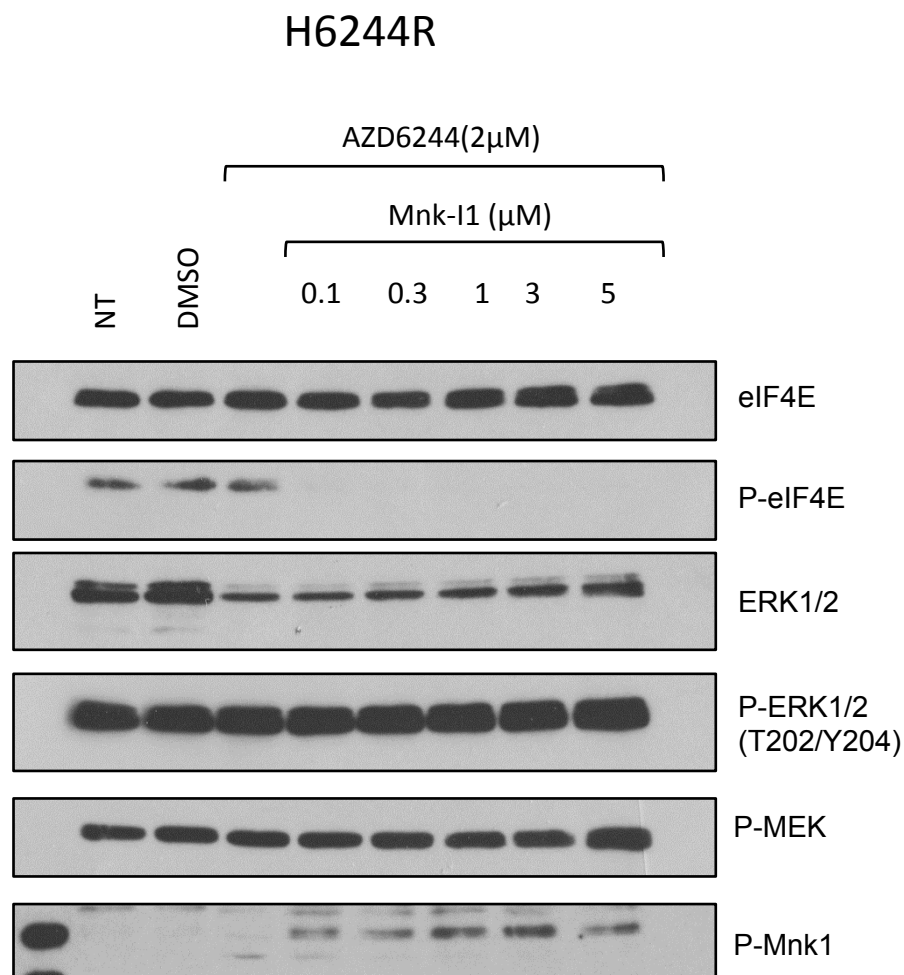
**B**

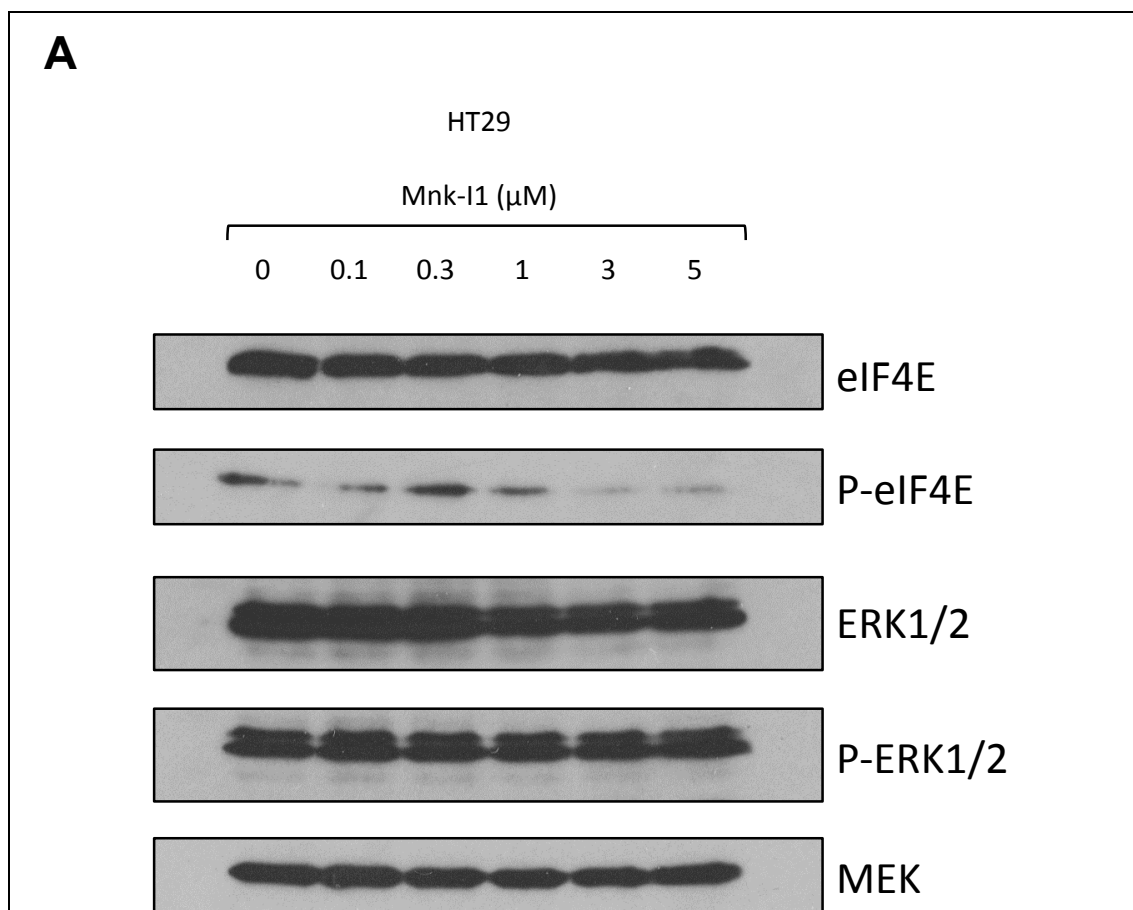
Figure 6.2 Mnk-I1, in combination with AZD6244 (2 $\mu$ M), inhibits eIF4E phosphorylation in AZD6244-resistant HCT116 (H6244R) cells

Western blots showing the effect of a 24h treatment with Mnk-I1 (0.1-5 $\mu$ M) on total expression levels and phosphorylation of eIF4E and ERK1/2 in (A) HCT116 cells and (B) H6244R cells – in combination with AZD6244 (2 $\mu$ M). The effects on MEK1/2 and Mnk-1 phosphorylation and total cyclin-D1 levels are also shown.

### 6.2.3 Mnk-I1 is effective at inhibiting Mnk kinase activity in HT29-AZD6244 resistant (HT29-6244R) cells

Mnk-I1 did not appear to be as effective at inhibiting Mnk kinase activity in the HT29-AZD6244 resistant cell line (**Figure 6.3B**). At least 3 $\mu$ M of Mnk-I1 was required to have a noticeable effect on reducing eIF4E phosphorylation.

As in COLO205 and HCT116, and their respective AZD6244-resistant cell lines, Mnk-I1 did not appear to affect phosphorylation of ERK1/2 in HT29 or HT29-AZD6244R cells (**Figures 6.3A & 6.3B**). In summary, Mnk-I1 was most effective at inhibiting Mnk kinase activity in the HCT116-AZD6244 resistant cell line, where even at concentrations as low as 0.1 $\mu$ M there was no detectable P-eIF4E.





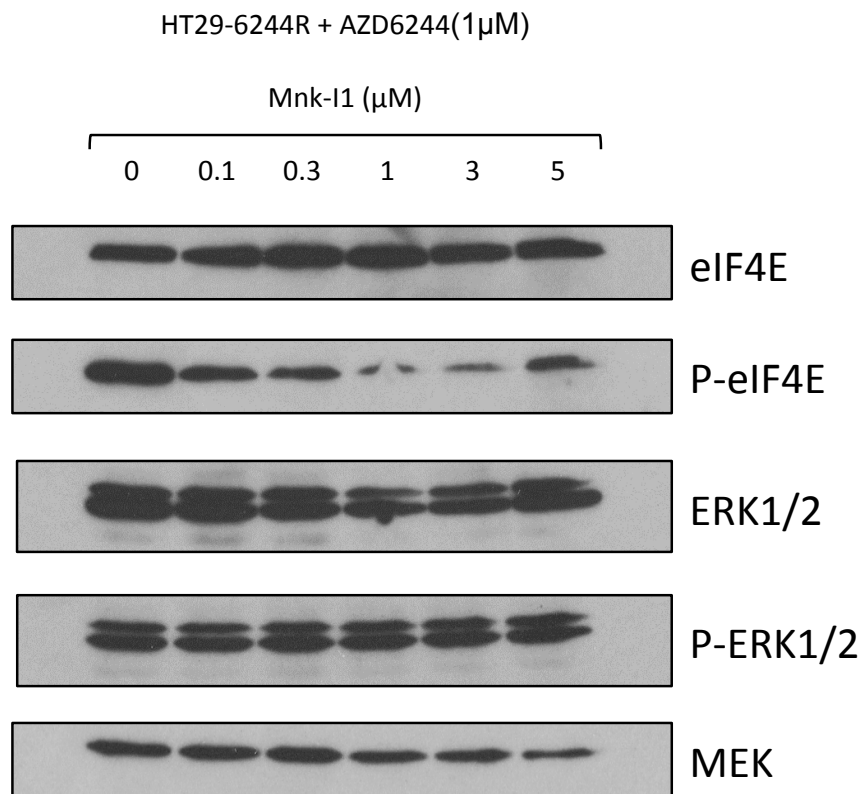
**B**

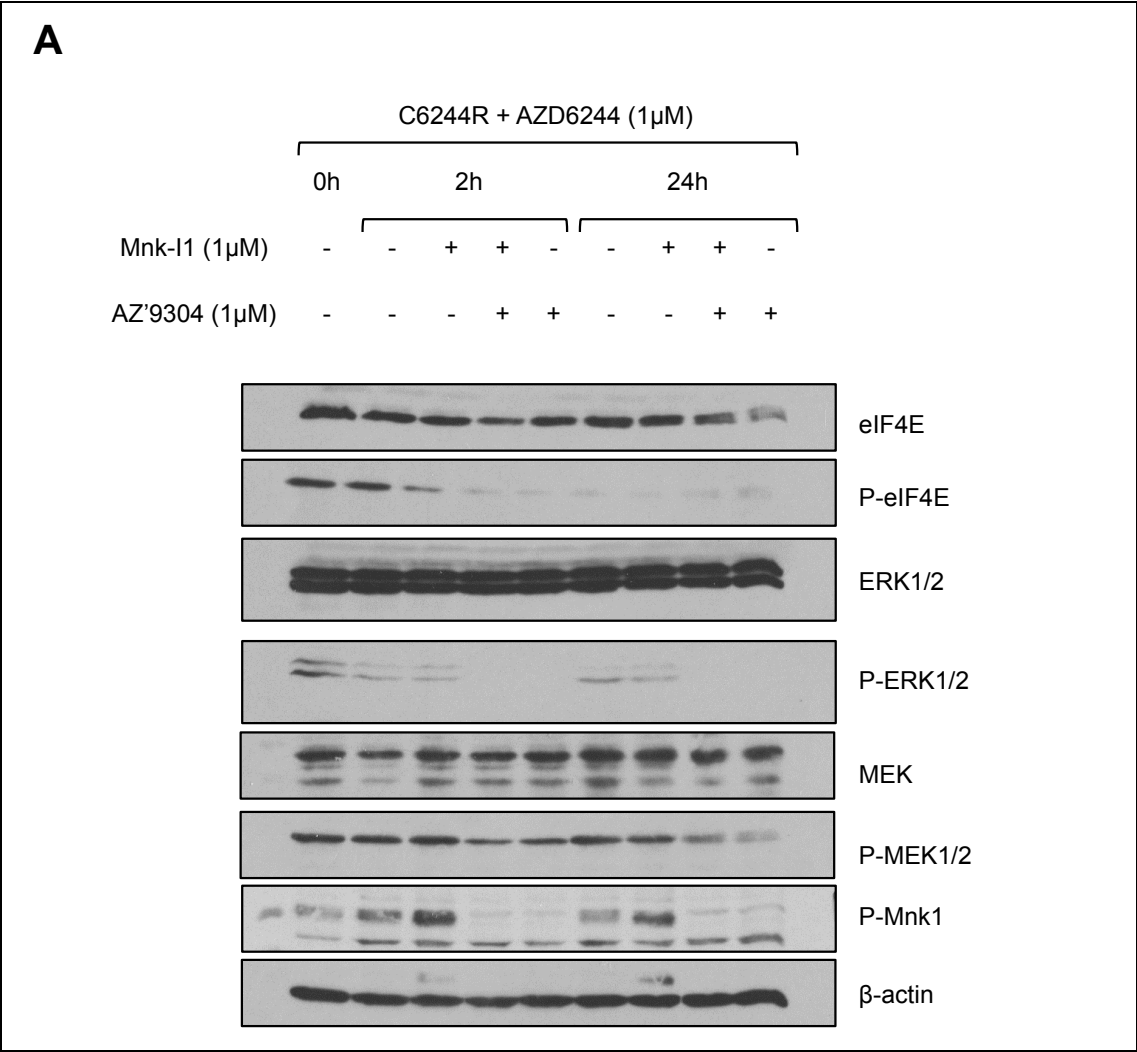
Figure 6.3 Mnk-I1, in combination with AZD6244 (1 $\mu$ M), inhibits eIF4E phosphorylation in AZD6244-resistant HT29 (HT29-6244R) cells

Western blots showing the effect of a 24h treatment with Mnk-I1 (0.1-5 $\mu$ M) on total expression levels and phosphorylation of eIF4E and ERK1/2 in (A) HT29 cells and (B) HT29-6244R cells – in combination with AZD6244 (1 $\mu$ M). The effect on total MEK levels is also shown.

#### 6.2.4 Mnk-I1 increases P-Mnk1 (Thr197/202) levels, but still inhibits eIF4E phosphorylation

In an attempt to work out the mechanism by which Mnk-I1 might be causing the observed increase in P-Mnk1, an experiment was carried out – in both the C6244R (**Figure 6.4A**) and H6244R (**Figure 6.4B**) cell lines - using a RAF inhibitor AZ9304. Interestingly, the addition of the RAF inhibitor prevents the increase in P-Mnk1 caused by Mnk-I1. The RAF inhibitor inhibits the phosphorylation of ERK1/2, but does not appear to affect P-MEK1/2. This suggests that the increase in P-Mnk1 caused by Mnk-I1 is dependent on

activity upstream of the Mnks. It does not however confirm whether this effect is due to an Mnk feedback loop; or, whether it is due to an allosteric effect of the phosphorylation site becoming protected from Mnk phosphatases. Both of these effects would be dependent on ERK1/2 activity, which is inhibited by the RAF inhibitor. One possible clue as to what might be going on here, can be found by comparing P-ERK1/2 levels, in the absence of any AZ'9304: here, the addition of Mnk-I1 does not appear to increase P-ERK1/2, but does increase P-Mnk1. This suggests that the increase in P-Mnk1 is independent of ERK1/2 activity. Although not conclusive, this suggests a direct effect of Mnk-I1 on Mnk1 structure is more likely to be the explanation behind this effect.



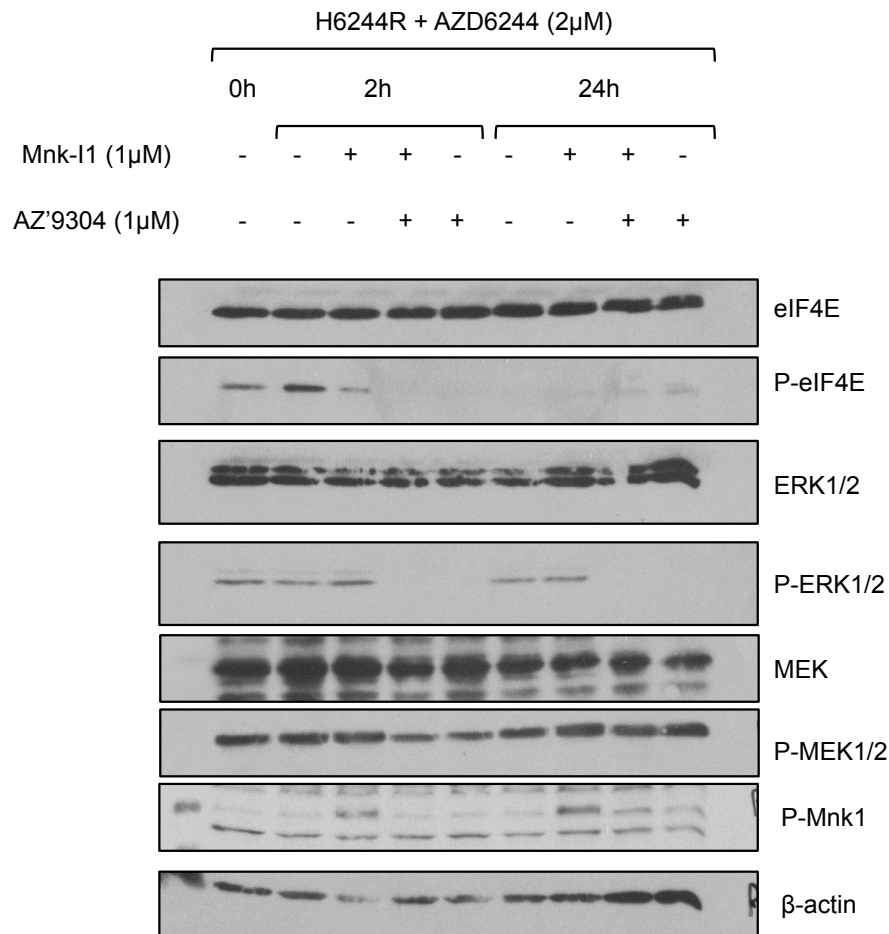
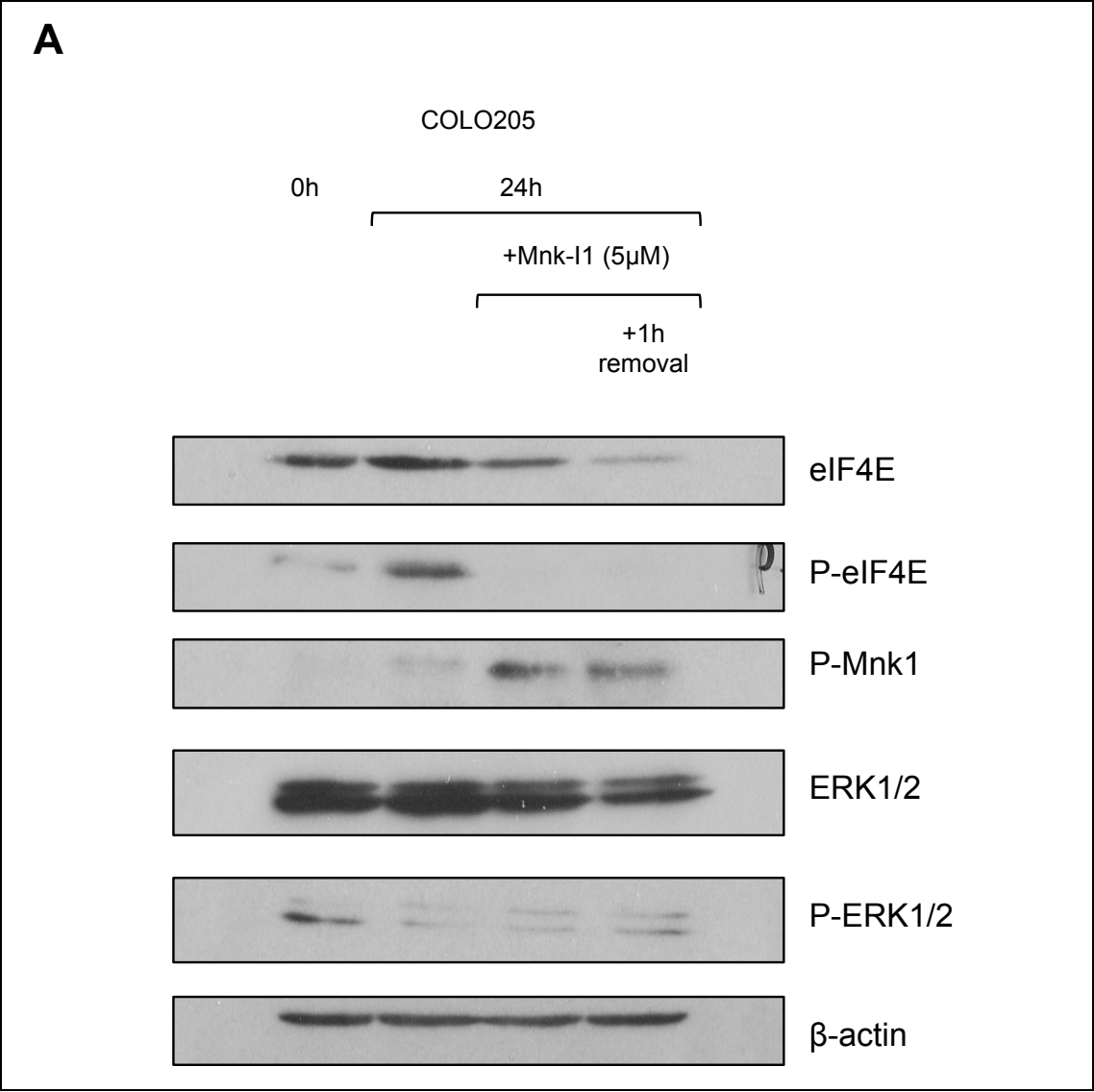
**B**

Figure 6.4 AZ'9304 inhibits the increase in P-Mnk1 caused by Mnk-I1

Western blots showing the effect of 2h and 24h treatments of Mnk-I1 (1μM) and AZ'9304 (1μM) alone, and in combination, on eIF4E, ERK1/2, MEK1/2 and Mnk1 phosphorylation in (A) C6244R and (B) H6244R cells.

To get an indication of how stable the phosphorylation of Mnk1 is, in response to Mnk-I1, an experiment was carried out that involved removing Mnk-I1 1 hour before harvesting the cells. COLO205 and C6244R cells (+/- AZD6244 (1μM)) were treated with Mnk-I1 (5μM) for 24 hours and then the Mnk-I1 – and AZD6244 for the C6244R cells – was removed for 1 hour before harvesting the cells (**Figures 6.5A & 6.5B**). Interestingly, the P-Mnk1 persisted for at least 1 hour after Mnk-I1 was removed across all conditions.

Despite the increase in P-Mnk1, Mnk-11 still appears to have a strong effect on inhibiting P-eIF4E in the AZD6244 resistant cell lines.



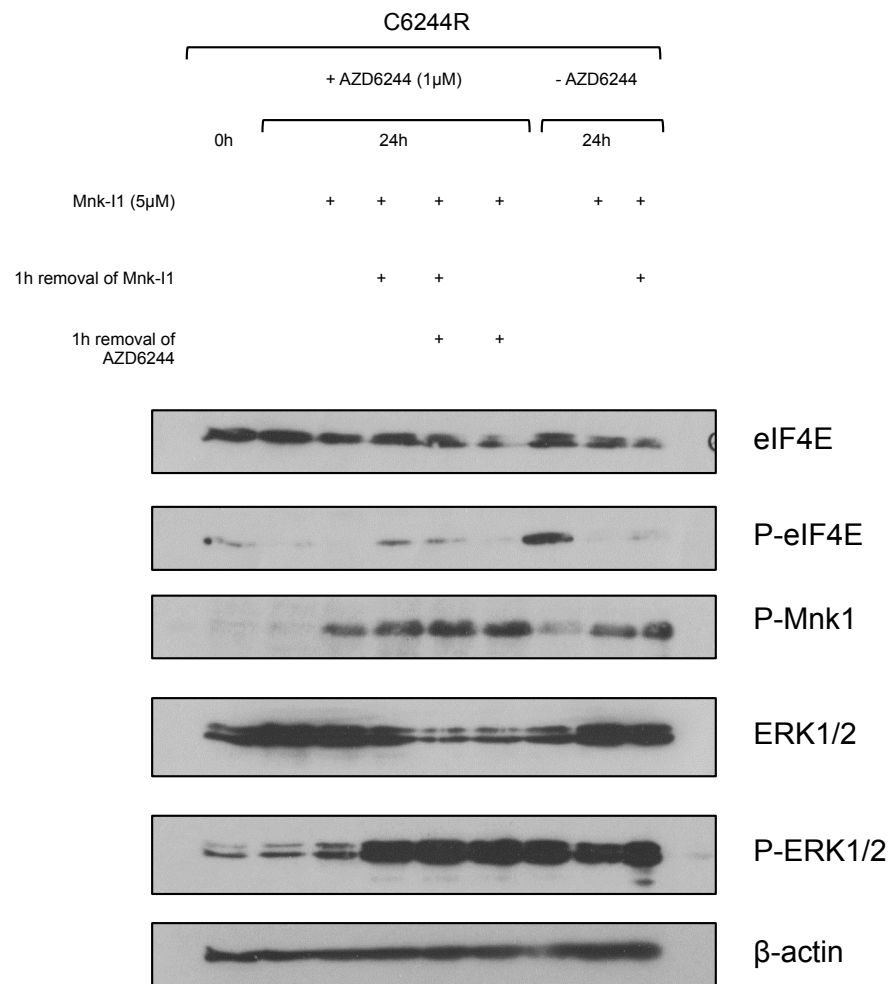
**B**

Figure 6.5 The Mnk-I1 dependent increase in P-Mnk1 persists for at least one hour after the removal of Mnk-I1.

(A) Western blot showing the effect of treating COLO205 cells with Mnk-I1 for 24 hours then removing Mnk-I1 for 1 hour on phosphorylation of eIF4E, ERK1/2 phosphorylation and Mnk1.

(B) Western blot showing the effect of treating C6244R cells with Mnk-I1 for 24 hours, in the presence and absence of AZD6244 (1 $\mu$ M), then removing Mnk-I1 and/or AZD6244 for 1 hour on phosphorylation of eIF4E, ERK1/2 phosphorylation and Mnk1.

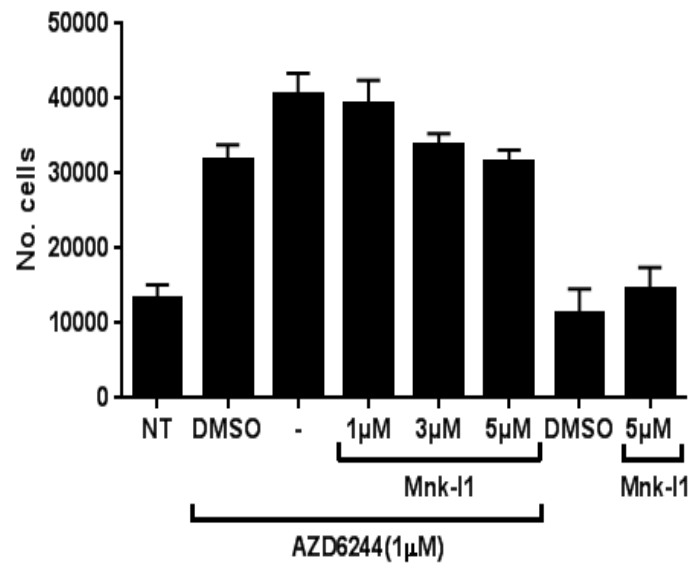
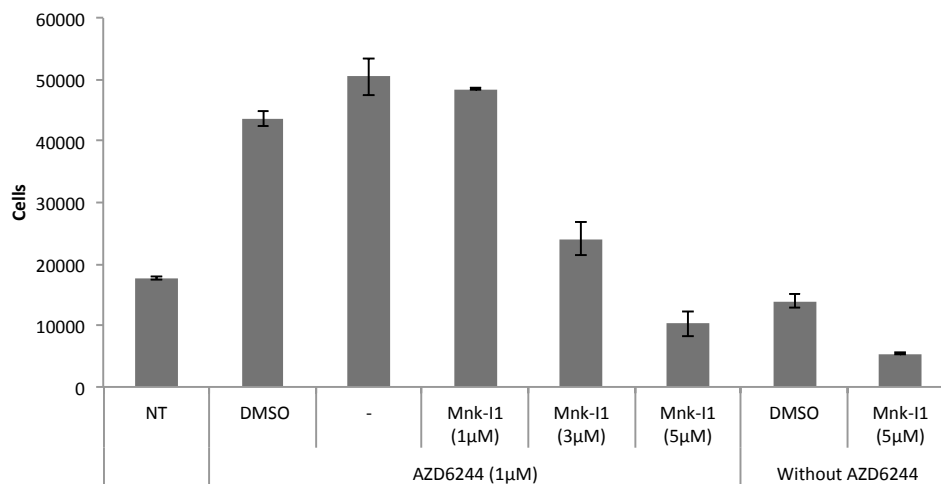
**A****Proliferation assay (24h): COLO205 AZD6244-resistant****B****Proliferation assay for COLO205-AZD6244R cells: 48h, in the presence of serum**

Figure 6.6 Mnk-I1 inhibits C6244R cell number over 48 hours, but does not have any effect over 24 hours.

(A) Proliferation assay to show the effects of 24-hour treatments of Mnk-I1 alone and in combination with AZD6244 (1µM) on C6244R cell number.

(B) Proliferation assay to show the effects of 48-hour treatments of Mnk-I1 alone and in combination with AZD6244 (1µM) on C6244R cell number.

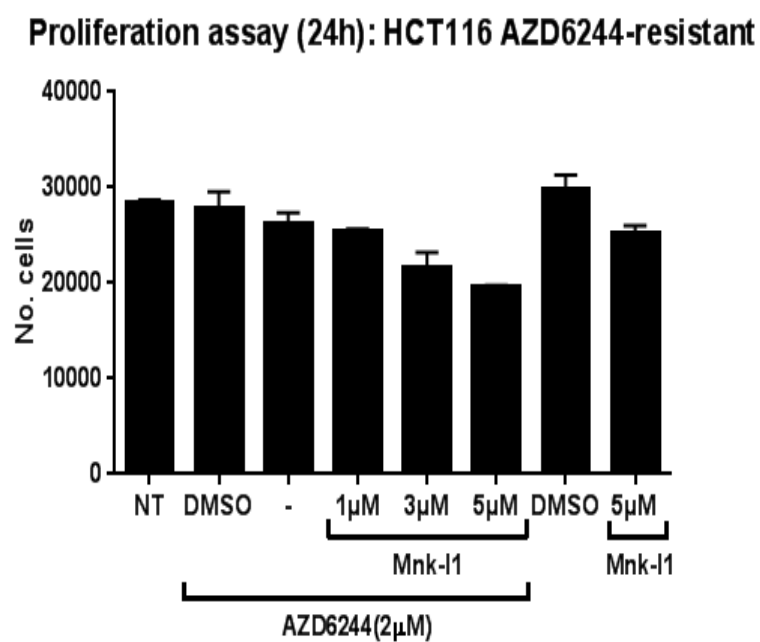
**A**

Figure 6.7 Mnk-I1, in combination with AZD6244 (2µM), has a small effect on inhibiting H6244R cell number over 24 hours.

(A) Proliferation assay to show the effects of 24-hour treatments of Mnk-I1 alone and in combination with AZD6244 (1µM) on H6244R cell number.

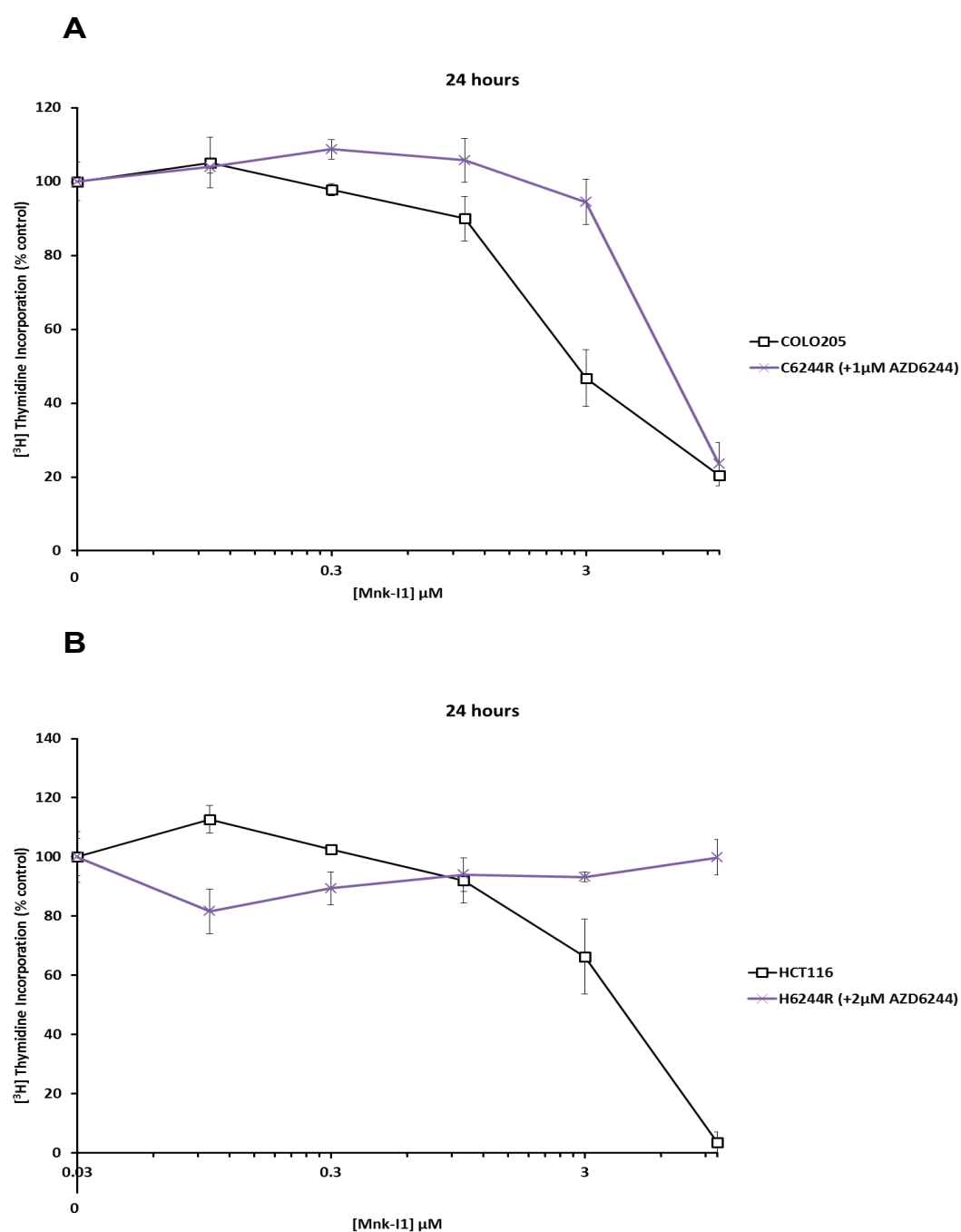


Figure 6.8 C6244R cells are sensitive to Mnk-I1, but H6244R are insensitive to Mnk-I1

(A)  $^3\text{H}$  Thymidine incorporation assay showing the effect of Mnk-I1 on COLO205 proliferation and a combination of Mnk-I1 and AZD6244 on C6244R proliferation.

(B)  $^3\text{H}$  Thymidine incorporation assay showing the effect of Mnk-I1 on HCT116 proliferation and a combination of Mnk-I1 and AZD6244 on H6244R proliferation.



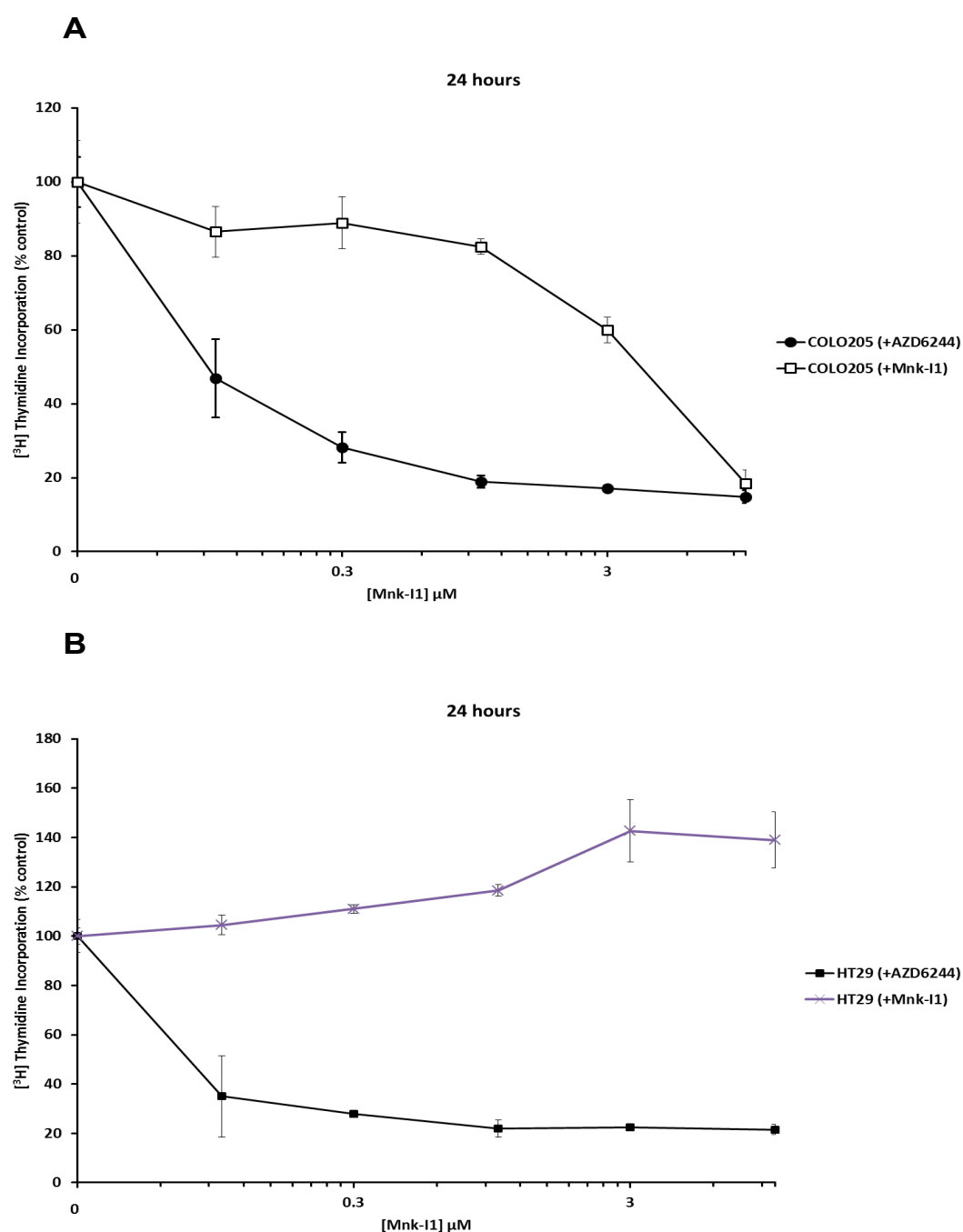


Figure 6.9 COLO205 cells are sensitive to Mnk-I1 and AZD6244; HT29 cells are sensitive to AZD6244, but not Mnk-I1.

(A)  $^3\text{H}$  Thymidine incorporation assay showing the effect of Mnk-I1 and AZD6244 on COLO205 proliferation.

(B)  $^3\text{H}$  Thymidine incorporation assay showing the effect of Mnk-I1 and AZD6244 on HT29 proliferation.

The next step was to see whether Mnk-I1 would have any effect on cell proliferation. Mnk-I1, above 3 $\mu$ M and in combination with AZD6244 (1 $\mu$ M), appeared to significantly inhibit the increase in cell number of C6244R cells over 48 hours (**Figure 6.6B**). Mnk-I1 (5 $\mu$ M) alone also appeared to inhibit C6244R cell number over 48 hours (**Figure 6.6B**). Over 24 hours, Mnk-I1, in combination with AZD6244 (1 $\mu$ M) and alone, didn't appear to have any effect on cell number beyond that of the DMSO vehicle in C6244R cells (**Figure 6.6A**). In H6244R cells, over 24 hours, there was a small effect of Mnk-I1 in combination with AZD6244 (2 $\mu$ M) (**Figure 6.7A**).

Thymidine incorporation assays, which measure the incorporation of radioactive thymidine into DNA, were also used to measure the effects of a range of Mnk-I1 doses on the rate of proliferation in the AZD6244 resistant cell lines. Interestingly, Mnk-I1 - in combination with AZD6244 (1 $\mu$ M) - appeared to have an inhibitory effect ( $IC_{50}$  = ~10 $\mu$ M) in the C6244R cells, but not the H6244R cells (**Figures 6.8A & 6.8B**). Both the parental COLO205 ( $IC_{50}$  = ~3 $\mu$ M) and HCT116 ( $IC_{50}$  = ~5 $\mu$ M) were sensitive to Mnk-I1 (**Figures 6.8A & 6.8B**). Interestingly, whilst the proliferation of HT29 cells was inhibited by AZD6244 (1 $\mu$ M) they appeared to be insensitive to Mnk-I1 across the concentration range tested (**Figures 6.9A & 6.9B**).

### 6.3 Discussion

The results presented in this chapter show that whilst Mnk-I1 appears to be effective at inhibiting eIF4E phosphorylation in AZD6244-resistant cell lines, there is not a consistent effect on inhibiting proliferation in these cell lines. Where Mnk-I1 is effective at inhibiting proliferation, in the C6244R cell line, the  $IC_{50}$  is about  $3\mu M$ , which is quite high.

Whilst there is increasing interest in developing inhibitors that act further downstream the RAS-RAF-ERK-MEK pathway, it could be the case that the Mnks are too far downstream. Several ERK inhibitors have been brought into clinical trials for their efficacy in overcoming resistance to MEK and RAF inhibitors, which is driven by resumption of ERK activity. Given that the Mnks are downstream of ERK, it is reasonable to think that an Mnk inhibitor would similarly block some of this activity. However, the problem is whilst indeed an Mnk inhibitor might block the effects of increased ERK signaling on the translation of a subset of mRNAs, ERK has many other substrates out-with the Mnks. Logic would suggest that these would be unaffected by an Mnk inhibitor, unless of course the Mnks turn out to be involved in a feedback loop acting upstream of ERK in which case it might actually lead to increased ERK activity. The Mnk inhibitor tested in this chapter, Mnk-I1, seems to be effective in stopping Mnk activity – indicated by reduced eIF4E phosphorylation levels – in the AZD6244 resistant cell lines. It is perhaps not surprising, given that the PKB pathway and others have been shown to be overactive in KRAS<sup>13D</sup>-amplified cells, that Mnk-I1 had no effect on the HCT116-AZD6244R cells (Little et al., 2011). The diminutive effect of Mnk-I1 on proliferation in the COLO205-AZD6244R cells, which have a BRAF<sup>V600E</sup>

amplification, could be explained by the reason given above: ERK is still activating its other substrates – such as AP1, which increases transcription of cyclin D1 - many of which encourage progression through the cell cycle (Sherr and McCormick, 2002).

An interesting and unexpected finding was that Mnk-I1 appears to increase P-Mnk1 in both the parental and AZD6244 resistant cell lines. Whilst it wasn't possible to interrogate this effect during this project, a few clues are provided to suggest that this effect is independent of ERK and upstream activity. A possible explanation, which has been observed in the context of Akt inhibitors, is that Mnk-I1 could be having an allosteric effect on Mnk1, such that it prevents Mnk phosphatases from gaining access to the Thr197/202 phosphorylation site (Lin et al., 2012). A simpler explanation could be that Mnk-I1 is causing a conformational change, which prevents Mnk1 from phosphorylating eIF4E, but doesn't stop ERK1/2 from phosphorylating Mnk1.

This chapter shows that Mnk inhibitors might have some effect on inhibiting the proliferation of cancer cell lines that have acquired resistance via a BRAF<sup>V600E</sup> amplification; however, it would seem, based on this evidence, unlikely that this effect would translate into an effect in the clinic. Further work is required, before the Mnks can be ruled out as a possible target for overcoming acquired resistance to AZD6244.

## 7. Final discussion

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The Mnks seem like an ideal cancer target. Knocking out both the Mnks appears to reduce tumourigenesis in mice, but has no effect on normal growth and development (Ueda et al., 2010b). On this basis, if a selective Mnk inhibitor could be produced it might also selectively inhibit cancer growth. This has been the trouble. Until very recently, there haven't been any selective and potent inhibitors of the Mnks (Beggs et al., 2015) (Sunita et al., 2015) (Teo et al., 2015a, Teo et al., 2015b). Not only might selective Mnk inhibitors be useful as therapeutics, but also as research tools for helping to elucidate exactly what role the Mnks are playing in cancer cells.

Numerous studies have linked the Mnks to cell proliferation and pro-survival, both hallmarks of cancer: however, a lot of this work has been based on the use of Mnk inhibitors, such as CGP57380 and cercosporamide, which inhibit other kinases with similar potency (Bain et al., 2007, Bianchini et al., 2008a, Grzmil et al., 2011) (Robichaud et al., 2014, Konicek et al., 2011). Presented here, are the results of experiments carried out with a novel and, importantly, selective Mnk inhibitor Mnk-I1 (patent WO 2011/104340 A1) (Beggs et al., 2015). The key findings are also supported with the results from experiments using MEFs that have had the Mnks genetically knocked out. The results provide little evidence to support a role for the Mnks in cell proliferation and pro-survival; inhibiting the Mnks may have a partial effect in reducing cell proliferation in some BRAF driven MEK inhibitor resistant cell lines, but the results are far from conclusive. What the results do show, however, is that the Mnks appear to play a key role in another important process in tumourigenesis: cancer cell migration. This finding is supported by several other publications, which were published whilst the data presented here was

being prepared for publication (Beggs et al., 2015, Robichaud et al., 2014, Ramalingam et al., 2014).

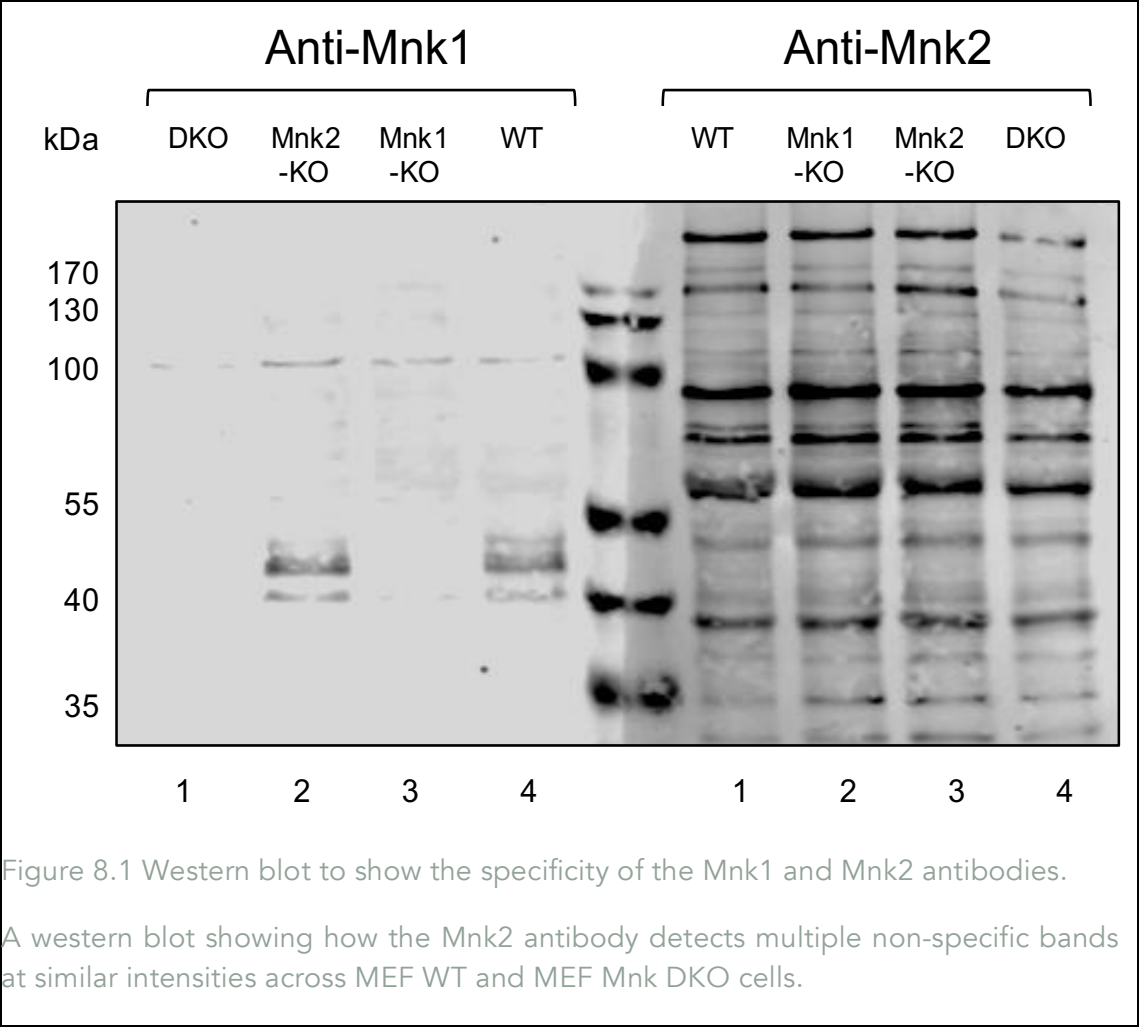
Cancer cell migration is an important part of the process of metastasis, which is responsible for 90% of deaths caused by cancer (Chaffer and Weinberg, 2011). Based on the data presented here and in other publications, showing that Mnk kinase activity is important for cancer cell migration, it might seem that the Mnks would be an attractive target for designing anti-metastatic cancer drugs. Whilst the Mnks could well be an effective anti-metastatic target, the problem is – as the drug companies would probably argue – that metastasis occurs too late in tumourigenesis for it to provide a basis for the design of effective treatments. If it was possible to design a drug to prevent a patients cancer from metastasising the patient would have to take this drug for a very long time, if not indefinitely. Any drug candidates would have to have very low toxicity to avoid any cumulative effect on the patient. Clinical trials would have to be designed to test the effects of the long treatment, which would make them very costly and difficult to manage. The lengthy trials would also reduce the amount of time a drug company has to recoup its investment in R&D (Research and Development) before the patent on such a drug expires.

The results presented in this chapter show that not only do the Mnks appear to play a role in cancer cell migration, but cell migration as a whole. Given that cell migration is an important process in wound healing, immunity and development the Mnks could be useful targets for other diseases, aside from cancer. Further research would be required to explore just how widely the Mnks are involved in cell migration.

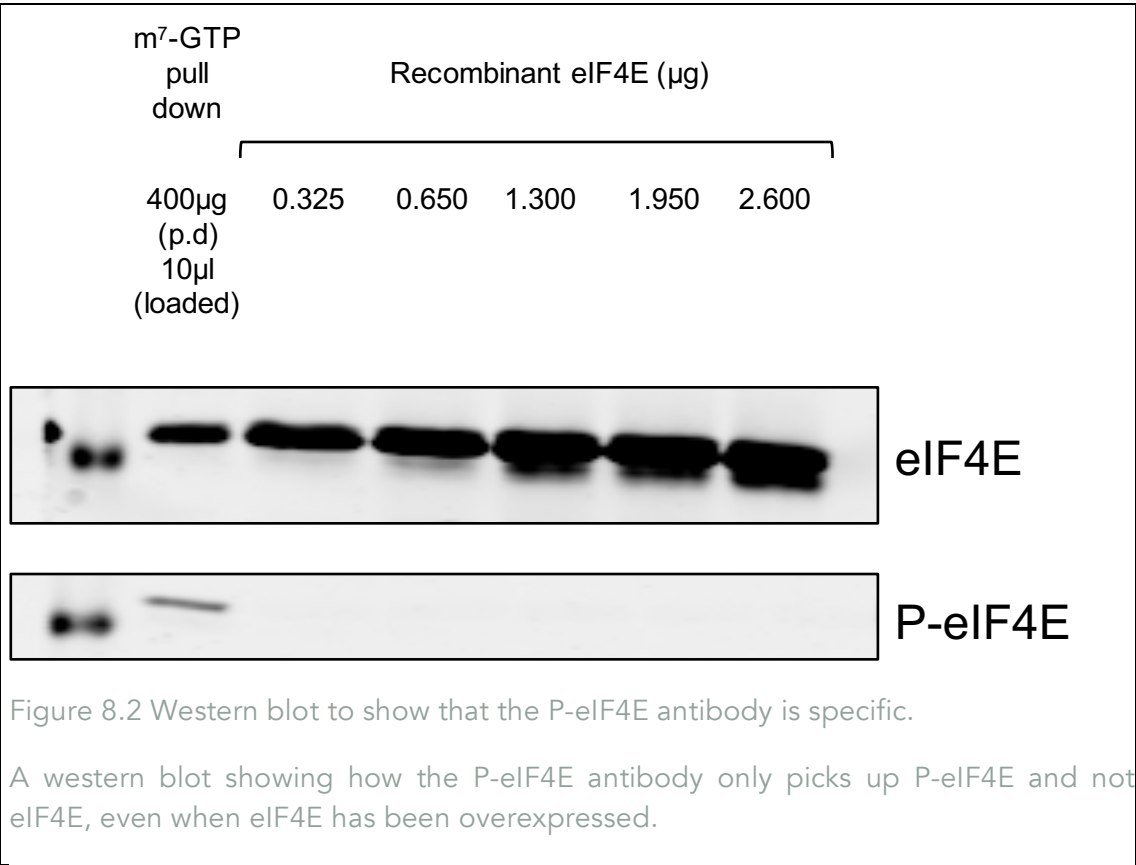
It is not possible to rule out a role for the Mnks in cell proliferation and survival on the basis of the results presented in this thesis. The reason is that a large part of the project was focused on establishing the role of the Mnks in cell migration. This meant that the proliferation assays were carried out over 24 hours: to make sure that an effect of the Mnk inhibitors, or genetic knockout on proliferation was not distorting the results of the migration assays. The proliferation assays would need to be carried out over longer time periods (e.g. 72 hours) before it can be certain whether inhibiting or knocking out the Mnks has an effect on cell proliferation. For instance, a recent study has shown that a 72-hour treatment with a type III Mnk inhibitor - believed to be more specific than earlier type I and type II inhibitors - had an anti-proliferative effect on a leukaemia cell line (Sunita et al., 2015).

In conclusion, the Mnks are still worth investigating as an anti-cancer target. More work is required to work out exactly how Mnk dependent phosphorylation of eIF4E appears to affect the translation of particular mRNAs involved in cancer. There could be additional mechanisms other than that involving the CYFIP1-FMRP complex. The new type III Mnk inhibitors should help elucidate other roles for the Mnks. More specific inhibitors may help uncover additional mRNAs whose translation may be influenced by the Mnks and perhaps additional protein targets. The new inhibitors could also be used to validate some of the earlier studies that showed, using non-selective Mnk inhibitors such as CGP57380, how inhibiting the Mnks has an anti-proliferative effect in cancer cells.

# 8. Appendix







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