UNIVERSITY OF ADELAIDE

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Novel Roles of the MAP kinase-interacting kinases

Thesis for the degree of Doctor of Philosophy

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Novel roles of the MAP kinase-interacting kinases (MNKs)

by Shuye Tian

The MAP kinase-interacting kinases (MNKs) are ubiquitously expressed in mammalian cells. They are activated through MAP kinase pathways and can phosphorylate the eukaryotic translation initiation factor 4E (eIF4E) at a single site. eIF4E plays a key role in protein synthesis and its control. eIF4E and its phosphorylation play important roles in cancer and tumorigenesis. As MNKs play important roles in several diseases, but are not essential to animal development, they may be good targets for cancer therapy.

Our recent studies show that the MNKs contribute to the migration of cancer cells. The cytoplasmic FMRP-interacting protein 1 (CYFIP1) is reported as an eIF4E binding partner, and suppresses translation by binding with fragile X mental retardation protein (FMRP) and eIF4E. Our research demonstrates that inhibition of MNKs can also inhibit release of CYFIP1 from eIF4E, which may repress translation of certain mRNAs related to cell migration, including matrix metalloproteinases (MMP3, MMP9) and Vimentin (which serves as a marker of the epithelial-mesenchymal transition). Also, the MNKs were found to be involved in regulating the expression and phosphorylation of N-Myc Downstream Regulated 1 (NDRG1), a protein which is a well-known metastasis suppressor in breast cancers.

In addition to functions in cell migration, our previous research shows that MNK2 affects fat mass of mice on a high fat diet. In addition, MNK2KO mice fed a high fat diet are protected from adipose tissue inflammation. Consequently, we focused on MNK functions in inflammation, and this thesis describes the effect by MNK inhibition on the morphology and other features of RAW macrophage cells.

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Abbreviations

4E-BP	eukaryotic translation initiation factor 4E-binding protein
AP-1	activator protein 1
ARE	the AU-rich regulatory element
AT	Advanced tumour
ATP	adenosine triphosphate
BDNF	the brain-derived neurotropic factor
BMDM	bone marrow-derived macrophage
BSA	bovine serum albumin
CaMK-II	calmodulin kinase 2 phosphorylation
CCR2	C-C chemokine receptor type 2
CD	Cluster of Differentiation
CK-II	a casein kinase II
cPLA2	cytoplasmic phospholipase A2
CYFIP1	the cytoplasmic FMRP-interacting protein 1
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulphoxide
Dp44mT	the di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone
DpC	di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbazone
ECM	extracellular matrix
eEF	eukarytotic elongation factor
EGFR	epidermal growth factor receptor
eIF	eukaryotic initiation factor
EMT	the epithelial-to-mesenchymal transition
eRF	eukaryotic release factor
ERK	extracellular signal-regulated kinase
ET	Early tumour
F4/80	EGF-like module-containing mucin-like hormone receptor-like 1
FAK	Focal adhesion kinase
FBS	foetal bovine serum
FMRP	the fragile X mental retardation protein
FXS	the fragile X syndrome
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	guanosine diphosphate
GSK3	glycogen synthase kinase 3
GTP	guanosine triphosphate
h	hours
H/E	Hyperplasia-epithelial neoplasia
HBSS	Hanks' Balanced Salt Solution
HEK 293	human Embryonic kidney 293

HER	human epidermal growth factor receptor
HFD	high-fat diet
HIF1a	hypoxia-inducible factor 1 alpha
hnRNPA1	heterogeneous nuclear RNA-binding protein A1
IFN	interferon
IL	interleukin
IRES	ribosome entry site
IκBα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
LPS	Lipopolysaccharide
LRP6	LDL receptor related protein 6
LT	Late tumour
m7GTP	7-methyl-GTP
MAPK	mitogen-ativated protein kinase
MCF 7	Michigan Cancer Foundation-7
MCP-1	monocyte chemo-attractant protein-1
MEF	mouse embryonic fibroblast
Min	minutes
MMP	the matrix metalloproteinase
MNK	the MAP kinase interacting kinase
mRNA	messenger ribonucleic acids
MT	Medium tumour
mTORC1	the mammalian target of rapamycin complex 1
NCKAP1	NCK-associated protein 1
NDRG1	N-Myc Downstream Regulated 1
NEDD4	neural precursor cell expressed developmentally down-regulated protein 4
NES	nuclear export signal
NF-κB	nuclear factor-κB
NLS	nuclear localization signal
NT	no treatment
ODC	ornithine decarboxylase
p38	p38 mitogen-activated protein kinase
p53	Tumor suppressor protein that protects from DNA damage
PABP	poly (A)-binding protein
PBS	phosphate-buffered saline
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP3	phosphatidylinositol-3-trisphosphate
PKB	protein kinase B
PKC	protein kinase C
PSF	the PTB (polypyrimidine tract-binding protein)-associated splicing factor
PTEN	phosphatase and tensin homolog
qPCR	quantitative real time polymerase chain reaction
Rac	Ras-related C3 botulinum toxin substrate
rRNA	ribosomal RNA
S6	small subunit ribosomal protein S6

S6K	p70 ribosomal protein S6 kinase
SCC25	human oral squamous carcinoma cell
SG	stress granules
SGK1	serum/glucocorticoid regulated kinase 1
siRNA	small interfering RNA
SMAD	Contraction of Sma and Mad (Mothers against decapentaplegic)
Spry2	Sprouty homolog 2
Src	proto-oncogene tyrosine-protein kinase
TGF-β	transforming growth factor beta
TLR	Toll like receptor
TNBC	triple-negative breast cancer
TNF-α	the tumour necrosis factor alpha
TOP	terminal oligopyrimidine
tRNA	aminoacyl-transfer RNA
UTR	untranslated region
VEGF	vascular endothelial growth factor
WRC	the WAVE Regulatory Complex

Chapter 1

Introduction

1.1 Introduction to translational control

Proteins make up the largest proportion of biological macromolecules in the human body. They not only give structure to cells and tissues, but also perform a wide range of specialized tasks, such as catalysing reactions and transmitting signals. Therefore, protein synthesis has to be a critical process. Translational control is the regulation of proteins synthesised from their messenger ribonucleic acids (mRNAs). During this process, mRNA — produced by transcription from DNA — is decoded by specialized intracellular organelles called ribosomes to produce amino acid chains, or polypeptides. Control of mRNA translation allows regulation of the synthesis of specific proteins, which is of essential importance to cell growth, proliferation and development [1].

A large number of proteins, termed as translation factors, are involved in the complicated process of mRNA translation. The activity or expression levels of these proteins can not only control overall translational rates, but also regulate the translation of specific mRNAs [1, 2].

Translation can be divided into three stages: initiation, elongation and termination.

1.1.1 Translation initiation

In the stage of initiation, the ribosomes assemble around the target mRNA. In eukaryotes, the small ribosomal subunit 40S binds indirectly (via translation initiation factors) to structure at the 5’ end of mRNA. According to the different structure that 40S binds to, two modes of translation initiation have been described below.

1.1.1.1 Cap-dependent initiation

Cap-dependent initiation is responsible of mediating the translation of the majority of mRNA transcripts within a eukaryotic cell. Most of mRNAs have a cap-structure at their 5'-end, which consists of a guanine nucleotide connected to mRNA via an unusual 5′ to 5′ triphosphate linkage. This guanosine is methylated on the 7 position directly after capping *in vivo* by a methyltransferase [3]. This cap structure is bound by eIF4E which will, by interacting with other translation factors, will recruit the small ribosomal complex to the 5’-end of the mRNA, following by the scanning for the start codon.[4-6]

Translation initiation is a cellular process of great complexity involving a number of non-ribosomal proteins, which are termed as eukaryotic initiation factors (eIFs) (a brief summary is provided in Table 1.1).

Table 1.1 Eukaryotic initiation factors

Initiation factor	Subunits	Functions
eIF1	Monomer	Stimulates the formation of the 48S pre-initiation complex and drives the movement of the ribosome subunit during scanning
eIF1A	Monomer	Stabilises Met-tRNAi to mRNA, and enhances the activity of eIF5B
eIF2	α, β, γ	Recruits Met-tRNAi to the 43S pre-initiation complex
eIF2B	α, β, γ, δ, ε	Guanine nucleotide (GDP:GTP) exchange factor for eIF2
eIF3	multimeric	Dissociation factor of the 40S and 60S ribosomal subunits. Binds to eIF4G to from the 48S pre-initiation complex.
eIF4A	Monomer	ATP-dependent RNA helicase. Binds to eIF4G to be part of the eIF4F complex. Melts the secondary structure of the 5’ UTR of an mRNA.
eIF4B	Monomer	Enhances the helicase activity of eIF4A
eIF4E	Monomer	Binds to the cap structure of mRNA
eIF4G (I and II)	Monomer	Scaffolding protein which bridges eIF4E and eIF4A to form eIF4F complex
eIF4H	Monomer	Stimulates the helicase activity of the eIF4F complex
eIF5	Monomer	Induces the hydrolysis of eIF2-bound GTP and releases eIF2-GDP from the 48S pre-initiation complex.
eIF5B	Monomer	Acts along with eIF5 to induce the binding of 60S subunit to the 48S pre-initiation complex

At first stage of initiation, eukaryotic initiation factor 3 (eIF3) first binds to the 40S ribosome subunit and blocks the association of the 40S and the 60S subunits, to provide a supply of 'free' subunits. Once the 80S ribosome dissociates to form two subunits, the 40S subunit is free to bind to eIF1A and the ternary complex (eIF2-GTP- Met-tRNAi) to yield the 43S pre-initiation complex [7]. When bound to GDP, eIF2 cannot bind to the Met-tRNAi. The guanine nucleotide exchange factor eIF2B is required to recycle eIF2-GDP back to active eIF2-GTP [8]. eIF1A and eIF1 are required for mRNA binding and facilitate the scanning of the 43S complex along the 5' UTR [9]. eIF2 is a heterotrimer with three subunits (α, β and γ). The α subunit of eIF2 is implicated in AUG recognition. It can be phosphorylated at Ser51 by eIF2 kinases, which are activated in response to cellular stresses. The β subunit promotes the binding of eIF2 to the initiator tRNA, by inducing the GTPase activity of eIF2γ [8, 10].

After the formation of the 43S pre-initiation complex, it will be recruited to the 5' end of the mRNA. The essential factor mediated this process is the eIF4F complex (Figure 1.1). eIF4F is a heterotrimeric complex, which is composed of the RNA helicase eIF4A, the cap-binding protein eIF4E, and the scaffold protein eIF4G [2, 11, 12]. eIF4A belongs to the DEA (D/H)-box RNA helicase family. During initiation, eIF4A unwinds secondary structure in the 5' UTR of mRNAs, and promotes scanning by the 40S ribosomal subunit for the initiation codon [13]. eIF4A itself has weak, poor processive helicase activity, but this can be greatly enhanced upon binding to eIF4G and eIF4B [13]. After binding to eIF4A, eIF4B activates eIF4A and interacts with the 18S ribosomal RNA (rRNA) to promote the recruitment of the 40S ribosomal subunit to the single-strand region of the mRNA [6]. eIF4E specifically binds to the cap structure in the 5' end of mRNAs and thereby initiate cap-dependent translation. eIF4G has two binding sites for eIF4A and one for eIF4E. It can bind both eIF4E and eIF4A and thereby forms the eIF4F complex. Mammalian target of rapamycin complex 1 (mTORC1) signalling pathway is also involved in this process. It inhibits activity of eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), which shares similar binding sites to eIF4G and is therefore competitive against eIF4G to bind to eIF4E, resulting in the inhibition of eIF4F assembly [14]. At the same time, eIF4G binds to eIF3 to form the 48S pre-initiation complex [2, 15-17]. As a result, the 40S ribosomal subunit is recruited to the cap structure of the mRNA.

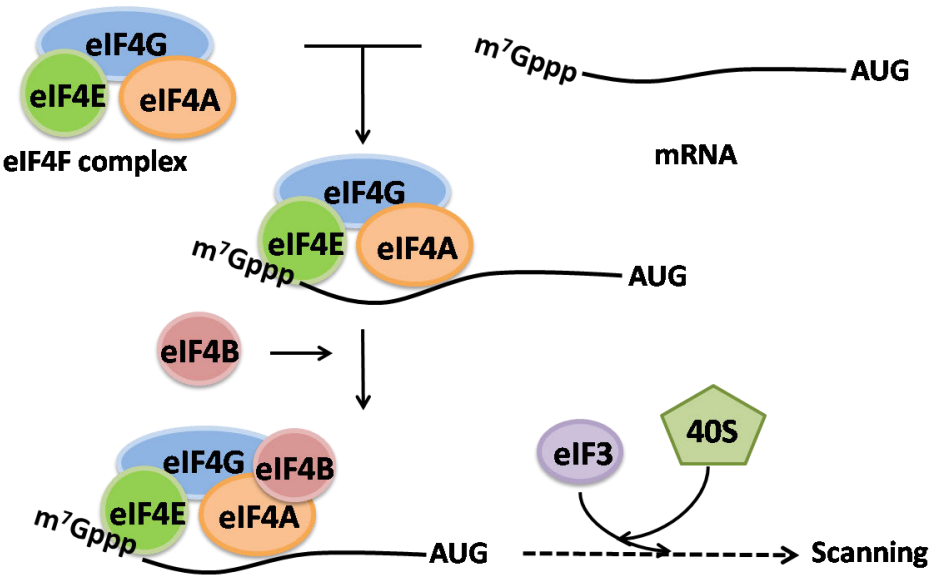


Figure 1.1 mRNA recruitment during translation initiation

Once the 40S is recruited to the 5' end of the mRNA, the 43S pre-initiation complex starts to scan along the 5'-UTR to search for the start codon [2]. eIF4B and eIF4H enhance helicase activity of the eIF4F complex, which helps the melting of any secondary structure of the mRNA in this process. During this step, eIF1A binds to the A site of the ribosome to stabilise the binding of the 43S pre-initiation complex to the mRNA at the P site, while eIF1 promotes the formation of the 48S pre-initiation complex [9].

The start codon (AUG) stably binds to the ribosome through the RNA-RNA interaction and stops the ribosome scanning. eIF5, a GTPase activating protein, induces the hydrolysis of eIF2-bound GTP and releases eIF2-GDP from the 48S complex [18]. The release of eIF2-GDP also triggers the disassembly of the 48S complex from the AUG triplets and subsequently leads to the dissociation of eIF3 [19]. Once released, the inactive eIF2-GDP is recycled to produce active eIF2-GTP by the guanine nucleotide exchange factor eIF2B, a process which can be blocked upon the phosphorylation of eIF2 [8]. Interestingly, the presence of eIF1 in the 43S complex inhibits eIF5-induced GTP hydrolysis, so that codon-anticodon base pairing in the 48S complex will keep binding tightly with hydrolysis of eIF2-bound GTP [19]. Finally, eIF5B regulates the association of 60S and the 40S ribosomal subunits to form a protein synthesis-competent 80S ribosome [20, 21], while eIF2-GTP is active and ready to bind to the Met-tRNAi to start a new round of translation initiation.

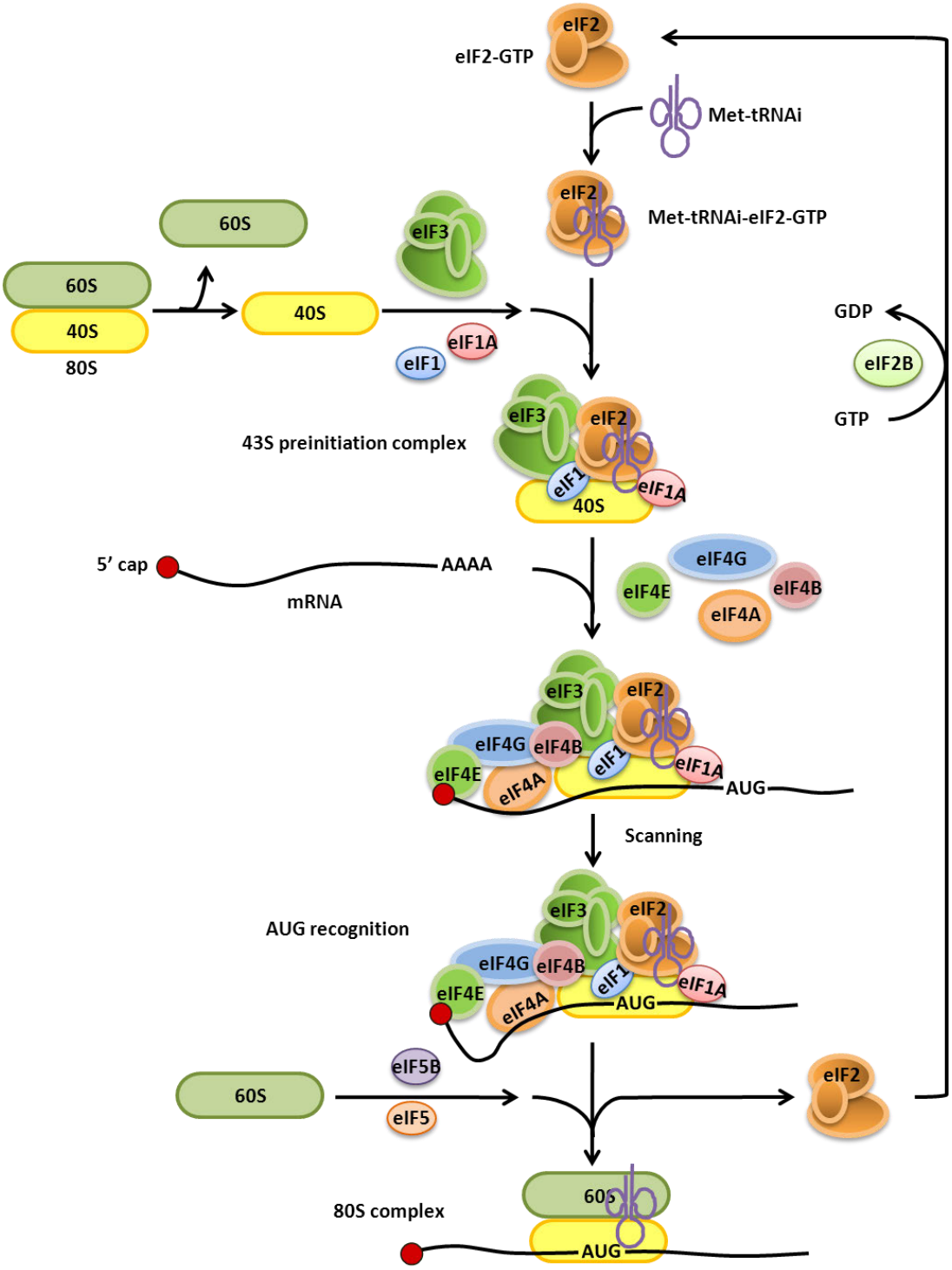


Figure 1.2 Mechanism of cap-dependent initiation in eukaryotes

The scanning mechanism described above mediates initiation of translation of most of the mRNAs, but in some cases, mRNAs can also go through other special cap-dependent initiation mechanisms, such as leaky scanning [22], ribosomal shunting [23],

and termination-reinitiation [24]. These alternative ways allow the initiation complex to overcome some limitation imposed by the 5'UTR. Recently, eIF3d was reported as a cap binding protein, which can recognize the cap-structure on eIF3-dependent mRNAs such as the cell proliferation regulator c-Jun [25, 26]. This mechanism allows cells to control protein synthesis when eIF4E is inactivated.

1.1.1.2 Cap-independent initiation

Although cap-dependent initiation is considered as the major way of mRNA translation in eukaryotes, it cannot account for the translation of all prokaryotic, eukaryotic, and especially viral RNAs.

Many viruses, including several important pathogens to human and livestock, do not require the methylguanosine cap motif attached to the 5' terminus of their RNAs, but they rather use an alternative, cap-independent mechanism that substitutes the RNA structure for the cap for the production of their proteins. This mechanism was first reported in 1988, when both poliovirus (PV) and encephalomyocarditis virus (EMCV) were found to use cap-independent initiation to translate their RNAs [27, 28]. During cap-independent initiation, the ribosome is recruited to the mRNA by special sequences in the 5'-UTR of their mRNA that form distinct secondary or tertiary structures. These special sequences are usually referred to as internal ribosome entry sites (IRES) [27, 29, 30].

Since their discovery, more than 80 IRES elements have been identified in eukaryotic mRNAs, including in yeast, *Drosophila*, and mammals [31, 32], making this cap-independent initiation an important part of mRNA translation and cell process regulation in eukaryotes. Many of these IRES elements are located in mRNAs encoding proteins that protect cells from stress, when cap-dependent translation is compromised [33, 34]. IRES-mediated initiation is also reported to be involved in cell development, differentiation, cell cycle progression, cell growth, and cell death [33-36].

1.1.1.3 Special structured mRNAs

Apart from the regulation of global mRNA translation initiation, there are several selected groups of mRNAs of which translation can be initiate through special mechanisms [37].

Translation of some mRNAs, such as cyclins [38], VEGF [39] and ornithine decarboxylase (ODC) [40], is more dependent on the levels of eIF4E in cells. Unlike normal housekeeping genes, these 'eIF4E-sensitive' mRNAs contain long and structured 5'UTR [41, 42], which makes them more dependent on the unwinding activity of eIF4A within the eIF4F complex [43]. The mammalian target of rapamycin complex 1 (mTORC1) is considered to be particularly important for the translation of these 'eIF4E-sensitive' mRNAs. In addition to its control of eIF4F assembly through 4E-BP1, mTORC1 can also regulate the activity of eIF4A via phosphorylation by the p70 ribosomal protein S6 kinases (S6Ks) [44], which lies downstream of mTORC1 [44]. Some other mRNAs contain a terminal oligopyrimidine (TOP) tract at their 5' end, which consists of a cytosine at the penultimate nucleotide position followed by a stretch of 4–14 pyrimidines [45, 46]. These mRNAs encode for components of the translational apparatus, such as ribosomal proteins and elongation factors [45, 46]. Recent studies showed that the translation of TOP mRNAs is highly sensitive to mTOR inhibitors [47, 48], as well as depending on the regulation of eIF4E by 4E-BPs, In contrast, a study from Miloslavski, et al. also discovered that hypoxia and other stresses suppress the translation of TOP mRNAs independently of the 4E-BPs [49].

1.1.2 Translation elongation

Translation elongation is the process of polymerizing amino acids into a polypeptide chain. Eukaryotic elongation factors are relied on to make sure that the elongation proceeds accurately. Three elongation factors are involved in eukaryotes: eEF1A, eEF1B, and eEF2 [50, 51].

In the presence of GTP, eEF1A is activated and binds to amino acid-containing tRNA molecules (aminoacyl-tRNAs). A ternary complex (eEF1A-aminoacyl-tRNA-GTP) is formed to deliver the aminoacyl-tRNA into a free A site on the ribosome [52]. After the complex delivered to the A site, GTP is hydrolysed and inactive eEF1A dissociates

from the ribosome. The guanine nucleotide exchange factor for eEF1A (eEF1B), which catalyses the release of GDP from eEF1A, is required for its reactivation [53]. On the ribosome, the anticodon of the incoming aminoacyl-tRNA is matched against the mRNA codon in the A site. Occasionally, the non-cognated anticodons may also bind to the mis-matched tRNAs, resulting in the mispaired aminoacyl-tRNAs, which may finally cause a loss of protein structure or function [54].Numerous quality control mechanisms are taking place to prevent this incorrect attachment [55-57]. Generally, after the binding of a non-cognate amino acid to the mis-matched tRNA, GTP hydrolysation will be stopped by eEF1-α, which leads the release of the erroneous anticodons from the ribosome [58, 59]. Once the right aminoacyl-tRNA enters the A site, a peptide bond formed between the aminoacyl group and the petidyl group in the P site. Following the formation of the peptide bond, tRNAs in the P site release its polypeptide chain and a new peptidyl-tRNA is formed in the A site. Then the ribosome moves forward one codon along the mRNA, a step which is facilitated by the activation ofeEF2 [60, 61]. This translocation of ribosome leads to the movement of the empty tRNA from the P site to the E site. Therefore, an empty A site is available for the next new incoming aminoacyl-tRNA.

1.1.3 Translation termination

Translation will be terminated when a ribosome reaches a stop codon (UAG, UAA, or UGA) on an mRNA strand. Two polypeptide chain release factors (eRF1 and eRF3) are required for translation termination in eukaryotic cells [62, 63]. eRF1 has two main functions associated with different domains on its protein respectively [64]. The core domain binds to the ribosome and triggers the hydrolysis of peptidyl-tRNA, so that the peptide chain can be released from the ribosome. The eRF3 binding domain is at the C terminal of eRF1, which allows eRF1 bind to and activate eRF3. The active eRF3 serves as a GTPase to enhance translation termination [65].

1.2 The MAP kinase-interacting kinases (MNKs)

The MAP kinase interacting kinases (MNKs) are ubiquitously expressed in mammalian cells. They lie at the nexus of major intracellular signalling pathways, including the

mammalian target of rapamycin complex 1 (mTOR1) and ERK pathways, which are involved in functions of both normal and cancer cells. The best-known and only fully validated MNK substrate is eukaryotic translation initiation factor 4E (eIF4E), which plays an important role in protein synthesis. [66]

1.2.1 Discovery and structure

The MNKs were first identified in 1997, when MNK1 and MNK2 were found to bind tightly to the growth factor-regulated MAP kinases (MAPKs), ERK1 and ERK2, and can be activated by the ERK or p38 MAP signalling pathways [67, 68].

The MNKs are products of two genes, MKNK1 and MKNK2. In human cells, each gene gives rise, by alternative splicing, to two mRNAs encoding distinct proteins, and these distinct proteins are differed at their C-termini [69, 70]. The longer forms of the human proteins are termed as MNK1a and MNK2a, while the shorter ones are MNK1b and MNK2b. In mice, the only isoforms identified are similar to the longer MNK 'a' forms in human have been identified.

Sequence alignment analysis shows that all four proteins contain catalytic domains, which display approximately 70% sequence identity between MNK1 and MNK2, and an N-terminal polybasic sequence which can interact with eIF4G (Figure 1.3). Both of the longer a-forms contain a MAP kinase-binding region in their C-terminal domain. This motif recruits the classical MAPK, ERK, and stress-activated p38 MAPKs (only binds to MNK1a), so that the MNKs can be phosphorylated at two threonine residues in their activation loops within the catalytic domain by these MAP kinases [71]. The short isoforms, MNK1b and MNK2b lack this domain and can only bind to ERK. So far, it is not clear how the b-forms are regulated.

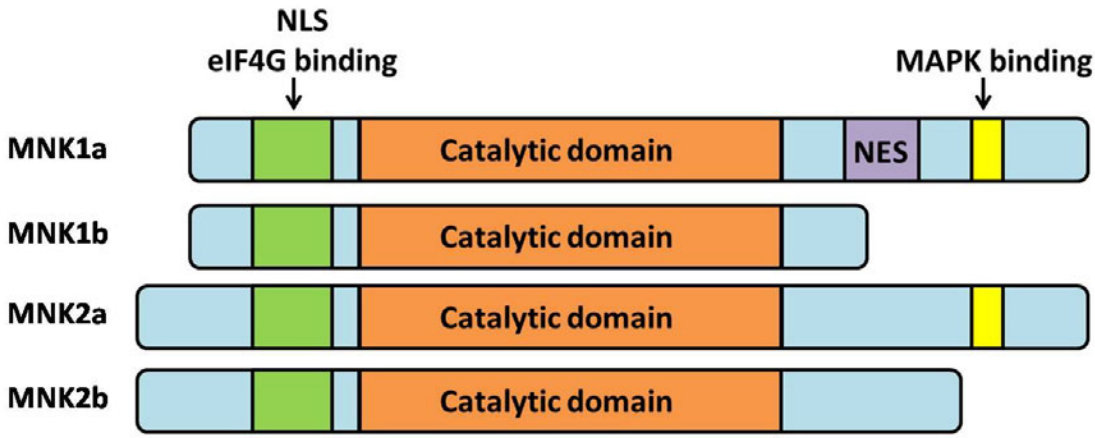


Figure 1.3 The arrangement of the known functional domains of four isoforms of MNKs

NLS: nuclear localization signal; NES: nuclear export signal.

There is an important difference between the longer isoforms MNK1a and MNK2a in terms of their binding to MAP kinases. MNK1a has quite low activity in serum-starved cells and can be activated strongly by ERK or p38 α/β MAPK [68, 72, 73]; while MNK2a shows high basal activity which is only enhanced slightly by activated ERK. This high activity of MNK2a probably reflects its ability to bind phosphorylated, activated ERK [74], allowing it to be continually activated and thus constitutively active. MNK1a cannot bind active ERK; also, while MNK1a binds ERK and p38 MAPK α and β, MNK2a only binds ERK. Also, as they lack the MAP kinase-binding sites, MNK1b and MNK2b show high and low activities, respectively, which are not affected by MAP kinase signalling [75]

1.2.2 Substrates of the MNKs

Several proteins have been reported as MNK substrates. Although they have important biological functions in cells (reviewed in [75]), they have not yet been validated fully as genuine physiological MNK substrates, except eIF4E.

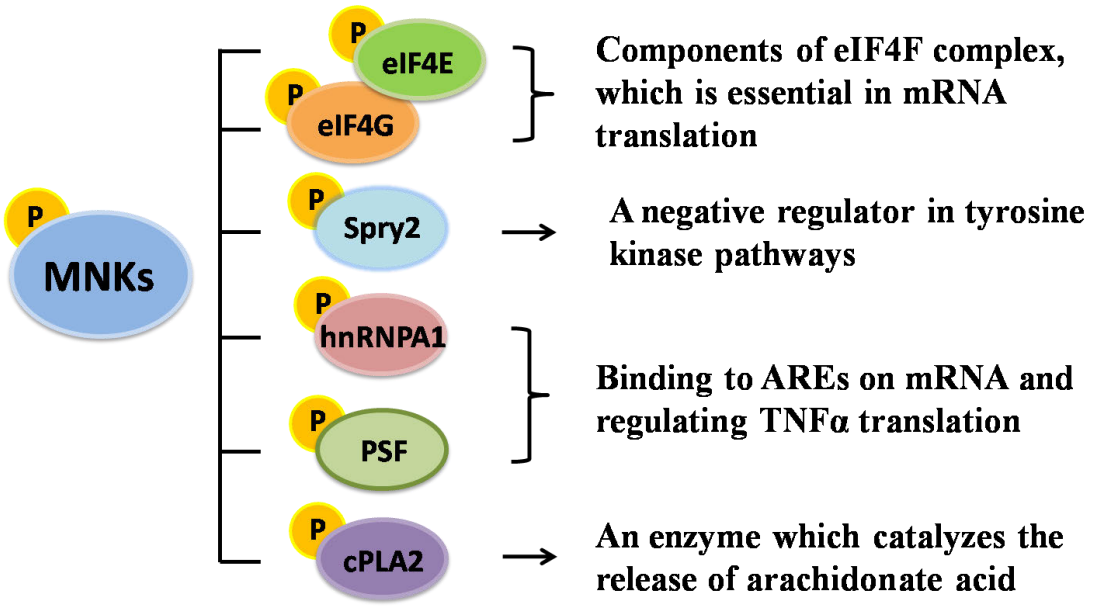
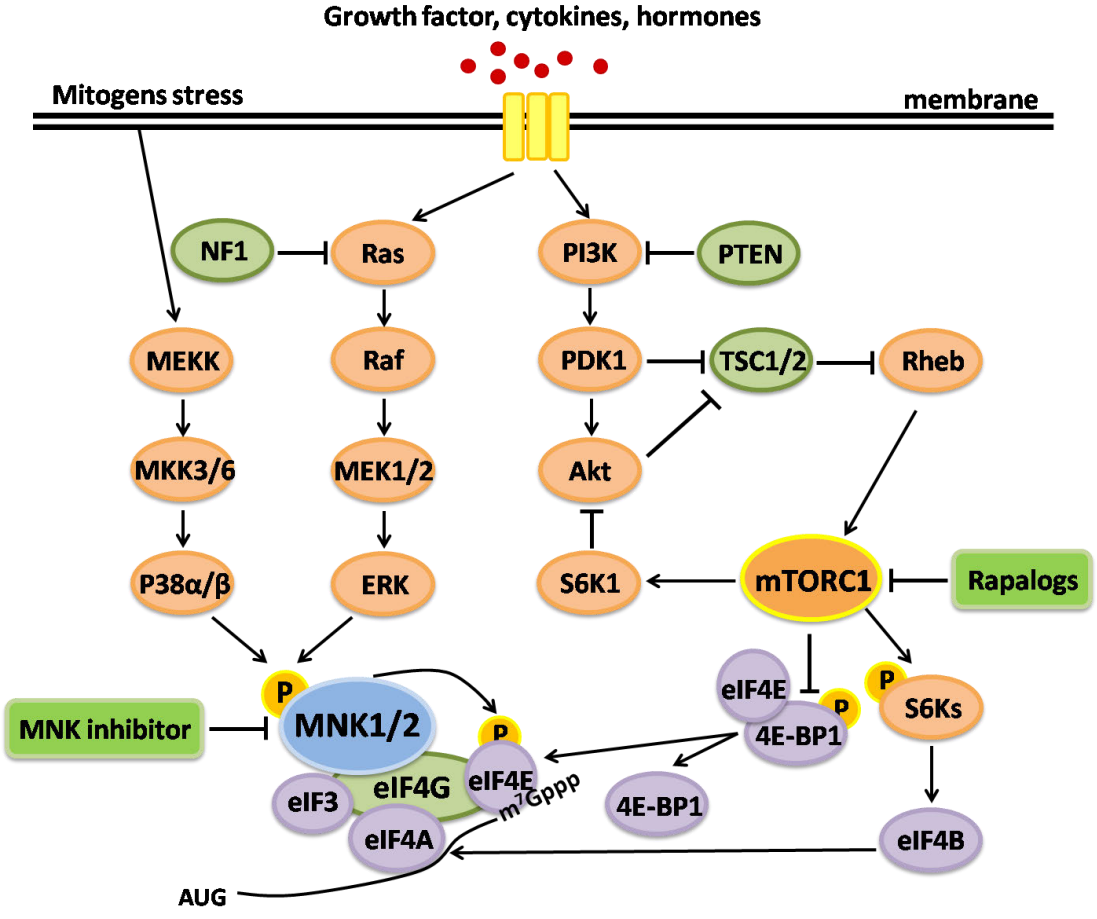


Figure 1.4 The MNK substrates and their biological functions

1.2.2.1 eIF4E

Eukaryotic initiation factor eIF4E is the best known MNK substrate. It binds to eIF4G, a. scaffold protein. It has been shown that phosphorylation of eIF4E promotes its rate of dissociation, resulting in the significant reduction on its affinity to the capped mRNA [76]. MNKs are associated with eIF4G isoforms which serves to bring eIF4E in close proximity to the bound MNKs, thereby allowing the latter to phosphorylate eIF4E at Ser209 [73, 77, 78]. The availability of eIF4E to bind eIF4G is regulated by eIF4E-binding proteins (4E-BPs), and the best-studied one is 4E-BP1 because it is ubiquitously expressed in all cell types. 4E-BPs and eIF4G interact with overlapping sites on eIF4E, so they compete for binding to eIF4E. Hyperphosphorylated 4E-BP1 cannot bind to eIF4E, so that eIF4G can bind to eIF4E instead allowing translation initiation to continue, as well as facilitating the phosphorylation of eIF4E by the MNKs. The phosphorylation of 4E-BPs is catalysed by mTORC1, which can be activated by

the Ras/Raf/MAPK or the PI3K/Akt signalling pathways



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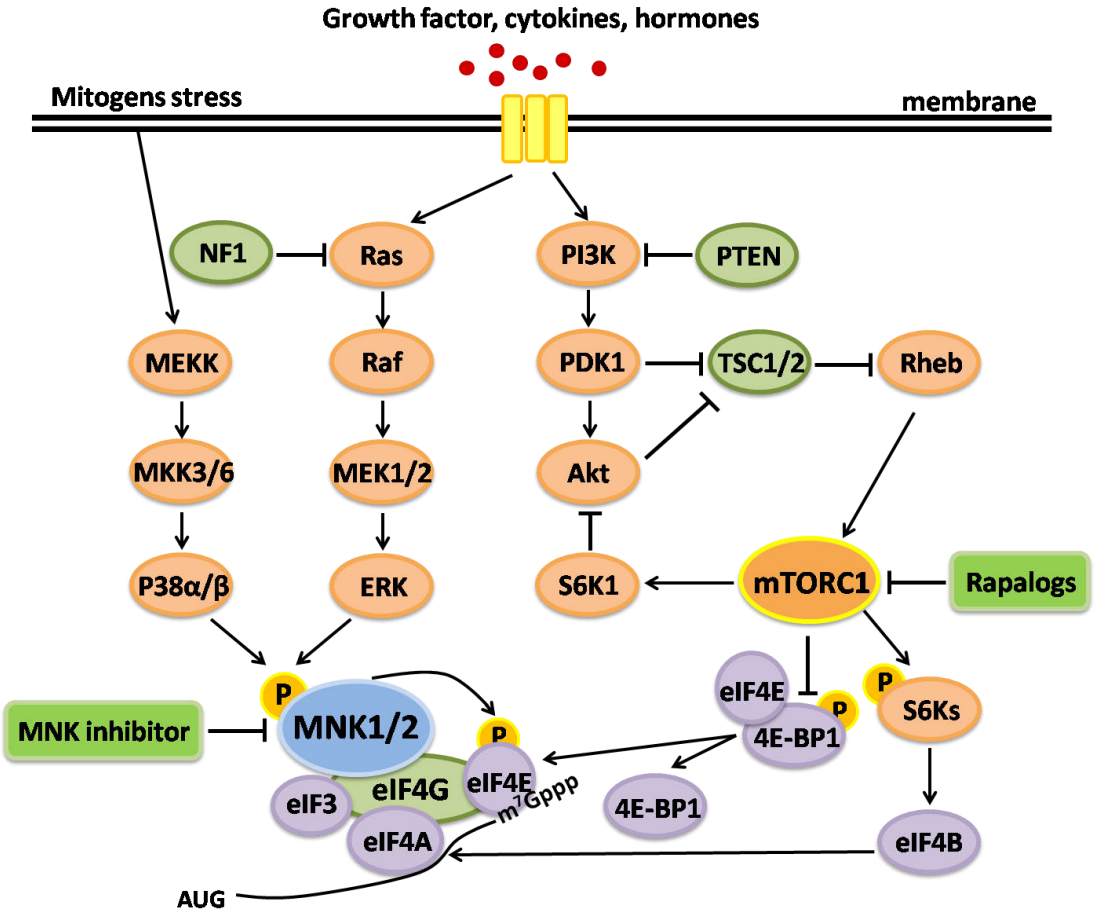


Figure 1.5 Intracellular signalling pathways involving eIF4E

1.2.2.2 Sprouty

Sprouty 2 (Spry2) is served as a negative regulator of multiple receptors in tyrosine kinase pathways. It is reported to be phosphorylated by the MNKs at Ser112 and Ser121, but the reports are controversial regarding the effects of Spry2 phosphorylation on these sites depending upon cell lines [82, 83]. The study from DaSilva *et al.* [82] showed that the phosphorylation of Spry2 by the MNKs increases Spry2 stability. In contrast, another study provided evidence that the phosphorylation of Spry2 by MNK2 promotes Spry2's interaction with the E3 ubiquitin ligase, which leads to Spry2 degradation[83].

1.2.2.3 hnRNP

Heterogeneous nuclear RNA-binding protein A1 (hnRNPA1) binds to the AU-rich regulatory element (ARE) of the tumour necrosis factor α (TNF-α) mRNA. TNF-α is mainly secreted by activated macrophages and T lymphocytes and plays important roles in inflammation [84]. The AU rich elements (AREs) from the 3' UTR of hnRNPA1 regulate its nuclear export, mRNA stability and mRNA translation [85-87]. It has been reported that hnRNPA1 can be phosphorylated by the MNKs at Ser192 and Ser310/311/312, which leads to its dissociation from the TNF-α 3' UTR [88]. This release may contribute to the derepression of the translation of the TNFα mRNA, although the exact mechanisms by which hnRNPA1, and other ARE-binding proteins, controls translation remain to be elucidated.

Stress granules (SGs) are cytoplasmic domains that mRNAs accumulate on when cells are exposed to a variety of stresses, such as oxidative stress, genotoxic stress, hyperosmotic stress, or heat shock. It has been reported that the stress-induced phosphorylation of hnRNPA1 will prevent its interaction with transportin, which results in its accumulation in the SGs [89]. Depletion of MNK kinases decreases the recovery of cells from stresses, suggesting that the MNKs are involved in this effect [89].

1.2.2.4 PSF

PSF [the PTB (polypyrimidine tract-binding protein)-associated splicing factor], along with its partner p54 (nrb), was found to bind mRNAs containing AREs in their 3'-UTRs. One study identified PSF as a potential MNK substrate, and showed that it can be phosphorylated on Ser8 and Ser283 by MNK2 *in vitro* [90]. MNK-mediated phosphorylation of PSF was found to enhance its binding to the AREs of the TNFα mRNA [90], suggesting a potential role of the MNKs in inflammation.

1.2.2.5 cPLA2

Cytoplasmic phospholipase A2 (cPLA2) is an enzyme which catalyses the release of arachidonate acid from glycerophospholipids to provide the precursor of the eicosanoids. Eicosanoids, which act as important secondary messenger molecules, play important roles in immunity, inflammation and central nervous system regulation [91]. cPLA2 needs to be activated by increased cytosolic calcium. MNK1 was found to

phosphorylate cPLA2 on Ser727, which will enhance its enzymatic activity [92]. Thus, the MNKs can be involved in arachidonate acid release and eicosanoid signalling through mediating the activity of cPLA2.

1.2.3 Cellular functions of the MNKs

1.2.3.1 Roles of the MNKs in translational control

As mentioned, eIF4E can bind to the 5'-cap structure of eukaryotic mRNAs and is essential for cap-dependent translation initiation. During this process, eIF4G binds to both eIF4E and eIF4A to assemble an eIF4F complex, which recruits the 40S ribosomal subunit to the mRNA to allow translation to commence [2, 11, 12, 93].

Given this, the major roles of the MNKs and eIF4E phosphorylation are so far thought to be involved in oncogenesis and tumour growth, through regulating the translation of specific mRNAs. A number of such mRNAs have been identified, but further investigation is still needed [94-96].

1.2.3.2 Roles of the MNKs in cancer.

eIF4E is a proto-oncogene, whose level is increased in many types of tumours and cancer cell lines [97-101], and it is responsible for cancer cell transformation [102, 103]. Clinical studies have indicated that the phosphorylation of eIF4E may also contribute to tumour progression. Elevated eIF4E phosphorylation levels have been observed in lymphomas and various solid tumours, and it negatively correlates with the survival prognosis of these cancer patients [103-105]. Recent research shows MNK1 and MNK2 double deficiency can delay tumorigenesis in $PTEN^{-/-}$ mice, and mouse embryonic fibroblast (MEF) cells from these mice are resistant to Ras-induced transformation, which is also supported by the clinical data [106]. Several mRNAs encoding pro-tumorigenic factors are less efficiently translated when eIF4E is unphosphorylated [95]. The chemokine Ccl2, the matrix metalloproteinases (MMPs), baculoviral IAP repeat-containing protein 2 (BIRC2) and the growth factor VEGFC are a few examples which mRNA translation are greatly dependent on the phosphorylation of eIF4E. Targeting Ccl2 with a neutralizing antibody caused prostate tumour regression [107, 108], MMP3 and MMP9 are highly expressed in PC3 prostate cancer

cells [109], BIRC2 and VEGFC serve as inhibitors of cancer cell apoptosis. All of them could contribute to tumorigenesis.

Recently, it has been demonstrated that the MNK2a/MNK2b ratio is much lower in breast tumours than in normal tissues, and that the splicing factor SRSF1 (SF2/ASF) (an oncoprotein), which reduces the expression of MNK2a whereas increases that of MNK2b [110], is also elevated in most of the tumours. MNK2a can enhance p38α MAPK-mediated cell apoptosis, which acts as a tumour suppressor both *in vitro* and *in vivo*, whereas MNK2b is pro-oncogenic [111].

1.2.3.3 Roles of the MNKs in innate immunity

Pro-inflammatory cytokines play an important role in mediating an effective immune response to pathogens, and the regulation of their expression is associated with multiple disorders such as auto-immune diseases, allergies, neurological disorders, cardiovascular diseases and obesity [112-114]. Recent studies have demonstrated the roles of MNKs in regulating inflammatory cytokine production.

Studies using non-phosphorylatable eIF4E (Ser209A) knock-in mice and have shown that these transgenic mice exhibited increased type I interferon immune response, which protected them against viral infection [115]. Also, eIF4E was found to regulate the translation of IκBα, which serves as a NF-κB suppressor, leading to less IFN-β production [115]. As the only known kinase of eIF4E, this study suggests that the MNKs may also play a role in antiviral host defence through eIF4E phosphorylation.

As mentioned above, MNKs are potential kinase of hnRNPA1 and PSF, which are involved in maintaining TNF-α mRNA stability and promoting its translation [88, 90]. Also, pharmacological inhibition of the MNKs was reported to block the production of pro-inflammatory cytokines, such as TNF-α, IL-6, monocyte chemo-attractant protein-1 (MCP-1), as well as to promote the yield of anti-inflammatory cytokine IL-10 in macrophages stimulated with multiple Toll like receptor (TLR) agonists [116, 117]. However, conclusion from these studies were drawn mainly by using CGP57380 as an MNK inhibitor, which has been shown to have some off target effects on other kinases [118]. The effects of MNKs on pro-inflammatory cytokines still need further studies.

Furthermore, our previous research found out that MNK2-KO mice showed less weight gain, improved insulin sensitivity and significantly decreased inflammation on adipose tissue under high-fat diet (HFD) compared to their wild-type littermates [113]. Bone marrow-derived macrophages (BMDMs) from MNK2-KO animals showed elevated basal levels of anti-inflammatory markers (*IL-10* and *IL-5*) in response to IL-4. This suggests that MNK2-KO mice represent an increased tendency towards M2 polarisation of BMDMs [113].

1.3 Cancer cell metastasis

1.3.1 Overview of cancer metastasis

In response to physiological or pathological stresses, cells may respond in different ways to adapt to cope with these conditions. Cancer occurs after a single cell is progressively and genetically damaged to produce cells with uncontrolled proliferation, which produces a primary heterogeneic tumour. The cells which constitute the primary tumor eventually undergo metaplasia, followed by dysplasia and then anaplasia, resulting in a malignant phenotype. This malignancy allows for cancer cell invasion into the circulation, followed by invasion to a second site for *de novo* tumourigenesis. The spreading of tumour cells from a primary tumour to a secondary site throughout the human body is known as tumour metastasis. Most of the cancer patients die from the detrimental effects of the resulting secondary tumours, rather than the primary lesion.

The cascade of metastasis can be classified into two major phases: During the first phase, cancer cells physically translocate from the primary tumour to a distant tissue, and the second phase commences with the colonization at that distant site [119]. First of all, tumour cells detach from the primary tumour and bind to the proteins involving in the surrounding extracellular matrix (ECM). Cancer cells will escape by degrading these proteins and breach the ECM. Then, they undergo extravasation into blood or lymphatic vessels and disseminate in the blood stream or the lymphatic system. Finally, they invade and grow at a secondary site. Each of these steps is highly regulated and requires a distinct molecular program.[120-122]

Metastasis is one of the hallmarks of an aggressive tumour, which distinguishes it from benign tumours. As noted, it is responsible for almost 90% of cancer-associated mortality, but its molecular process is still poorly understood.

1.3.2 The epithelial-to-mescenchymal transition

The epithelial-to-mesenchymal transition (EMT) is currently in the limelight of investigating cancer cell migration, invasion and metastatic dissemination. EMT contributes to the differentiation of many tissues and organs, and plays critical roles in early embryonic morphogenesis. During EMT, epithelial cells lose their cell-cell adhesion and polarity, and promote their migratory and invasive properties, resulting in the conversion to mesenchymal-type of cells.

Epithelial and mesenchymal cells are quite different in both phenotype and function. Epithelial cells attach to each other closely, by tight junctions, gap junctions and adherent junctions, they have an apico-basal polarity, and are bound by a basal lamina at their basal surface. On the other hand, mesenchymal cells lack this polarization, but they rather have a spindle-shaped morphology and interact with each other only through focal points. Epithelial cells express high levels of E-cadherin, whereas mesenchymal cells express those of N-cadherin, fibronectin and vimentin. [123]

Several proteins which repress E-cadherin transcription have been identified. They can be classified into two groups depending on their effects on the E-cadherin promoter. The Snail, Zeb, E47, and KLF8 factors bind to and repress the activity of the E-cadherin promoter whereas factors such as Twist, Goosecoid, E2.2, and FoxC2 repress E-cadherin transcription indirectly [124-127].

Several signalling pathways, such as TGF-beta, EGF, HGF, Wnt/beta-catenin and Notch) have been reported to be involved in the EMT. Ras-MAPK pathway has been shown to activate Snail and Slug, which comprises the first and essential phase of the EMT process [128]. Activation of the phosphatidylinositol 3' kinase (PI3K)/AKT axis is emerging as a central feature of EMT [129]. Wnt signalling pathway regulates EMT in gastrulation, cardiac valve formation and cancer. The EMT regulator SNAIL will be induced when the Wnt pathway is activated in breast cancer cells, following by the upregulation of the mesenchymal marker, vimentin [130].

Recent studies also show that EMT is not only involved in cancer cell metastasis, but also participates highly in tumour progression. EMT can confer resistance to cell death and to oncogene-induced premature senescence [131, 132]. Also, it has been reported that Neu-driven tumours can escape immune surveillance through EMT [133]. Further studies revealed that EMT induces immunosupression by targeting Snail [134]. This may provide an interaction between the cancer progression and immunosuppression during EMT [135].

1.3.3 The cytoplasmic FMRP-interacting protein 1 (CYFIP1)

The fragile X mental retardation protein (FMRP) is almost ubiquitously expressed. Its absence in the brain causes the fragile X syndrome (FXS), which is the most common form of inherited intellectual disabilities. In brain, FMRP can enhance the stability of selected mRNAs and also block their translation [136-138]. In addition to its function in neuronal cells, FMRP has also been recently reported to play an important role in cancer cell metastasis. Recent research shows that FMRP is highly expressed in human breast cancer, and increased expression of FMRP can enhance the metastatic spreading of breast tumour cells to the lungs [139]. FMRP also regulates the expression of two proteins involving in the EMT: E-cadherin, which is a marker of epithelial cells; and vimentin which is regarded as a mesenchymal marker.

The cytoplasmic FMRP-interacting protein 1, CYFIP1, is a key partner of FMRP. It is also known as “specific Rac1-activated” protein (Sra1) in other tissues [128]. CYFIP1 is part of two different complexes in cells, which play roles in two apparently unrelated processes (Figure 1.6). Firstly, together with the fragile X protein (FMRP), CYFIP1 binds to the cap-binding protein eIF4E, preventing the initiation of translation of a number of mRNAs [140]. The brain-derived neurotropic factor (BDNF) can induce protein synthesis and cytoskeletal rearrangements [141, 142]. Upon stimulation by BDNF, CYFIP1 switches to a planar conformation and is released from eIF4E. After that, active GTP-Rac1 stimulates the binding of CYFIP1 to NCKAP1 (NCK-associated protein 1) to form a different complex termed as the WAVE Regulatory Complex (WRC). WRC is implicated in remodelling the cytoskeleton, and hence plays an important role in cell movement. [139, 143, 144]

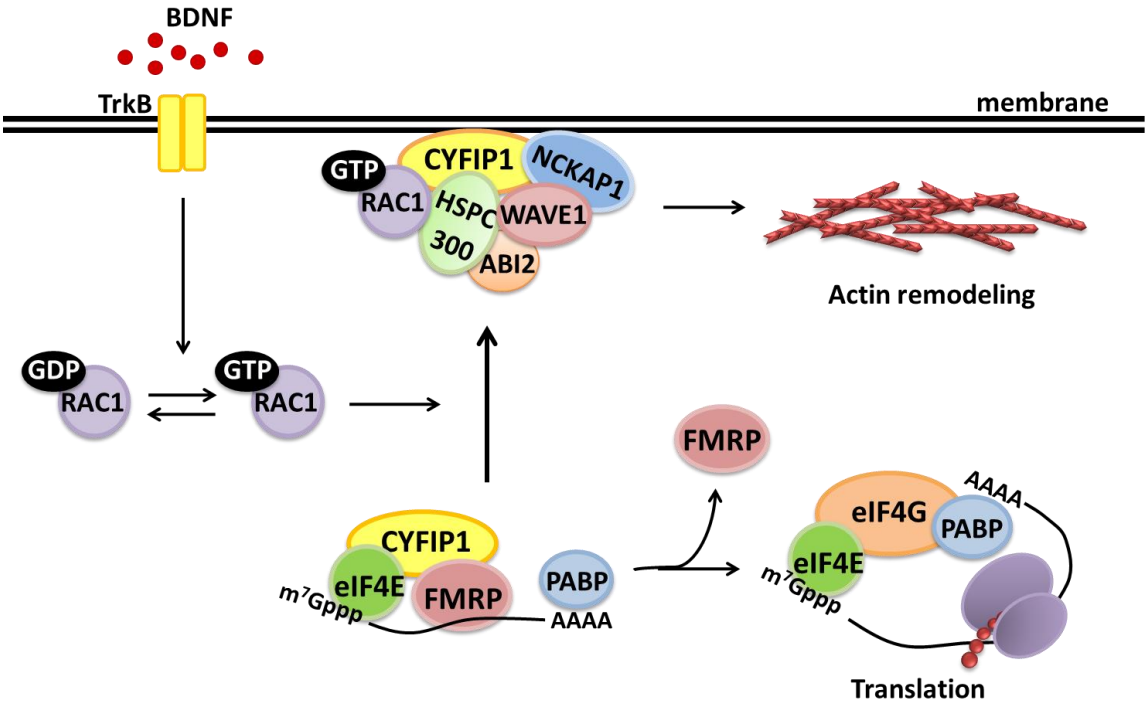


Figure 1.6 The proposed model for the interplay of CYFIP1 complexes

1.3.4 N-myc downstream regulated gene 1 (NDRG1)

NDRG1 (also known as Drg-1 or Cap43) was firstly identified due to its significant expression changes during the differentiation of colon carcinoma cells *in vitro* [145]. Its mRNA is ubiquitously expressed in most human tissues, but is particularly high in the prostate, brain, kidney, placental tissues and intestinal tissues [146, 147]. NDRG1 has been reported to play important roles in many physiological processes, such as tumour growth, cell cycle and T-cell inflammation [148-151]. Among these functions, it has been well described as a metastatic suppressor in several cancers including prostate, colon and breast cancers [146, 152-154]. Clinical studies on prognosis of latter neoplasms demonstrated that, comparing patients with low NDRG1 levels, those with higher NDRG1 levels show a significantly better outcome [148, 155, 156]. Therefore, NDRG1 is a potential molecular target for these common and fatal cancers.

1.3.4.1 Protein structure

The NDRG1 gene is a member of the human NDRG family which is highly conserved among organisms. Chromosome mapping studies have demonstrated that the NDRG1 gene is located on chromosome 8 and encodes a 3kb mRNA which is translated into a 43kDa protein [147, 154].

The NDRG family belongs to the α/β hydrolase group of enzymes; however, NDRG1 lacks a hydrolytic catalytic site, resulting in the deficiency of this hydrolytic enzyme activity [157-159]. Apart from the catalytic domain, NDRG1 also lacks a transmembrane domain, signal sequence or endoplasmic reticulum sequence [146, 147].

Specially, there are three tandem repeats of 10 hydrophilic amino acids (GTRSRSHTSE) in the C-terminus of NDRG1, which are not present in the other proteins of the NDRG family [146]. It probably indicates the unique function of NDRG1 amongst other family members. A recent study shows that this NDRG1-specific motif is crucial for the binding of heavy metal ions like nickel and copper [160].

NDRG1 is a multiphosphorylated protein that contains five calmodulin kinase 2 phosphorylation (CaMK-II) sites, three serine phosphorylation sites, one casein kinase II (CK-II) site, five myristoylation sites, three protein kinase C (PKC) phosphorylation sites, one thioesterase site, one tyrosine phosphorylation site and one phosphopantotheine attachment site [161, 162]. NDRG1 has been reported to be phosphorylated by several upstream kinases, such as serum/glucocorticoid regulated kinase 1 (SGK1) and glycogen synthase kinase 3 (GSK3) [163]. However, the role of NDRG1 phosphorylation is still not clear, although some recent studies indicated that NDRG1 phosphorylation is related to several cellular processes. Phosphorylation level of NDRG1 was found to be spatially and temporally regulated during the cell cycle, and phosphorylated NDRG1 was also found to be localized with centromeres and β-tubulin bundles during cytokinesis [164].

1.3.4.2 Regulation and function of NDRG1 as a metastasis suppressor

NDRG1 has been demonstrated to significantly inhibit metastasis both *in vivo* and *in vitro* [149, 165, 166]. Studies *in vivo* showed that NDRG1 prevented the metastasis of rat prostate cancer AT6.1 cells to the lungs [165], through reducing cell-matrix and cell-cell adhesion [166]. Further studies have shown that NDRG1 prevents metastasis by its regulation of a number of signalling pathways [149, 167-169]. (Shown in Figure 1.7)

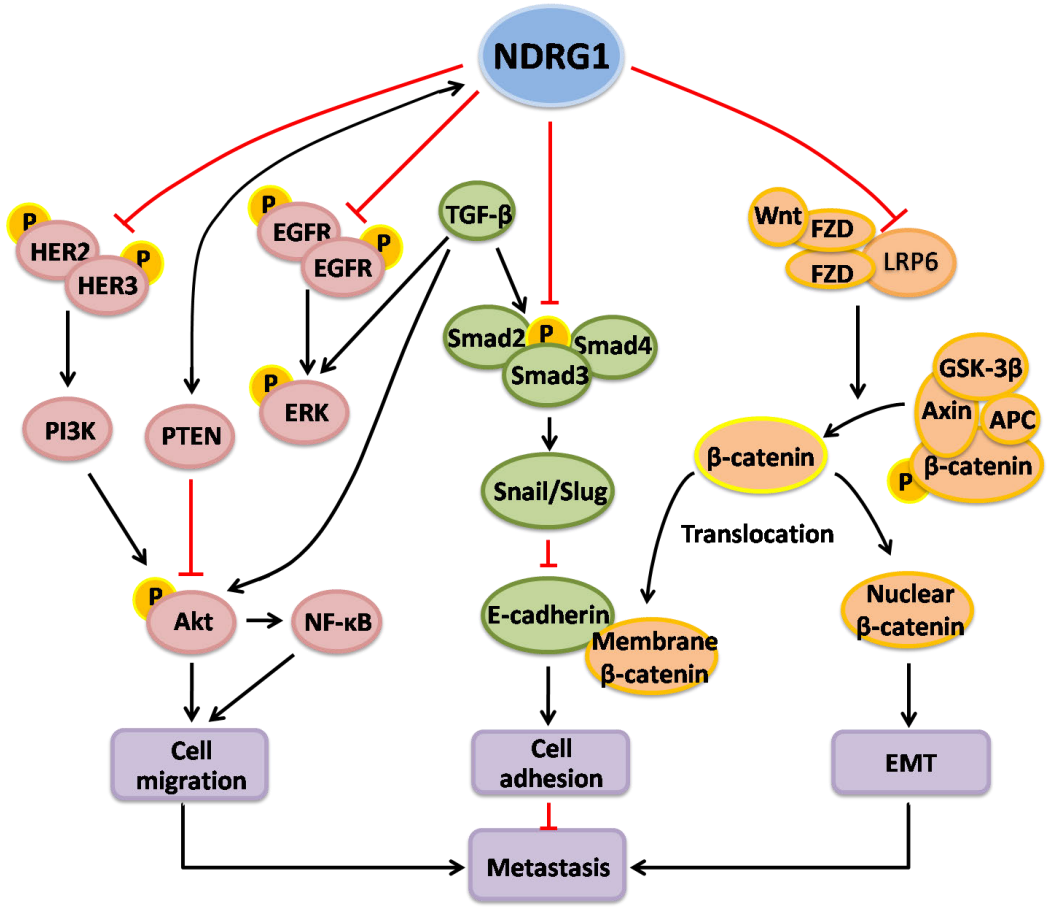


Figure 1.7 The signalling pathways that NDRG1 is involved in metastasis

1.3.4.2.1 NDRG1 and TGF-β pathway

Recent studies have also demonstrated that NDRG1 inhibits TGF-β signalling pathway [149, 168]. The TGF-β pathway is a major signalling cascade in regulating EMT in many cell types [170]. After activation by proteolytic enzymes, such as insulin-like

growth factor, matrix metalloprotease and integrin [171], active TGF-β binds to cell-surface receptors to trigger signal transduction through either the canonical or the non-canonical pathways.

The canonical TGF-β pathway is transduced through mothers against decapentaplegic homolog, Smads. TβRI, a TGF-β receptor, can phosphorylate Smad2 and 3, both of which bind to Smad4 to form functional heterotrimers. These heterotrimers translocate into the nucleus, where they interact with DNA-binding transcription factors and activate transcription of genes relevant to EMT, such as Snail and Slug [172]. NDRG1 was shown to inhibit Smad2 at the protein level as well as inducing the phosphorylation of Smad3, resulting in the inhibition on Slug expression, which is responsible for E-cadherin repression [149, 168].

1.3.4.2.2 NDRG1 and Wnt/β-catenin pathway

NDRG1 has also been shown to be involved is the Wnt/β-catenin pathway. Apart from the essential roles for embryogenesis and normal physiology, the Wnt signalling pathway is also important in the primary tumourigenesis and the metastatic progression [173, 174]. Wnt activation promotes the dephosphorylation of GSK3β, which then allows β-catenin escape from phosphorylation and translocate to the nucleus or the membrane [175]. When expressed at the cell membrane, β-catenin and another EMT marker E-cadherin bind together, which plays in important role in cell adhesion [176]. The disruption of E-cadherin/β-catenin complex formation causes the dysfunction of cell-cell junction, followed by triggering cancer invasion and metastasis [176]. NDRG1 has recently been demonstrated to inhibit the Wnt signalling pathway by interacting with the Wnt receptor, LRP6 [167]. It was also reported that NDRG1 induces translocalization of β-catenin to the plasma membrane, following with the upregulation of E-cadherin and β-catenin in the membrane, which causes the inhibition of cancer metastasis. Taken together, these studies have indicated that NDRG1 plays an important role in cell migration [149].

1.3.4.2.3 NDRG1 and the ErbB family

EGFR, HER2 and HER3, which belong to the ErbB family, are considered as crucial up-stream regulators of multiple signalling pathways in cancer cells [177, 178]. Each of

these receptors is associated with carcinogenesis [178-181] and plays important roles in the activation and regulation of multiple cell responses [177, 178]. Upon ligand-binding, dimerization of the ErbB receptors leads to auto-phosphorylation on their cytoplasmic domains and subsequently results in the activation of downstream pathways, including the PI3K, Ras, MAPK, Wnt, TGF-β, NF-κB and c-Src pathways [178, 182], all of which have been shown to be involved in cancer metastasis as described above. Clinical specimens also showed that high expression of EGFR in triple-negative breast cancer (TNBC) indicates a poor prognosis [183]. Recent studies showed that NDRG1 significantly inhibits both EGFR protein expression and its translocation to the plasma membrane. It also blocks the dimerization and thus reduces the activity of EGFR, resulting in a decreased activation of its downstream target MAPKK [169]. The activation of HER2 and HER3 in response to the EGF ligand is reduced by NDRG1, following by a decrease on the heterodimer formation by EGFR/HER2 and HER2/HER3 [169]. Furthermore, recent study also described that the degradation of HER3 increased upon NDRG1 activation and subsequent enhancement of the association between NEDD4 and HER3 [183].

1.3.4.2.4 NDRG1 and other signalling pathways

In addition to the involvement of Smads, TGF-β also stimulates signalling response through other pathways, such as ERK, c-jun N-terminal kinase, PI3K/AKT [184]. The activation of these pathways in response to TGF-β is related to EMT through their regulation of distinct processes, such as cell survival, cytoskeleton organization or migration [185]. It has been reported that NDRG1 is inhibitory to the PI3K/AKT pathway via upregulating the protein expression of phosphatase and tensin homolog (PTEN), through its induction on the transforming growth factor-β (TGF-β) pathway [149, 168]. PTEN is a tumour suppressor that dephosphorylates phosphatidylinositol-3-trisphosphate (PIP3) to yield PIP2 and consequently leads to a decrease in AKT phosphorylation [186]. Studies showed that the overexpression of NDRG1 upregulates PTEN protein levels in several cell types [156, 168]. On the other hand, NDRG1 protein expression can also be increased by PTEN, and will be restored by a PI3K inhibitor, LY-294002 [187], suggesting the existence of a positive feedback loop between these two proteins.

Moreover, NDRG1 was demonstrated to promote the expression of Smad4, resulting in the inhibition of ERK phosphorylation [168].

In summary, NDRG1 could perhaps be used as a target to block cell migration and invasion, resulting in the final inhibition of metastatic potential. Two anticancer agents, the di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT) and di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbazone (DpC) have been shown to significantly promote NDRG1 expression in multiple cancer types *in vitro* and *in vivo* [146, 149, 167, 188].

1.3.4.3 Other cellular processes that NDRG1 is involved

Apart from metastasis, NDRG1 has been reported to be involved in many cellular processes, such as cell proliferation, cell differentiation and cell cycle, angiogenesis and T cell inflammation [148-151].

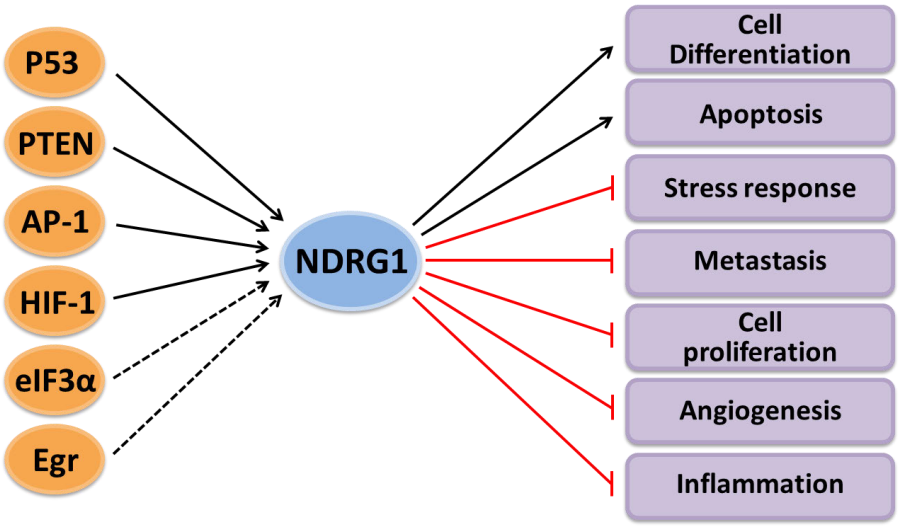


Figure 1.8 Regulations and functions of NDRG1

（Dotted lines mean the regulation happens under specific stimulations）

1.3.4.3.1 The role of NDRG1 in apoptosis

p53 is a tumour suppressor gene known to be mutated or reduced in variety of cancers. P53 induces cell cycle arrest and programmed cell death (apoptosis) and thereby

prevents the propagation of cells that maybe undergoing malignant transformation [189]. It has been reported that p53 induces NDRG1 activation in specific type of cells, which leads to cell proliferation, caspase activation and apoptosis [160, 190]. In the colon cancer cell line DLD-1, p53 binds to the NDRG1 promoter, resulting in the activation of NDRG1 [160]. It was also shown that expression of NDRG1 can also be induced by DNA damaging agents, which is dependent on p53.

1.3.4.3.2 The role of NDRG1 in stress response

Expression of NDRG1 is induced by a range of stimuli which induces stress, such as hypoxia, nickel, androgens, calcium and iron depletion [160, 190, 191]. Hypoxia promotes the generation of mitochondrial reactive oxygen species and plays an essential role in solid tumour formation [192]. The hypoxia-inducible transcription factor HIF1α helps cells adapt quickly to the low oxygen concentrations and survive [193]. Studies have shown that HIF1 upregulates NDRG1 transcriptional expression through its binding to the NDRG1 promoter [194]. NDRG1 can still be induced in HIF1α knock-out cells under long term hypoxia [195]. In addition, Erg-1 was also demonstrated to regulate NDRG1 expression under hypoxic conditions [196].

Stress granules are intracellular complexes formed when cells are exposed to stressors such as hypoxia and oxidative stress. Eukaryotic initiation factor 3α (eIF3α) is considered as a constituent of stress granules. NDRG1 expression has been shown to be positively regulated by eIF3α under iron-depleted conditions [191], which suggests an important role of NDRG1 in the cellular response to stress.

1.3.4.3.3 The role of NDRG1 in angiogenesis

The NDRG1 gene is important in regulating angiogenesis [148, 197, 198]. Interestingly, overexpression of NDRG1 leads to a reduction in the mRNA levels of two angiogenic factors, vascular endothelial growth factor (VEGF-1) and interleukin-8 (IL-8) [148]. Pancreatic cancer cells with high NDRG1 expression levels had a significant decrease in both VEGF-1 and IL-8 proteins [197], which results in the inhibition of angiogenesis. Also, NDRG1 expression is related to tumour microvascular density. It negatively regulates tumour microvascular density via the inhibition of NF-κB, chemokines and VEGF-A [199], while another study has demonstrated that in cervical cancer patients,

high expression levels of NDRG1 are correlated with high tumour microvessel densities [200].

1.3.4.3.4 The role of NDRG1 in inflammation

T cell clonal anergy is a tolerance mechanism in which T lymphocytes are functionally inactivated following T-cell receptor (TCR) engagement, but remains alive for an extended period of time at a hyporesponsive state [201]. Calcium signalling following TCR stimulation leads to the activation and induction of the transcription factors NFAT and Egr2/3. In addition, a costimulatory receptor CD28 activates other pathways (such as PI3K) during T cell stimulation, resulting in the increase of expression levels of NF-κB and AP-1 in the nucleus [202]. Anergic T cells show poor proliferation and produce little interleukin (IL) -2 on subsequent TCR stimulation, even in the presence of costimulation [201]. NDRG1 was identified as a novel anergy factor. Importantly, ablation of NDRG1 causes the T cells insensitive to clonal anergy induction, but didn't affect naive T cell reactivity [151]. NDRG1 is upregulated under anergy-inducing conditions by newly synthesized Egr-2, and is inactivated through its phosphorylation and degraded by CD28 signalling pathway [151]. This suggests how NDRG1 contributes to the prevention of autoimmunity by clonal anergy.

It has also been reported that NDRG1 can inhibit the NF-κB pathway by blocking the phosphorylation of IκBα, which serves as an inhibitor of NF-κB [197]. Interestingly, a recent study showed that NDRG1 phosphorylation at Ser330 and Thr346 by SGK1 was required for its inhibitory effect on the NF-κB pathway [203].

1.4 Aims of this study

The MNKs are not essential to animal development, but still play important roles in specific diseases processes such as cancer and metabolic disease, which makes them potential targets for therapy of certain diseases, such as cancer and metabolic disease/type II diabetes. However, the mechanism through which the MNKs are

involved in these diseases is not clear. Studies in this thesis were designed to address some of the outstanding questions regarding MNKs' roles in cell function:

1. The wide-used MNK inhibitor, CGP57380 [118], is not a potent MNK inhibitor, and has been reported to show some off targets on other signalling pathways, such as S6K. Finding a specific MNK inhibitor is important for both *in vitro* and *in vivo* basic science studies, and potential use e.g., in cancer therapy. In the thesis, a specific and potent MNK inhibitor, MNK-I1 was characterised and used to specifically block MNK activities in many settings.

2. To explore the role of MNKs in migration of cancer cells; MNK inhibition is found to block cancer cell migration in this thesis.

3. To find out the mechanism by which MNKs play a role in cancer cell migration. Further studies prove the links between MNKs and other metastasis-related proteins, including CYFIP1 and NDRG1.

4. Previous studies showed lack of MNK2 can protect mice against high-fat-diet-induced glucose intolerance and insulin resistance, and can decrease the inflammation level in adipose tissues [113]. Following these data, studies in this thesis were undertaken to examine the effects of MNK inhibition on macrophages in response to LPS. This will ultimately give a better understanding of MNK functions in immune and inflammation.

5. Given the roles of the MNKs in disease biology [113, 204, 205], we wanted to identify other substrates for the MNKs which may be involved. In this thesis, a powerful chemical biology approach [206] was developed for use to identify new MNK substrates.

Chapter 2

Materials and Methods

2.1 Cell biology

2.1.1 Cell lines and culture

Unless described otherwise, all cell lines were from Prof. Chris Proud's lab stock. The human breast cancer cell lines MDA-MB453, MCF7, MCF10A were a kind gift from Dr. Marina Kochetkova, Centre for Cancer Biology, University of South Australia. All cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies), supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin/streptomycin (PS) at 37°C in humidified air with 5% CO_2.

Primary mouse embryonic fibroblasts (MEFs) were prepared from 13.5 day-old mouse embryos and maintained in DMEM (Life Technologies) supplemented with 10% (v/v) FBS. MEFs were not used in experiments beyond passage 4.

Primary bone marrow-derived macrophages (BMDMs) were isolated from MNK wild-type and knockout mice from Prof. Chris Proud's lab, and maintained in Complete Macrophage Medium (CMM). This method is described in more detail in section 2.1.6.

2.1.2 Cell treatment

The MNK inhibitors used were MNK-I1, documented in patent WO 2011/104340 A1, and CGP57380 [20]. Agents were added to the medium in DMSO vehicle at the appropriate concentration (always <1% v/v DMSO). The amount of vehicle added was normalized across the different treatment concentrations within each experiment.

RAW264.7 cells were treated with 200ng/ml LPS for different time within each experiment.

2.1.3 Transient transfection

HEK293 cells were transfected using the calcium phosphate method. For transfection of 10 cm plates, 6 μg of MNK1 vector was added to 450 μl of autoclaved water and 50 μl of 0.25 μM $CaCl_2$, then vortexed. 500 μl of 2xBES was added and vortexed for a further 20 secs. The sample was incubated at room temperature for 10 mins before being added to cells in a dropwise manner. Cells were incubated at 37°C in a

humidified incubator with 5% CO_2 for 24-48h before they were utilised for experimentation.

MDA-MB-231 cells were transfected using Lipofectamine 3000 Transfection Reagent (Invitrogen), according to the manufacturer's instructions. 10µg of myc-flag tagged CYFIP1 and 8µg HA-tagged wild type eIF4E, or indicated mutants were added for each 10cm plate. The general ratios for different plates were used as below. Cells were then incubated at 37 °C for 48h.

Plate	Vol. of OPTI medium	DNA	Reagent 3000	Lipofectamine 3000
6 well	2 x 125µl	2µg	5µl	3.5µl
10cm plate	2 x 500µl	10µg	20µl	14µl

2.1.4 Gene silencing by small interfering RNA

The gene expression of human NDRG1 and SGK1 was transiently knocked down with small interfering RNA (siRNA) in MDA-MB-231 cells. siRNA was transfected into cells using Lipofectamine™ 3000 Transfection Reagent as described in section *2.1.3*. A scramble siRNA with the same nucleotide composition was used as a negative control.

Table 2.0.1 Sequences for primers used for gene silencing

Target gene	siRNA sense sequence
human NDRG1	5'- CAUCGAGACUUUACAUGGCUCUGUU -3'
human SGK1	5'- GGAGCUGUCUUGUAUGAGA-3'
scramble	5'- UCUCCAGAAGGCUUAAGUCUUAGGA-3'

2.1.6 Isolation of bone marrow-derived macrophages (BMDMs)

BMDMs were isolated from MNK-knockout mice. The femur, tibia bones were isolated and associated muscle and tendons were cleared. The clean bones were placed in sterile Hank's Balanced Salt Solution (HBSS), transferred to 70% ethanol for <5 minutes then returned to HBSS for a further 5 minutes. The top and bottom of the

bones were then cut until the red bone marrow could just be seen. Bone marrow was flushed out of the femur, tibia and hip bones with Ca^{2+} and Mg^{2+}-free HBSS (Life Technologies) using a 10 ml syringe with a 25G 5/8'' 0.5x16mm needle. Cell clusters were disrupted by pipetting and passing the suspension through a 40 μm cell strainer (Thermo Fisher). The cell suspension was centrifuged at 400g for 10 mins and the resulting cell pellet resuspended in CMM. Cells were then seeded at $2.5x10^5$/ml in 12 ml bacterial-grade petri dishes and cultured at 37°C in humidified air with 5% CO_2 for 7 days. The adherent macrophages were harvested with a cell scraper and grown in CMM with 20ng/ml macrophage colony-stimulating factor (M-CSF) (PreproTech) for utilization in future experiments.

For long-term storage, macrophages were cryopreserved in 90% FBS and 10% DMSO media and stored in lipid nitrogen (5 million cells/vial).

2.1.7 Wound healing assay

MDA-MB231 cells were grown to 70-80% confluence in 6-well plates then serum-starved overnight. The next morning cells were treated with 100ng/ml mytomycin C (Sigma) for 3 hours to inhibit cell growth. The medium was then removed, washed once with PBS and replaced with growth media supplemented with appropriate treatments. A 200μl tip was used to scratch the centre of each well with a straight line. Cells were maintained at 37^oC and images of the scratch were acquired at 0, 16, 24, 40 hours.

2.1.7 Transwell migration/invasion assays

Transwell migration/invasion assays were performed using 24-well 6.5mm Diameter Inserts with an 8.0μm pore size (Costar). Cells were serum-starved overnight and pretreated with 5μM MNK-I1 or 10μM SGKi for 1 hour before the assay.

For migration assays towards collagen, wells of a 24-well plate were pre-coated with 10μg/ml collagen I (Life Technologies) in PBS at 37 °C for 2 hours. The PBS was removed and replaced with 500μl serum free medium. The transwell insert was placed into wells and incubated for 30 mins at 37 °C. $3x10^5$ cells were resuspended in 200μl serum free medium and added to the top of the insert. Cells were incubated at 37 °C and migration was assessed after 24 hours. Media was removed from wells and cells

were stained at 37°C for 45mins with 2μg/ml calcein AM (Life Technologies) diluted in 450μl serum free medium. The staining solution was removed, cells detached with 500μl 0.25% trypsin for approximately 15mins, resuspended and assayed with Nanoluc Luciferase Reader (Promega).

For invasion assays towards serum, DMEM supplemented 10% FBS was used as the chemoattractant. 20μg/ml gelatin (Sigma) was used to coat the top of insert at 37 °C for 2 hours. $1.5x10^4$ cells (resuspended in 200 μL serum free DMEM) were added to the top of the insert. Cells were incubated at 37 °C and migration was assessed after 24 hours. Media was removed from the top and bottom of the wells and cells on the insert were fixed with 3.7% formaldehyde for 2 mins followed by 20 mins with 100% methanol. Cells were then stained with DAPI (5μg/μl) for 15min, protected from light, washed twice in PBS and cells on the top were removed with a cotton stick. At least five photographs were acquired for each sample using a fluorescent microscope (Nikon) and the number of cells was analyzed using image J.

2.1.8 Adhesion assay

96 well black Assay plates (Costar) were coated with 2% gelatin (Sigma) for 1 h, or with poly-L-lysine (Invitrogen) for 10min. RAW 264.7 cells were pretreated with MNK-I1 for 1h, following by 200 ng/ml LPS for another hour. $5x10^4$ cells were seeded into each well (with DMSO or MNK-I1 treated) for 0.5, 1, or 2 h. Then remove the unattached cells and wash twice with PBS. Adhesive cells were then stained with calcein AM (Life Technologies) for 45 min and measured with Nanoluc Luciferase Reader (Promega).

2.1.9 Immunofluorescence

Cover slips were coated with 4% poly-L-lysine (Sigma) at room temperature for 5min before the RAW264.7 cells seeded on. After treatment with LPS for 24h, cells were fixed with 4% paraformaldehyde for 10min at room temperature, permeablised with 0.1% Triton X100 in PBS for 5min and blocked with 5% goat serum in PBS for 30min at room temperature. The cover slips were immunostained using mouse anti tubulin antibody (Sigma) overnight followed by staining with anti-mouse Alexa Fluor® 488 (Molecular probes) and Phalloidin Rohadamine to stain for F-actin (Life Technologies)

for 2hrs next day. The cells were then stained with DAPI to mark nuclei for 15 minutes and mounted on slides with Prolong Gold Antifade Mount (Life Technologies). Immunofluorescence was visualised using a confocal laser scanning microscope (Leica SP5). The results were based on three independent analyses.

2.2 Molecular biology

2.2.1 Vectors

Vectors for HA-tagged eIF4E, HA-eIF4E-Ser209Ala, GST-tagged-MNK1 and MNK2 were acquired from Prof. Chris Proud's lab stock [68, 73]. Other mutants of HA-eIF4E and MNKs were generated using Quick-Change Site-Directed Mutagenesis as described in Section *2.2.4.* Myc-flag tagged CYFIP1 was generously provided by Prof. C. Bagni, Leuven, Belgium. Flag-tagged SGK1 was a kind gift from Lab of Professor Roger Daly from Monash University. HA-tagged mouse NDRG1 WT was a gift from Prof. Kyungho Choi in Korea [151].

2.2.2 Transformation

For heat-shock transformation, DH5α competent cells were thawed on ice for 5 mins. Approximately 100 ng DNA was added gently to the cells, and incubated on ice for 30 mins. Cells were heat-shocked at 42 °C for 45 secs and then returned to ice for a further 2 mins. Cells transformed with vectors conferring ampicillin resistance were immediately plated onto LB-agar containing 100 μg/ml ampicillin. Cells transformed with vectors conferring kanamycin resistance were recovered in LB media at 37 ºC for 60 min, and then pelleted at 200 x g for 1 min. Cell pellets were resuspended in LB media and plated onto LB-agar containing 50 μg/ml kanamycin. LB-agar plates were incubated at 37 ºC for 16 h, before subsequent plasmid DNA purification.

2.2.3 Plasmid purification

Single bacterial colonies resulting from transformation of DNA vector were picked from LB-agar plates and grown in LB culture media containing the appropriate selection antibiotic (100 μg/ml ampicillin or 50 μg/ml kanamycin) at 37 ºC for 16 h.

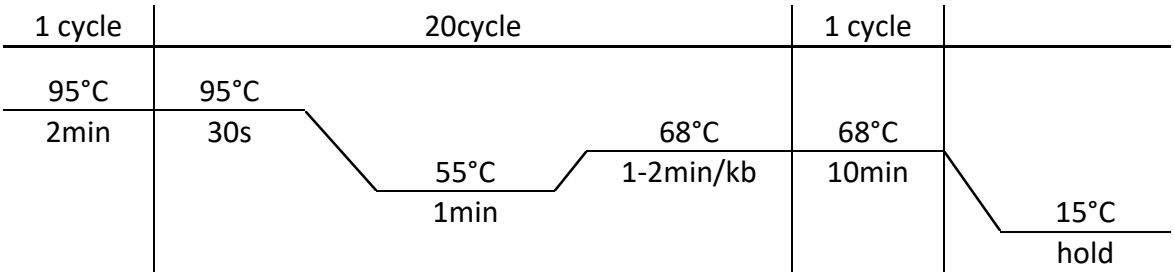
Plasmid DNA was purified using the PurElute™ IEX Plasmid Maxiprep Kit (EdgeBio, Gaithersburg, MD, USA), according to the manufacturer's instructions. Briefly, bacteria were pelleted by centrifugation, resuspended in a suitable buffer and lysed. The bacterial lysate was cleared by loading into an equilibrated filter whilst DNA was bound to a resin. Plasmid DNA was eluted from the resin and resuspended in autoclaved water. Concentration and purity were measured on the Nanodrop 8000 (Thermo scientific).

2.2.4 Mutagenesis

Quick-Change Site-Directed Mutagenesis was performed by mixing the following components in PCR reaction tubes on ice.

	Volume (µl)
H_2O	39
10 x Pfu buffer	5
template dsDNA (50ng/µl)	1
10µM forward primer (diluted in water)	1
10µM reverse primer (diluted in water)	1
10mM dNTP	2
Pfu turbo DNA polymerase (2.5U/µl)	1

Reaction tubes were transferred to a PCR thermocycler with the following reaction conditions.



Following PCR, reaction tubes were transferred to ice and digested with 1 µl *Dpn*1 for 1 hour at 37°C. Digestion products (10µL) were visualized on a 1% agarose gel. Products conforming to the expected size were transformed into competent cells and plasmid DNA was purified as described in Section *2.2.2* and *2.2.3*, respectively. The purified DNA was analysed by Sanger sequencing.

Table 2.0.2 Sequences for primers used for mutagenesis

mutant	Sequence (5' to 3')
HA-eIF4E S209C	F: CACAGCTACTAAGAGCGGCTGCACCACTAAAAATAGGTTTG
	R: CAAACCTATTTTTAGTGGTGCAGCCGCTCTTAGTAGCTGTG
HA-eIF4E S209D	F:CACAGCTACTAAGAGCGGCGACACCACTAAAAATAGGTTTG
	R: CAAACCTATTTTTAGTGGTGTCGCCGCTCTTAGTAGCTGTG
HA-eIF4E S209E	F: ACACAGCTACTAAGAGCGGCGAGACCACTAAAAATAGGTTTGTTG
	R: CAACAAACCTATTTTTAGTGGTCTCGCCGCTCTTAGTAGCTGTGT
MNK1a S353A	F: CTCCAGAGGAACAGCGCCACAATGGACCTGACG
	R: CGTCAGGTCCATTGTGGCGCTGTTCCTCTGGAG
MNK1a S372A	F: CCTTAACCGCCAGCTAGCTCAGCACGAAGAGAAC
	R: GTTCTCTTCGTGCTGAGCTAGCTGGCGGTTAAGG

2.2.5 RNA isolation

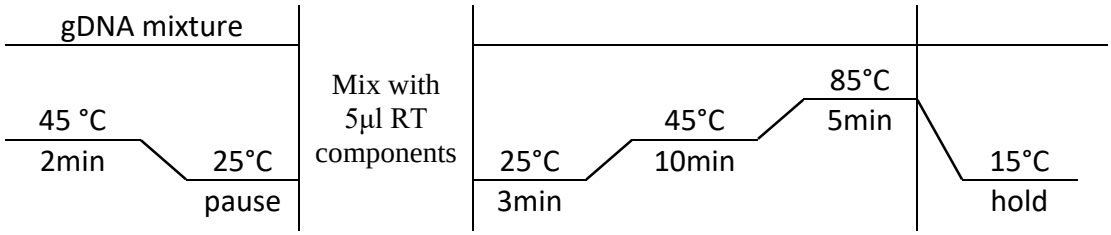
Trizol Reagent (Sigma) was used to harvest cells grown in 6-well plates. 1mL of Trizol was added to each well to lyse cells directly. The lysate was collected into Eppendorf tubes, then 200µl of chloroform was added, vortexed thoroughly for 15 seconds and incubated at room temperature for 15min. The lysate was centrifuged at 12,000g for 15min at 4 ^{o}C separating the mixture into 3 phases. The top aqueous layer (containing RNA) was transferred to a new tube. 500µl isopropanol was added, mixed well and allowed to incubate for 10 minutes at room temperature to precipitate the RNA. The mixture was centrifuged at 12,000g for 10min at 4 ^{o}C to collect an RNA pellet on the bottom of the tube. The supernatant was removed, pellet washed with 1ml 70% ethanol then centrifuged at 7,500g for 5min. Following centrifugation, the supernatant was removed and the pellet left to air dry. Once dried completely, the pellet was dissolved in 30µl DEPC water and concentration and purity were measured on the Nanodrop 8000 (Thermo scientific).

2.2.6 Reverse transcription PCR

Reverse transcription of NDGR1 and SGK1 RNA in Chapter 4 was performed using the QuantiNova Reverse Transcription Kit (QIAGEN), according to the manufacturer's instructions. Briefly, 1µg RNA was mixed with 2µL genomic DNA (gDNA) Removal Mix and incubated at 45^{o}C for 2 minutes in a thermocycler to remove any contaminating gDNA. 4µL of Reverse Transcription Mix and 1 µL Reverse Transcriptase was added to gDNA treated RNA then transferred to a thermocycler with the following reaction conditions.

gDNA mixture (15µl)	
Competent	**Volume**
gDNA Removal Mix	2 µl
Template mRNA	1 µg
Water	to 15 µl total

RT compotents (5 µl)	
Competent	**Volume**
Reverse transcription Mix	4 µl
Reverse Transcription Enzyme	1 µl



Reverse transcription of RNA in Chapter 5 was performed using the ImProm-II™ Reverse Transcription System (Promega) according to the manufacturer's protocol. Briefly, RNA was mixed with cDNA primers in nuclease-free water to a volume of 5µL. The RNA/primer mix was thermally denatured at 70°C for 5 min in a thermocycler and chilled on ice for at least 5 min. A reverse transcription (RT) reaction mix was then prepared by combining nuclease-free water, reaction buffer, reverse transcriptase, magnesium chloride, dNTPs and ribonuclease inhibitor on ice. The RT reaction mix was added to the RNA/primer mix on ice before being transferred to a thermocycler with the following conditions: initial annealing at 25°C for 5 min, RT at 42°C for 1 hr, inactivation of RT at 70 °C for 15 mins.

cDNA from both kits was diluted 1:10 with 180 µl water and used immediately or stored at -20 °C.

2.2.7 Real-time PCR

Applied Biosystem (Fast) was used for Real-time PCR in this thesis. Reaction was mixed as below:

Compotent	Volume (μl)
Water	4.6
Fast SYBR Green master mix (Applied Biosystem)	8.4
Forward primer (1 μM)	1
Reverse primer (1 μM)	1
cDNA (1:10 diluted in water)	5

Table 3.3 Sequences for primers used in qPCR analysis

Target		Sequence (from 5' to 3')
Mouse TNFα	Forward	TCTCAGCCTCTTCTCATTCCTGCT
	Reverse	AGAACTGATGAGAGGGAGGCCATT
Mouse MHC II	Forward	GACGCTCAACTTGTCCCAAAAC
	Reverse	GCAGCCGTGAACTTGTTGAAC
Mouse CCR2	Forward	ATTCTCCACACCCTGTTTCG
	Reverse	GATTCCTGGAAGGTGGTCAA
Mouse MCP-1	Forward	GCATCCACGTGTTGGCTCA
	Reverse	CTCCAGCCTACTCATTGGGATCA
Mouse MMP10	Forward	CCTGATGTTGGTGGCTTCAGT
	Reverse	CTGGTGTATAATTCACAATCCTGTAGGT
Mouse MMP12	Forward	TTGGATTATTGGAATGCTGC
	Reverse	GCACATTTTGATGAGGCAGA
Mouse GAPDH	Forward	GGTCCTCAGTGTAGCCCAAG
	Reverse	AATGTGTCCGTCGTGGATCT
Human NDRG1	Forward	GGCAACCTGCACCTGTTCATCAAT
	Reverse	TGAGGAGAGTGGTCTTTGTTGGGT
Human SGK1	Forward	CTCAGTCTCTTTTGGGCTCTTT
	Reverse	TTTCTTCTTCAGGATGGCTTTC
Human GAPDH	Forward	AGGGCTGCTTTTAACTCTGGT
	Reverse	CCCCACTTGATTTTGGAGGGA

2.3 Protein biochemistry

2.3.1 m7GTP affinity purification of eIF4E and associated proteins

γ-Aminohexyl-m7GTP agarose beads (Jena Bioscience, Germany) were washed twice with Buffer A containing 0.1% β-mercaptoethanol, 0.5mM $NaVO_3$ and protease inhibitors (Roche). 500 μg of cell protein lysate was incubated with the washed beads for 2 h at 4°C. Finally, beads were washed with 400 μl of Buffer A and bound proteins analysed by SDS-PAGE as described in Section *2.3.4*.

2.3.2 Immunoprecipitation (IP)

For CYFIP1 and SGK1 immunoprecipitation, 500μg of cell protein lysate was incubated with 4 μg CYFIP1 antibody or 3μl SGK1 antibody (Cell Signalling) at 4°C for 2 hours in Buffer A. Following antibody incubation, lysate was incubated for a further 2 hours with 20 μl Protein G Sepharose Beads (GE Healthcare) pre-washed in PBS. The beads were washed twice in lysis buffer before being analysed by Western blot.

For Flag and HA IP, 15 μl of Protein G Sepharose Beads (GE Healthcare) were incubated with 1 μg Flag antibody in PBS at 4°C for 2 h. The beads were incubated with 500 μg of cell lysate in Buffer A for 2 hours, and then washed twice with lysis buffer before being analysed by Western blot.

2.3.3 GST-tagged MNK1 purification

6 μg of GST-tagged-MNK1 vector was transfected into HEK293 cells in 10cm plates using calcium phosphate method (as described in Section *2.1.3*). Whole cell lysate was harvested from cells using Buffer A containing 0.1% mercaptoethanol, 0.5 mM NaVO3 and protease inhibitors (Roche). Whole cell lysate was incubated with 300μl of GST beads at 4 °C for 2 h. The beads were then centrifuged at 6,000 rpm for 30 seconds and washed twice in High Salt Buffer and a further two times in GST-Purification Washing Buffer. Protein was then eluted from GST-beads by incubating with 300μl elution buffer on ice for 15 mins. Elution was repeated twice more. The samples from each step were kept and analysed by SDS-PAGE.

The protein eluate was then dialysed using Slide-A-lyzer dialysis cassettes (Thermo Scientific) in dialysis buffer at 4°C overnight. Check the samples using SDS-PAGE gel together with a standard BSA curve, compare the bands with BSA to find the concentration. Aliquot the purified protein and store in -80 °C immediately.

Activated MNK-1 was purified from cells which were starved in serum-free medium overnight, and treated with 1 μM PMA for 30min.

2.3.4 SDS-PAGE electrophoresis and electrotransfer

Proteins analysed by SDS-PAGE were resolved on 12.5% (w/v) acrylamide gels using the Bio-Rad Electrophoresis System. Low molecular weight proteins (less than 20 kDa) such as 4E-BP1were resolved on 13.5% (w/v) acrylamide gels containing a higher concentration of 2% bisacrylamide [0.36% (v/v)].

Cell lysates or immunoprecipitated samples were mixed with SDS-PAGE Sample Buffer (5x), then heated at 97°C for 10 min. 30-40μg of protein was loaded into each lane of the SDS-PAGE gels, then placed in running buffer and run at a constant voltage (180 V) until the bromophenol blue dye was approximately 1cm from the bottom of the gel. Protein separated by SDS-PAGE was transferred to 0.45μm nitrocellulose membranes (Bio-Rad) using the Bio-Rad Electrotransfer system. Transfer was performed on ice at 100V for 1 hour.

Membranes were blocked in 5% (w/v) skim milk powder in PBS-0.05% (v/v) Tween 20 (PBST) for 1 h at room temperature. The membranes were probed overnight at 4°C with primary antibody diluted 1:1000 in PBST containing 2% (w/v) BSA. Unbound antibody was removed by washing the membrane three times with PBST for 5-10 minutes. The membrane was then incubated with fluorescently-tagged secondary antibody diluted 1:20,000 in PBST (Thermo Scientific) for one hour. Membranes were washed another three times in PBST for 5-10 minutes then scanned using the Licor Odyssey Imaging System (LI-COR Bioscience).

2.3.5 Kinase assay with radioactive ^{32}P in vitro

Purified MNK proteins

For SGK1 kinase assay, SGK1 was purified by SGK1 Immunoprecipitation as described in 2.3.2, and the beads were used directly as a kinase.

Compotent	Volume (µl)
SGK1 on beads	On beads
MNK proteins (0.1 µg/µl)	10
Kinase assay buffer	14.8
Unlabelled ATP (10mM)	0.2

The reaction was inbucated at 30°C for 10min, to remove background of MNK phosphorylation. Radioactive ATP (below) was then added and incubated for another 30min at 30°C. 7µl SDS sample buffer was added to stop the reaction.

Compotent	Volume (µl)
[γ-32P]-ATP, 3000Ci/mmol (PerkinElmer)	0.1
Kinase assay buffer	4.9

Samples were analysed by SDS-PAGE gel. Phosphor signal was scanned with Storage phosphor system (PerkinElmer), and protein loading levels were analysed by Coomassie blue staining. NDRG1 was immunoprecipitated as a positive control.

For MNK kinase assay, MNK proteins were purified as described in 2.3.3 from PMA stimulated cell lysates. 0.5µg MNK proteins were used as kinase and 0.6µg eIF4E protein were used as a positive control. Pre-incubating with unlabelled ATP is not necessary.

2.3.6 MNK kinase assay with N6-modified ATPγS in vitro

In vitro kinase assays using ATPγS analogs were performed using MNK WT or mutants (expressed and purified by GST pulldown from HEK293 cells as described in 2.3.5).

Compotent	Volume (µl)
MNKs on beads	On beads
Purifed eIF4E (200 ng/µl)	2

Kinase assay buffer	25
ATPγS or N6-modified ATPγS (10mM)	3

The mixture was incubated at 30°C for 30 min. Then 1µl PNBM (50mM) (Abcam) was added and incubate at room temperature for another hour. 7µl SDS sample buffer was added to stop the reaction and the samples were analyzed by western blot, using the specific thio(p) antibodies.

For the assay with cell lysate, the reaction was mixed as below:

Compotent	**Volume**
MNKs on beads	On beads
Cell lysate in lysis buffer	500µg
$MgCl_2$ (1M)	1 µl
ATPγS or N6-modified ATPγS (10mM)	4µl
Cell lysis buffer	To 400µl total

After incubation at 30°C for 30min, 20µl PNBM was added and incubated for a further 60min. The reaction was used for m7GTP pulldown to check eIF4E thio-phosphorylation as a positive control.

2.3.7 Flow cytometry

For staining with fluorochrome-tagged antibodies, cells ($2.5x10^5$) were distributed into FACS tubes and blocked with FcR(BD) at 4°C for 5 min. Cells were washed twice with FACS washing buffer (5% FBS, 0.2% sodium azide in PBS) and followed by staining with primary antibodies or the corresponding isotype control antibody at 4°C for 30 min in the dark. Cells were then washed twice with FACS washing buffer. Cells were isolated by centrifugation at 400 x*g* for 5 min in 4°C, and then gently resuspend with 500 µl FACS fixing buffer (2% of 1M glucose solution, 1% formaldehyde, 0.02% sodium azide in PBS). Cell surface markers were analysed on LSR Fortessa Flow Cytometer (BD Biosciences). Further analysis was performed by Flow Jo-V.10.0.8.

2.4 Buffers and chemicals

2.4.1 Chemicals and antibodies

Chemicals and antibodies are described specifically in each chapter.

2.4.2 Buffers and solutions

Complete macrophage medium (CMM):

50% DMEM (high glucose, with glutamax)

30% L929 cell conditioned medium (supernatant of L929 cells grown for 7 days in DMEM, 10% FBS)

20% fetal bovine serum (FBS)

50 μg/ml Pen/Strep (P/S)

2x BES

50 mM BES

280 mM NaCl

1.5 mM Na2HPO4

PH 6.96 with 1M NaOH

Cell lysis buffer A

25 mM Tris-HCl [pH 8.0]

50 mM KCl

50 mM β-glycerophosphate

0.2 mM EDTA

1% (v/v) Triton X-100

High salt buffer

50 mM Tris-HCl [pH7.5]

50 mM β-glycerophosphate

350 mM NaCl

1 mM MgCl2

0.5 mM EDTA

0.1 mM EGTA

1% (v/v) Triton X-100

GST-purification washing buffer

25 mM Tris-HCl [PH7.5]

50 mM KCl

5 mM mecaptoethanol

GST elution buffer

25 mM Tris-HCl [PH7.5]

50 mM KCl

40 mM L-Glutathione reduced (Sigma)

1 x protease inhibitors (Roche)

5 mM β-mecaptoethanol

Dialysis buffer

25 mM Tris-HCl [PH7.5]

50 mM KCl

5% glycerol

5 mM β-mecaptoethanol

MNK1 kinase assay buffer

25 mM Tris-HCl [pH 8.0]

50 mM KCl

2 mM $MgCl_2$

5 x Sample buffer

62.5 mM Tris-HCl [pH 6.8]

7% (w/v) SDS

20% (w/v) sucrose

0.01% (w/v) Bromophenol Blue

5% (w/v) β-mercaptoethanol

Coomassie Blue

0.1% (w/v) Coomassie Brilliant Blue

50% (v/v) Methanol

10% (v/v) Glacial Acetic Acid

40% (v/v) H_2O

Running buffer (for 1L)

10% (v/v) 10 x Tris/Glycine/SDS (Bio-Rad)

90% (v/v) H_2O

Transfer buffer (for 1L)

10% (v/v) 10 x Tris/Glycine (Bio-Rad)

10% (v/v) Methonal

1% (v/v) 20% SDS (Bio-Rad)

PBST

1x PBS

0.1% (v/v) Tween 20

Chapter 3

The MNKs regulate cell migration, vimentin expression and eIF4E/CYFIP1 binding

Chapter 4

The metastasis suppressor NDRG1 is regulated in multiple ways by MNK signalling

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Shuye Tian		
Contribution to the Paper	Designed experiments, performed analysis on all samples, interpreted data and wrote manuscript.		
Overall percentage (%)	90		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	13/12/2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

the candidate's stated contribution to the publication is accurate (as detailed above);

permission is granted for the candidate in include the publication in the thesis; and

the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Xuemin Wang		
Contribution to the Paper	Performed some of the experiments, helped plan the research and to analyse the data and prepare the manuscript.		
Signature		Date	14/12/2016

Name of Co-Author	Christopher G Proud		
Contribution to the Paper	Supervised the project, planned experiments, analysed data and played a major role in writing the paper.		
Signature		Date	14/12/2016

The metastasis suppressor NDRG1 is regulated in multiple ways by MNK signalling

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Abstract

The protein N-myc downregulated gene 1 (NDRG1) can repress tumour metastasis. It is phosphorylated at several sites by serum and glucocorticoid-regulated kinase 1 (SGK1). Here we show that NDRG1 is also regulated by signalling through the MAP kinase-interacting kinase (MNK) pathway.

Inhibition of MNKs induces an increase in the expression of the NDRG1 protein and its mRNA in breast cancer cells, which likely contributes to the inhibitory effect of blocking the MNKs on cell migration. MNK inhibition also decreases the phosphorylation of NDRG1. Decreased phosphorylation of NDRG1 is also seen in cells lacking MNK1 but not in MNK2-KO cells. Thus, NDRG1 is a specific target for control by MNK1, not MNK2. However, MNK1 cannot directly phosphorylate NDRG1 *in vitro,* indicating that additional signalling connections are involved. Taken together, our data indicate that MNK signalling regulates NDRG1 at transcriptional and post-translational levels.

Furthermore, SGK1 phosphorylates MNK1 at a conserved site, which represses its activity. NDRG1, SGK1 and the MNKs are implicated in processes linked to cell migration and metastasis.

Our data reveal novel connections between these proteins that may be important for tumour biology and relevant to the development of novel therapeutic approaches.

Introduction

The protein N-myc downstream regulated gene 1 (*NDRG1*) is widely expressed in mammalian cells, especially epithelial cells. NDRG1 has been reported to be a tumour suppressor and/or negative regulator of metastasis in various cancers, including breast cancer[1,2]. NDRG1 is also involved in other processes related to oncogenesis and tumour metastasis (reviewed[3]). For example, it is a differentiation marker in breast cancer[4].

NDRG1 undergoes phosphorylation at several sites including well-conserved ones by serum and glucocorticoid-regulated kinase 1 (SGK1)[2]. However, the role of NDRG1 phosphorylation remains unclear, as does its biological function[1,5]. In particular, it is unclear whether NDRG1 phosphorylation affects its role as an inhibitor of migration.

The MAP kinase-interacting kinases (MNKs[6-8]) also regulate cell migration, but in this case, they promote migration[9], although it remains to be determined how they do this.

There are two MNK genes in mammals; in humans, each gene transcript can be alternatively spliced, giving rise to two proteins. The longer forms, MNK1a/2a which differ from the shorter ones (MNK1b/2b) at their C-termini[8] contain a MAP kinase binding site.

Human MNK1a and its equivalent in mice, MNK1, are tightly regulated by the upstream kinases ERK and p38 MAP kinase[8,10,11] whereas MNK2a/MNK2 have high basal activity[10].

Although MNKs were discovered almost twenty years ago[6,7], there is only one *in vivo*-validated direct substrate for them, eukaryotic initiation factor (eIF) 4E. Both MNK1 and MNK2 phosphorylate this substrate[12], and there are so far no proteins that are controlled specifically by only one MNK. Other MNK substrates have been identified *in vitro* including some involved in gene expression or its control[13,14]. Further substrates may exist.

There is strong interest in the MNKs' role in tumorigenesis and tumor progression, as they are activated by oncogenic Ras/Raf/MAP kinase signalling[15]. Lack of the MNKs, or of eIF4E phosphorylation, delays development or progression of certain solid tumors[16,17]. However, little is known about the relevant molecular mechanisms and downstream targets of the MNKs involved.

We were interested to gain further insight into the mechanisms by which MNKs affect cell migration.

Materials and Methods

Cell culture and treatment

Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen), containing 10% (v/v) fetal bovine serum (FBS) and maintained at 37°C in humidified air with 5% CO_2.

The MNK inhibitor, MNK-I1, was described earlier[18]. GSK650394 (SGKi, Sigma) was used to inhibit SGK1. Drugs were dissolved in DMSO and control cells received this vehicle.

Gene silencing by small interfering RNA

Expression of NDRG1 and SGK1 was transiently knocked down with small-interfering RNAs (siRNA) in MDA-MB-231 cells (Suppl. Table 1). siRNAs were transfected into cells using Lipofectamine™ 3000 Transfection Reagent (Life Technologies). A scrambled siRNA was used as negative control.

Gel electrophoresis and immunoblotting

Proteins were separated by 12.5% SDS-PAGE gel and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked in 5% (w/v) fat-free milk powder at room temperature for 1 h and incubated with indicated primary antibodies (at 1:1000 dilution). Antibodies were from Cell Signalling Technology, except for NDRG1 and SGK1 (Division of Signal Transduction Therapy, University of Dundee), and p-eIF4E (Millipore). Immunoreactive bands were detected by conjugated secondary antibodies (ThermoFisher Scientific) and scanned by Li-Cor Odyssey® imager.

Cell transfection, mutagenesis and vectors

HEK293 cells were transfected using the calcium phosphate method[10].Vectors for GST-MNK1[19] and HA-NDRG1[20] have been described previously. Mutagenesis was performed by PCR using the QuikChange procedure.

RT-qPCR analysis

Total RNA was extracted from cells using Trizol (Sigma). Total RNA (1 μg) from each sample was used for cDNA synthesis using QuantiNova Reverse Transcription Kit (QIAGEN). T-PCR was performed using cDNA as template and Fast SYBR Green master mix (Applied Biosystems) on an Applied Biosystems detection system. Primers for qRT-PCR: NDRG1 forward: 5'-GGCAACCTGCACCTGTTCATCAAT-3', reverse: 5'-TGAGGAGAGTGGTCTTTGTTGGGT -3'; SGK1 forward: 5'-CTCAGTCTCTTTTGGGCTCTTT-3', reverse: 5'-TTTCTTCTTCAGGATGGCTTTC-3'. The relative amount of mRNA was normalized to *GAPDH*.

Wound healing, migration and invasion assays

These assays were performed as described previously[10].

Transwell migration/invasion assays were performed using 24-well 6.5 mm diameter Inserts with an 8.0 μm pore size (Costar). Cells were serum-starved overnight and pretreated with 5 μM MNK-I1 or 10 μM SGKi for 1 h before the assay.

The top of transwell was coated with 0.2 mg/ml gelatin (Sigma) at 37°C for 2 h. $1.5x10^4$ MDA-MB231 cells (resuspended in 200 μL serum-free DMEM) were added to the top of the insert. Cells were incubated at 37°C and migration was assessed after 24 h. Medium was removed from the top and bottom of the wells; cells on the insert were fixed with 3.7% formaldehyde for 2 min followed by 20 min with 100% methanol. Cells were stained with 4′,6-diamidino-2-phenylindole (DAPI; 5 μg/μl) for 15 min, protected from light, washed twice in PBS and cells on the top were removed with a cotton stick. At least five photographs were acquired for each sample using a fluorescent microscope (Nikon) and the number of cells was analysed using Image J software.

In vitro protein kinase assays

Recombinant glutathione *S*-transferase (GST)-MNK fusion proteins were purified by glutathione pull-down from lysates of appropriately-transfected HEK293 cells.

SGK1 protein was prepared by immunoprecipitation using anti-SGK1 antibodies[9]. For kinase assay with [γ-^{32}P]ATP, beads with SGK1 protein and 300 ng non-activated MNK were incubated with 0.1 μl [γ-^{32}P]ATP (where used), 3000 Ci/mmol (PerkinElmer) and 0.2 μl non-radioactive ATP (10 μM) (Sigma) in kinase assay buffer (25 mM HEPES pH7.5, 50 mM KCl and 2 mM $MgCl_2$) at 30°C for 30 min. Where indicated, HA-NDRG1 (immunoprecipitated from HEK293 cell lysates) or 250 ng eIF4E (expressed in *E. coli*) were used as test substrates. Products were analysed by SDS-PAGE. Coomassie blue staining was used to assess protein levels and a phosphorimager scanner (PerkinElmer) to visualise the radioactive signals.

For experiments assessing MNK1 activity, cells were stimulated with 1 μM phorbol myristate acetate (PMA) (Sigma) for 30 min before harvesting. Assays were performed as described above, using the indicated substrates, in some cases using only non-radioactive ATP.

Results

MNK inhibition regulates the overall expression and phosphorylation of NDRG1 in MDA-MB-231 breast cancer cells

Our earlier data showed that MNKs promote cell migration[9]. However, it is unclear how they do this. We therefore asked whether MNKs regulate NDRG1, a phosphoprotein that negatively affects cell migration and tumour metastasis[5].

Treatment of MDA-MB-231 or -453 (‘triple-negative’ metastatic) or MCF7 (steroid receptor-positive) cells with the potent and specific MNK inhibitor MNK-I1[9] decreased the phosphorylation of NDRG1 by 8 h. In MDA-MB-231 cells, phosphorylated NDRG1 recovered by 24 or 48 h. MNK-I1 treatment also increased the levels of NDRG1 protein (Fig. 1A). Taking this into account, it is clear that MNK-I1 still suppresses the phosphorylation of NDRG1 (normalised to total NDRG1) at later times, but less effectively than at 8 h (Fig. 1A). The recovery in P-NDRG1 levels and increase

in total NDRG1 upon extended treatment with MNK-I1 were also observed in MDA-MB-453 (Fig. 1B) but not in MCF7 (Suppl. Fig. 1A).

MNKs regulate the expression of the NDRG1 mRNA

Treating MDA-MB-231 or -453 cells with MNK-I1 increased the levels of *NDRG1* mRNA (assessed by RT-qPCR; Fig. 1C,D), an effect which roughly paralleled the increase in NDRG1 protein (Fig. 1A,B). In contrast, treating MCF7 cells with MNK-I1 did not affect *NDRG1* mRNA levels (Suppl. Fig. 1B).

MNK-I1 treatment did not alter the levels of SGK1 or *SGK1* mRNA in MDA-MB-231 cells, ruling out that this contributes to the increased P-NDRG1 seen at later times (data not shown).

MNK-I1's ability to increase *NDRG1* mRNA levels could be mediated through altered stability of this mRNA. To assess this, MDA-MB-231 cells were treated with MNK-I1 for 4 h and then, in some cases, with actinomycin D for a further 16 h to block mRNA transcription. As expected, actinomycin D blocked the marked increase in *NDRG1* mRNA levels caused by MNK-I1 (Fig. 1E). In the presence of actinomycin D, NDRG1 mRNA levels fell markedly, and similarly in the presence or absence of MNK-I1. These data show that the *NDRG1* mRNA is unstable and rules out that MNK-I1 increases *NDRG1* mRNA levels by stabilising it.

Thus, MNKs likely repress the expression of NDRG1 at the level of transcription.

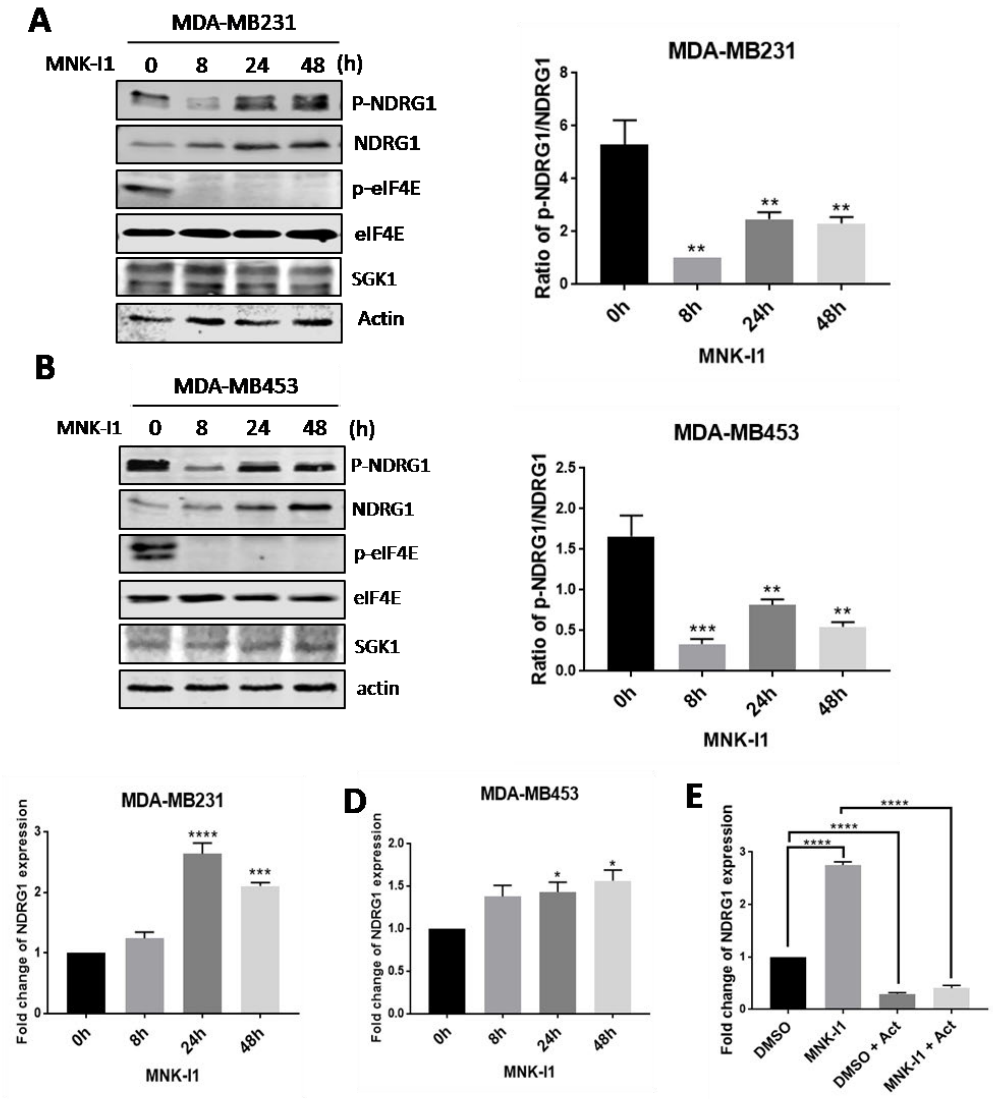

Figure 1. MNK inhibition regulates the phosphorylation and overall expression of NDRG1 in MDA-MB-231 breast cancer cells. Breast cancer cells were treated for different times with MNK-I1 (5 μM). Levels of specific proteins in **(A)** MDA-MB-231 and **(B)** MDA-MB-453 cells were analysed by western blot with indicated antibodies (the latter display two distinct bands for P-eIF4E). NDRG1 mRNA levels in **(C)** MDA-MB-231 and **(D)** MDA-MB-453 cells were analysed by RT-qPCR. **(E)** MDA-MB-231 cells were pretreated with MNK-I1 (5 μM) for 4 h and followed by 100 ng/ml actinomycin D for 16 h, where indicated. NDRG1 mRNA were analysed by qPCR. Data are shown as mean ± S.E.M. from three replicates. ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

The MNKs modulate the phosphorylation of NDRG1

Treatment of MDA-MB-231 cells with MNK-I1 for up to 8h (Fig. 2A) caused a rapid decrease in the phosphorylation of NDRG1 (the effect already being maximal by 2 h). This is consistent with their effects being mediated through inhibition of the MNKs, rather than down-regulation of another kinase acting on NDRG1. To study this further,

we used another small molecule MNK inhibitor, CGP57380. This was the first MNK inhibitor to be reported[21] but is weaker MNK-I1[10]. CGP57380 partially inhibited P-eIF4E and P-NDRG1 (Fig. 2B).

To confirm that the role of the MNKs in the phosphorylation of NDRG1， we employed cells in which one or both MNKs have been 'knocked out'[22]. NDRG1 phosphorylation was readily detected in mouse embryonic fibroblasts (MEFs) from wild-type mice but much lower in cells from MNK1+MNK2 double-knockout (DKO) animals (Fig. 2C). Knocking out MNK1 sharply decreased NDRG1 phosphorylation while, in contrast, MNK2 knock-out did not (although it did reduce P-eIF4E). Thus MNK2 is the major eIF4E kinase in MEFs (perhaps because, unlike MNK1, it is basally active[10]) , provide independent genetic evidence that MNKs modulate NDRG1 phosphorylation and show that it is primarily MNK1, which regulates NDRG1 phosphorylation. Lastly, the differing data for P-eIF4E and P-NDRG1 in single MNK-KO cells show that the effect of impairing MNK activity on NDRG1 phosphorylation is not a consequence of decreased phosphorylation of eIF4E.

To test the contribution of SGK1 to phosphorylation of NDRG1, we used siRNA to deplete SGK1 (Fig. 2D). This markedly decreased P-NDRG1, but a residual level did remain. MNK-I1 treatment almost completely eliminated the remaining P-NDRG1 (Fig. 2D), indicating that MNKs and SGK1 make independent inputs to phosphorylation of the same subset of sites in NDRG1.

To further assess the contributions of SGK1 and MNKs to controlling NDRG1 phosphorylation, we tested the effect of SGKi at a range of concentrations, in with or without MNK-I1, (Fig. 2E). At all SGKi concentrations, MNK-I1 further inhibited P-NDRG1. This is not because SGKi inhibits MNK function as it did not inhibit P-eIF4E showing that SGKi and MNKs make separate inputs to phosphorylation of NDRG1.

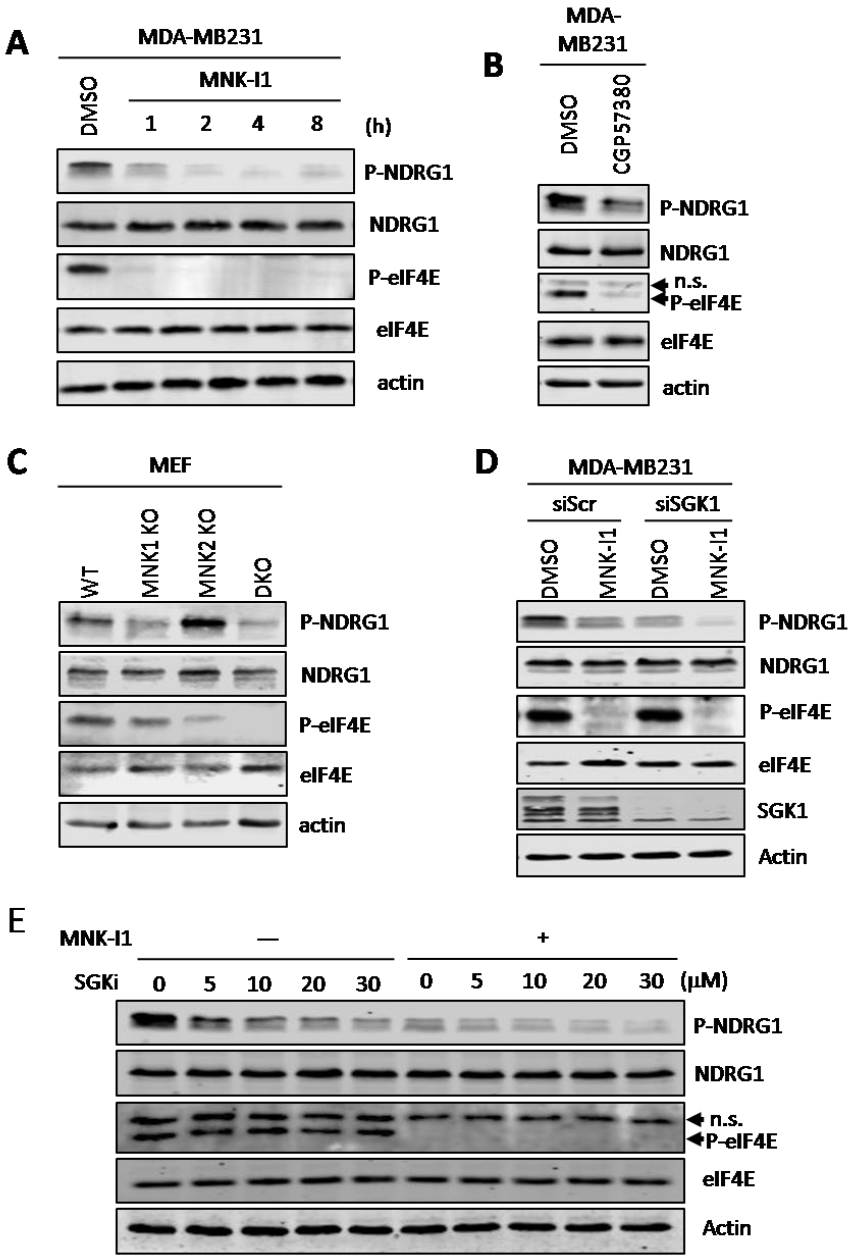

Figure 2. Pharmacological and genetic evidence that MNKs modulate the phosphorylation of NDRG1. (A) MDA-MB231 cells were treated with 5 μM MNK-I1 for the indicated times and lysates were then analysed by western blot. **(B)** MDA-MB231 cells were treated with 25 μM CGP57380 for 2 h. Lysates were analysed by western blot. **(C)** Lysates from wild type, MNK1-KO, MNK2-KO, and MNK1+MNK2 double knockout (DKO) MEFs were analysed by immunoblot with the indicated antibodies. **(D)** SGK1 was knocked down by an siRNA in MDA-MB231 cells and, where shown, cells were then treated with 5 μM MNK-I1 for 4 h. Lysates were analysed by western blot. **(E)** MDA-MB231 cells were treated with increasing concentrations of SGK inhibitor (SGKi) together with 5 μM MNK-I1, where indicated, for 4h. Lysates were analysed by western blot. In some cases, the antibody used for P-eIF4E recognises an additional band which appears to be a non-specific reaction and is not affected by the MNK inhibitors (indicated as 'n.s.' with an arrowhead in panels C and E).

Roles of SGK1 and MNKs in modulating the phosphorylation of NDRG1

In the absence of glucose, NDRG1 phosphorylation increases (Fig. 3A). When used alone, neither an SGK inhibitor (SGKi) nor MNK-I1 could block P-NDRG1, although, when used together, they almost completely eliminated it (Fig. 3A), again showing that SGK1 and MNK1 work in parallel to promote NDRG1 phosphorylation.

Treating cells with the proteasome inhibitor MG132 greatly increased SGK1 levels (see also Fig. 3B,C) and, presumably as a consequence, P-NDRG1. MG132 also caused the appearance of a slower-migrating form of NDRG1 which ran about 7-8 kDa 'larger' than the main species (Fig. 3B,C). The slower-migrating band was heavily phosphorylated (Fig. 3B,C). MNK-I1 decreased the proportion of the upper band (Fig. 3C) showing its appearance depends on MNK1 activity, presumably reflecting one or more MNK-dependent phosphorylation event(s). To test this, HA-NDRG1 was immunoprecipitated from MG132-treated cells, and then incubated with Antarctic phosphatase (AnP). This completely eliminated the upper band (and the signal for P-NDRG1; lower part of Fig. 3D) indicating the mobility shift is indeed due to phosphorylation and providing further evidence that MNKs promote NDRG1 phosphorylation.

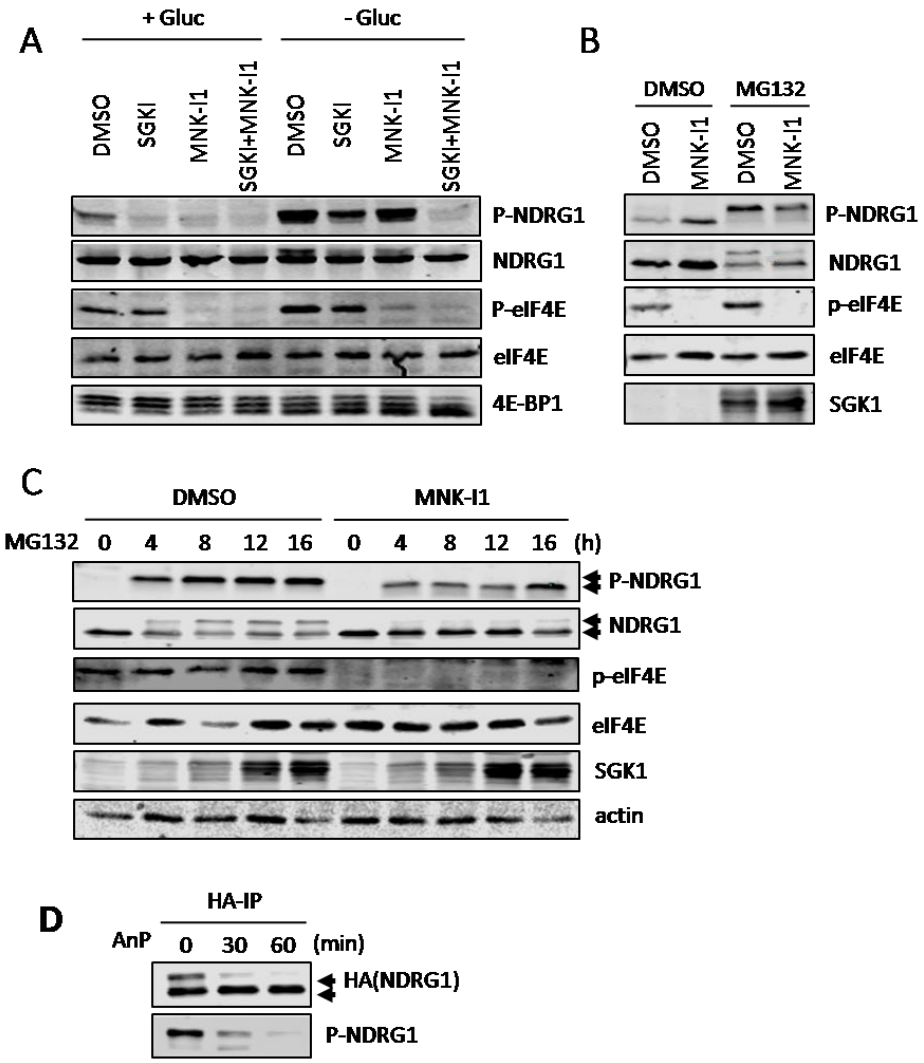

Figure 3. Roles of SGK1 and MNKs in modulating the phosphorylation of NDRG1. (A) Where indicated, MDA-MB231 cells were starved of glucose for 4 h in the presence, where shown, of 5 μM MNK-I1 and/or 10 μM SGKi. Lysates were analysed by western blot. **(B)** MDA-MB231 cells were treated with 5 μM MG132 and 5 μM MNK-I1 for 16 h and lysates were analysed by western blot. **(C)** MDA-MB231 cells were pretreated with 5 μM MNK-I1 for 1 h and then with 5 μM MG132 for the indicated times. Cell lysates were analysed by western blot. **(D)** MDA-MB231 cells were transfected with a vector for HA-tagged NDRG1 and then treated with 5 μM MG132 and 5 μM MNK-I1 for 12 h. HA-IP was performed to isolate NDRG1. Beads were then incubated with Antarctic phosphatase (AnP) at 37°C for 30 or 60 min. Reaction products were analysed by western blot.

MNK1 does not directly phosphorylate NDRG1 or SGK1

Taken together, the above data indicate MNK1 positively regulates NDRG1 phosphorylation. To test whether MNK1 directly phosphorylates NDRG1, we incubated active recombinant MNK1, expressed in HEK293 cells and purified on GSH

beads, with HA-tagged NDRG1 and radioactive [γ-^{32}P] ATP. MNK1 clearly phosphorylated purified recombinant eIF4E, a positive control. However, MNK1 did not phosphorylate HA-NDRG1 (Fig. 4A), although Western blot analysis confirmed that similar amounts of HA-tagged NDRG1 and HA-tagged eIF4E were used (Fig. 4A). MNK1 could not phosphorylate SGK1, even in this sensitive type of radioactive assay (Fig. 4A).

MNK1 is a substrate for SGK1 in vitro

Given the above connections between SGK1 signalling and MNK1, we tested whether SGK1 could phosphorylate MNK1. SGK1 reproducibly catalysed the incorporation of radiolabel into GST-MNK1a purified from HEK293 cells (Fig. 4B), above the background phosphorylation. Inspection of the sequences of MNK1a revealed two potential phosphorylation sites for SGK1 (Ser353 and Ser372), in a region that is absent from MNK1b. Its sequence is QV***L***Q***R***NS**S**TMDLTLFAAEAIA***L***N***R***QL**S**QHEENELA, where potential SGK1 target sites are shown bold/underlined and residues corresponding to the SGK1 recognition motif (LxRxxS/T[23]) are bold/italicised. Both sites are conserved, along with the main features of the adjacent sequence, in mouse MNK1, but not in human MNK2a or mouse MNK2.

We created mutants in which Ser353 or Ser372, or both, were converted to non-phosphorylatable alanine residues. Whereas wild-type MNK1 and MNK1 [S372A] were each radiolabelled by SGK1, it did not phosphorylate the S353A or S353A/S372A mutants (Fig. 4C). Thus, SGK1 phosphorylates MNK1 in a conserved C-terminal site, S353.

Mutating the SGK1 sites increased MNK1a activity (Fig. 4D), but did not affect the level of phosphorylation at its activation loop. Similar data were reproducibly obtained for the S353A/S372A double mutant (Fig. 4D) and the S353A mutant (not shown). This indicates that SGK1 provides an inhibitory input to MNK1 through phosphorylation in its C-terminus, and thus that SGK1 may negatively regulate eIF4E phosphorylation in cells where MNK1a is the major MNK isoform.

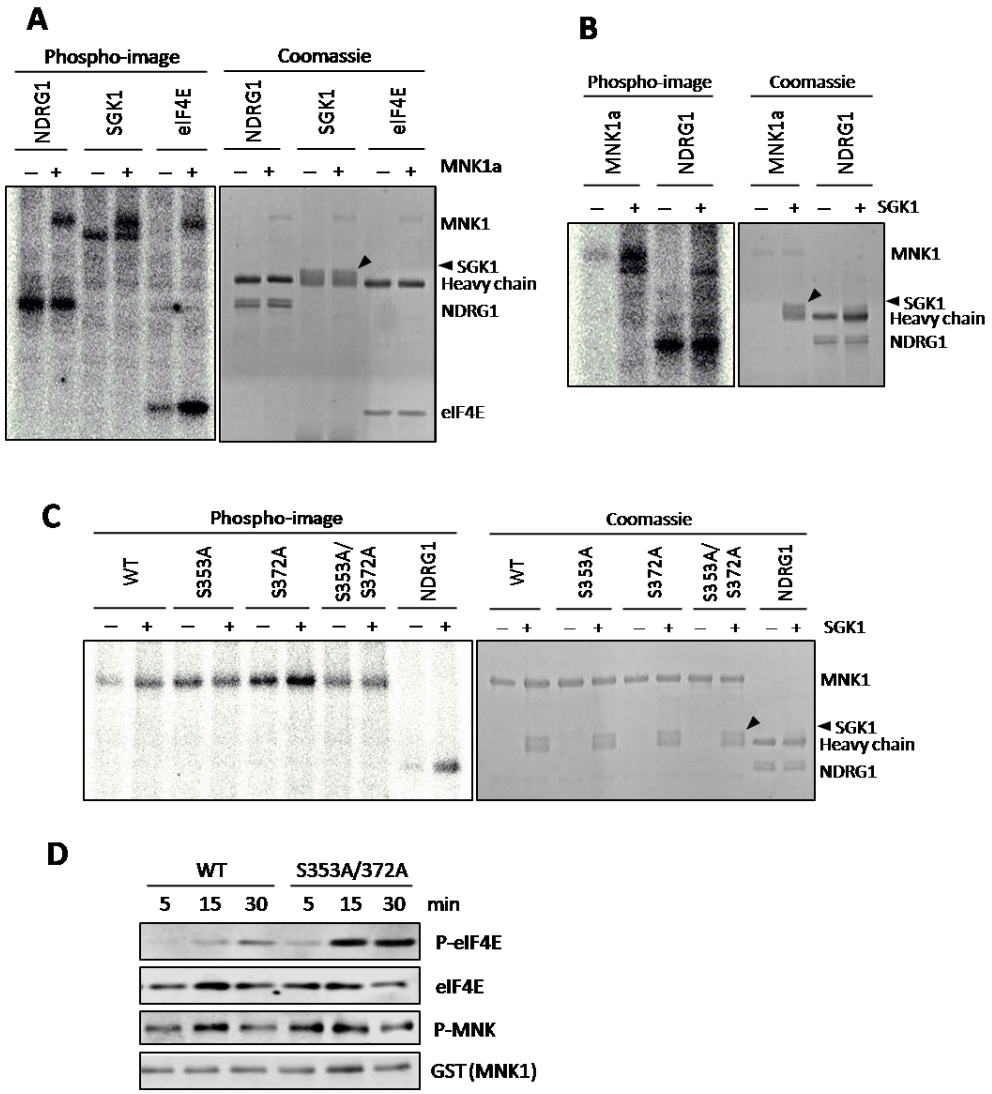

Figure 4. SGK1 phosphorylates MNK1 *in vitro*. **(A)** Kinase assay using [γ-^{32}P]ATP. NDRG1 or SGK1 was incubated with or without purified MNK1a at 30°C for 30 min. Incorporated radiolabel was assessed by phosphorimager (left). Protein levels were assessed by Coomassie blue staining (right). eIF4E was used as a positive control for MNK1a activity. **(B)** Kinase assays using [γ-^{32}P]ATP were performed with human MNK1a and beads (with/without SGK1) on the left (phosphorimage), and protein levels were assessed by Coomassie blue staining (on the right). NDRG1 was used as a positive control for SGK1 activity. **(C)** Kinase assay using [γ-^{32}P]ATP was used to test the ability of SGK1 to phosphorylate purified MNK1a protein and the indicated mutants. NDRG1 was used as a positive control. **(D)** The activity of activated MNK1a protein was assessed using non-radioactive ATP with eIF4E as substrate and samples were withdrawn at the indicated times. Samples were analysed by SDS-PAGE/immunoblot, using the indicated antibodies.

Roles of MNKs and SGK1in regulating cell migration/invasion do not require NDRG1

NDRG1 is generally reported to repress migration or metastasis[24-27], while MNKs promote migration of cancer cells such as MDA-MB-231[9]. The finding that MNK inhibition increases NDRG1 protein levels raised the possibility that inhibiting MNK1

impairs cell migration through this effect. We therefore examined the effect of NDRG1 knock-down on the ability of MNK-I1 to inhibit cell migration.

Knockdown of NDRG1 is efficient (Fig. 5A) and, as expected from the data in Fig. 1A,B, the MNK-I1 increases NDRG1 levels. Knockdown of NDRG1 enhanced cell migration, confirming that NDRG1 normally impairs this process (Fig. 5B; quantified in Fig. 5C). MNK-I1 inhibited migration of MDA-MB-231 cells to almost identical extents whether or not NDRG1 had been knocked down (Fig. 5B,C), indicating that MNK inhibition impairs migration independently of NDRG1. Thus, while regulation of NDRG1 protein levels by MNKs probably contributes to the MNKs' effects on cell migration, MNK-I1 also affects migration through additional mechanisms. This is consistent with the observation that, in MEFs, knocking out either MNK1 or MNK2 partially impairs cell migration[9], while only MNK1 plays a role in modulating NDRG1 phosphorylation (Fig. 2C).

Knocking down NDRG1 also promoted invasion of MDA-MB-231 cells (Fig. 5D). MNK-I1 almost completely blocked invasion in control and NDRG1-knockdown cells, similar to the situation for the effects of these manipulations on cell migration.

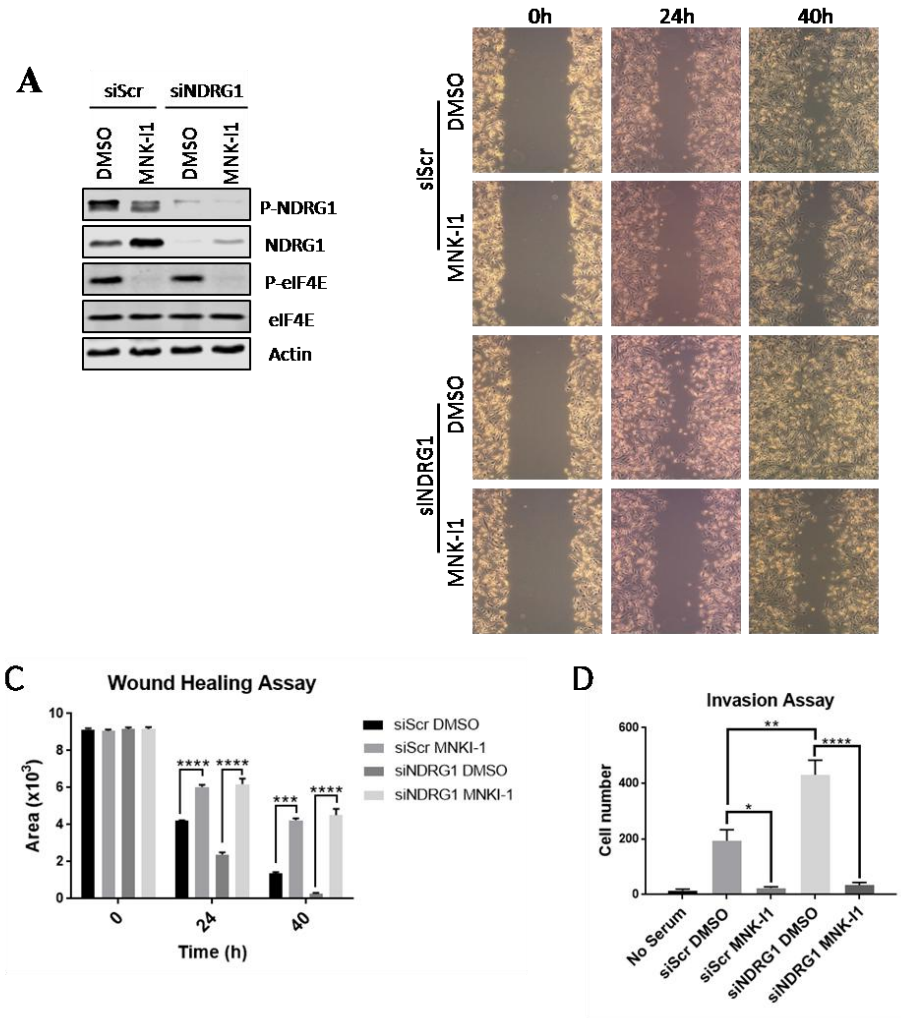

Figure 5. The regulation of cell migration by the MNKs does not require NDRG1. NDRG1 was knocked down by an siRNA in MDA-MB231 cells and cells were treated with 5 μM MNK-I1. A scrambled siRNA (siScr) was used as a negative control. **(A)** Cell lysates were analysed by western blot. Wound healing was tested in MDA-MB-231 cells in the presence of MNK-I1 (5 μM) where indicated. **(B)** Photographs were taken under a light microscope and **(C)** quantified using ImageJ. **(D)** A cell invasion assay was performed to measure the invasive ability of these cells. Data are shown as mean ± S.E.M. from three replicates. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

SGK1 affects the increased breast cancer cell migration induced by NDRG1 deficiency.

Given the potential link between SGK1, NDRG1 phosphorylation and cancer cell migration we used siRNA to deplete NDRG1 in MDA-MB-231 cells and then treated them with SGKi (Fig. 6A). As already noted (Fig. 5B,C), knock-down of NDRG1 enhanced cell migration. Interestingly, whereas SGKi did not affect the wound-healing ability of MDA-MB-231 cells under control conditions, it did block the increased migration induced by NDRG1 knock-down (Fig. 6B,C). This is consistent with data

showing that overexpressing or knocking out SGK1 can, respectively, accelerate or impede cell migration[28,29]. Our data indicate that SGK1 and the MNKs affect cell migration in distinct ways. SGKi caused a modest increase in total NDRG1 (Fig. 6A). This may reflect a role for phosphorylation in the degradation of NDRG1, but, given the present data, presumably does not explain the ability of this compound to impair cell migration.

NDRG1 knock-down also enhanced cell invasion. SGKi inhibited invasion by NDRG1-depleted but not control cells (Fig. 6B,C), indicating that SGK1 acts through targets other than NDRG1 to affect cell migration and invasion.

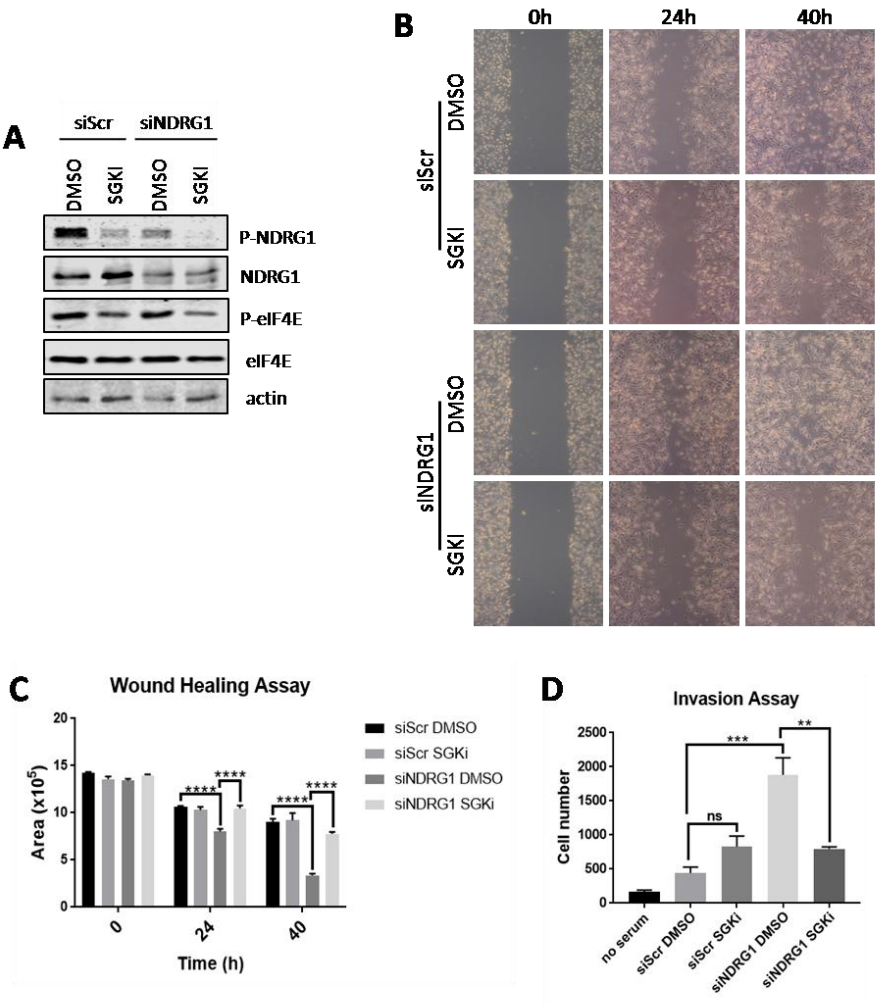

Figure 6. SGK1 inhibition impairs cell migration induced by NDRG1 knock-down. NDRG1 was knocked down by an siRNA in MDA-MB231 cells and cells were treated with 10 µM SGKi. A scrambled siRNA (siScr) was used as a negative control. **(A)** Cell lysates were analysed by western blot. Wound healing was tested in MDA-MB231 cells with or without the SGK inhibitor. **(B)** Photos were taken under a light microscope and **(C)** quantified with ImageJ. **(D)** Invasion assay was performed on MDA-MB-231 cells with/without SGK inhibitor. Data are shown as mean ± S.E.M. from three replicates. ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Discussion

Our data reveal that MNKs regulate both the cellular levels and the phosphorylation of the metastasis suppressor NDRG1. MNK inhibition impairs cell migration and invasion, while deleting NDRG1 increases these processes. Thus, it is likely that the control of NDRG1 protein levels by the MNKs contributes to their role in regulating cell migration/invasion. However, since MNK inhibition still impairs migration of cells depleted for NDRG1, the MNKs must also control migration via additional

mechanisms. Fig. 7 summarises the links between MNKs, SGKi and NDRG1 revealed by our data.

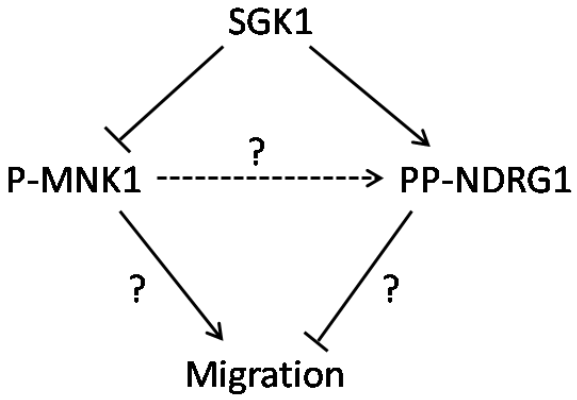

Fig. 7. Links between MNK1, SGK1 and NDRG1. '?' indicates links which are indirect or remain unclear. The dashed line indicates that MNK1 does not directly phosphorylate NDRG1. Please see the text for details.

MNK inhibition increases NDRG1 mRNA levels, especially in MDA-MB-231 cells. Our data indicate that this reflects increased transcription of the *NDRG1* gene, adding to the emerging but still limited information indicating that MNKs regulate gene transcription. For example, eIF4E phosphorylation controls the transcriptional activity of NF-κB[30], indirectly via translational control of expression of IκBα. The *NDRG1* gene is not a target for NF-κB. Instead, it is controlled by other factors including p53, hypoxia-inducible factor 1α, AP1 and Egr-1 (reviewed[31]). Since none of them is known to be controlled by the MNKs, MNKs presumably control *NDRG1* expression by a novel mechanism; further studies are needed to understand this.

SGK1 does not affect cell migration under normal conditions although inhibition of SGK1 does block the faster migration of MDA-MB-231 cells that is induced by knocking down NDRG1. This suggests that SGK1 regulates other proteins which influence cell migration. It will be important to identify the substrates for SGK1 and the MNKs which mediate their effects on cell migration.

Our data also show that SGK1 can phosphorylate MNK1 at Ser353 *in vitro* and suggest that this phosphorylation event inhibits MNK1. The interplay between NDRG1, SGK1 and MNK1 is therefore quite complex (Fig. 7). One implication of the data is that

SGK1 does not promote NDRG1 phosphorylation by activating MNK1, underscoring the conclusion that SGK1 and MNK1 make independent inputs to P-NDRG1.

Lastly, the use of NDRG1 phosphorylation as an indicator of the activity status of Akt/PKB and SGK1[32], or their common upstream regulators, mTORC2 and phosphoinositide signalling[33], must take into account this additional input from MNKs (mainly MNK1). MNK1 does not phosphorylate NDRG1 or SGK1 directly and the mechanism by which it affects NDRG1 phosphorylation remains to be clarified.

In summary, we describe a novel target for regulation by MNK1, i.e., NDRG1, which also provides the first example of a protein which is regulated by one MNK but by not the other. We have therefore identified novel links between the oncogenic MAP kinase and PI 3-kinase/mTORC2 signalling pathways, which control MNK1 and SGK1, respectively.

The very recent development[34] of an MNK inhibitor which exerts anti-tumour effects *in vivo* points to the importance of understanding more fully the roles of MNKs in solid tumour biology. This study makes a substantial contribution to achieving that aim. Our data show that the ability of SGK inhibition to impair cell migration depends on the NDRG1 expression levels in the cell, whereas inhibiting the MNKs blocks cell migration and invasion irrespective of the NDRG1 levels. Thus, an important conclusion from our studies is that MNK inhibitors may be more widely applicable for tackling tumour metastasis than inhibitors of SGK1.

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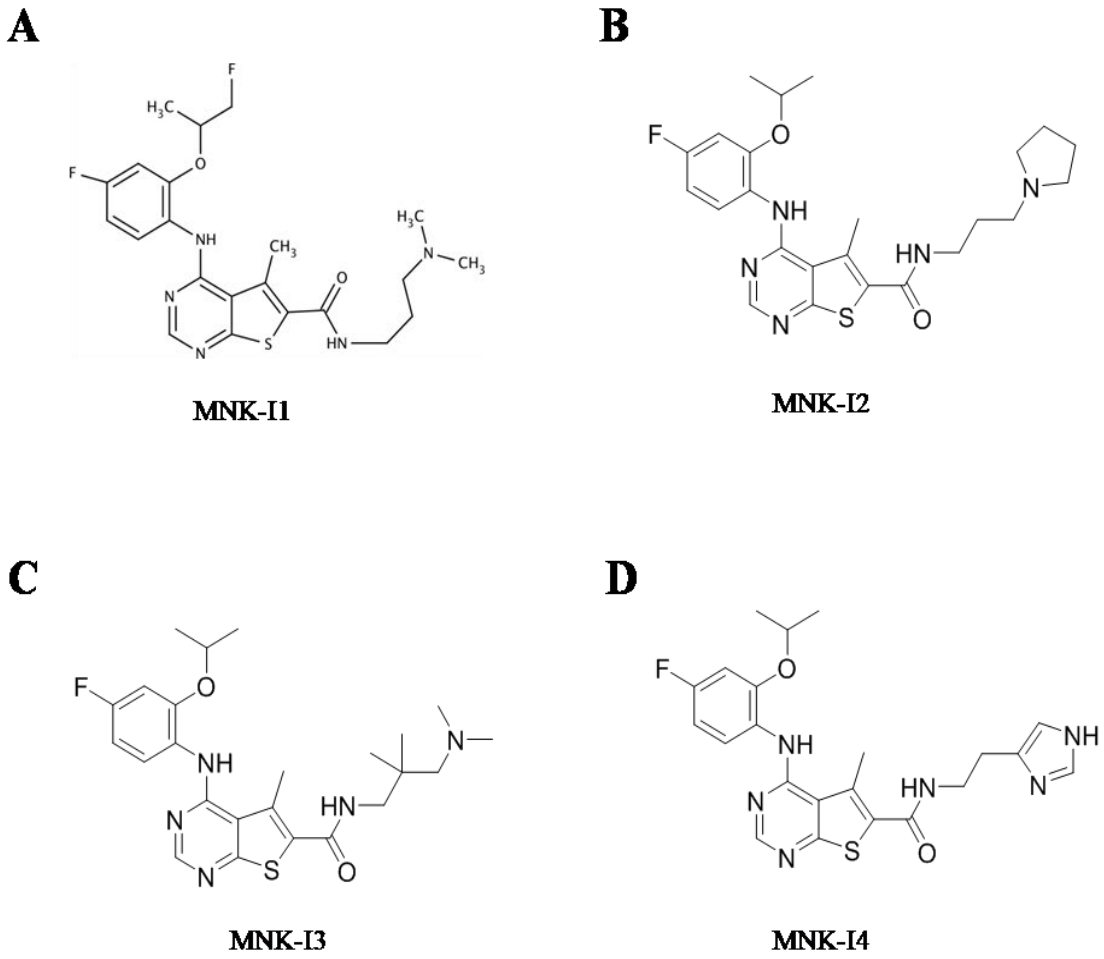
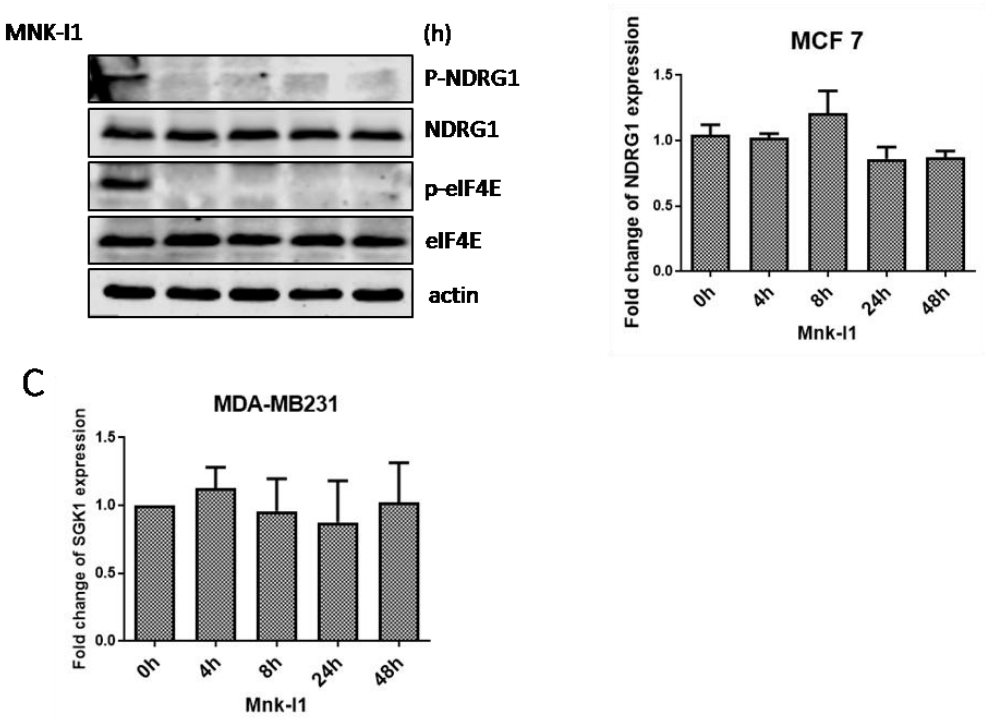
Supplementary Information



Suppl Figure 1. The structure of MNK inhibitors
(A) MNK-I1 **(B)** MNK-I2 **(C)** MNK-I3 **(D)** MNK-I4



Suppl Figure 2. MNK inhibition blocks NDRG1 phosphorylation but does not affect the expression of its mRNA in MCF7 cells.
MCF7 cells were treated with 5 μM MNK-I1 for 4, 8, 24 and 48 h. Protein level **(A)** were analysed by western blot with indicated antibodies, and its mRNA level **(B)** by qPCR. Data are shown as mean ± S.E.M. from three replicates.

Chapter 5

MNKs promote the adhesion and invasion of RAW 264.7 cells

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Shuye Tian		
Contribution to the Paper	Designed experiments, performed analysis on all samples, interpreted data and wrote manuscript.		
Overall percentage (%)	90		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	09/12/2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

the candidate's stated contribution to the publication is accurate (as detailed above);

permission is granted for the candidate in include the publication in the thesis; and

the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Maria Buxade		
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Contribution to the Paper	Performed some of the experiments, helped plan the research and to analyse the data and prepare the manuscript.		
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Contribution to the Paper	Supervised the project, planned experiments, analysed data and played a major role in writing the paper.		
Signature		Date	14/12/2016

MNKs promote the adhesion and invasion of RAW 264.7 cells

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Abstract

The MAP kinase-interacting kinases (MNK1 and MNK2) are activated by ERK and/or p38 MAP kinases and are implicated in controlling protein synthesis through its best-known substrate eIF4E. Migration of macrophages into tissue is an essential step in the host response to infection. Here we show that a selective MNK inhibitor, MNK-I1, impairs the lipopolysaccharide (LPS)-induced morphological change in RAW 264.7 macrophages, and inhibits their recruitment by monocyte chemo-attractant protein-1 (MCP-1). Interestingly, RAW 264.7 macrophages show less adhesive ability when treated with MNK-I1. Inhibition of MNKs decreases the activity of Src/FAK axis, which plays an important role in focal adhesion and actin remodelling. Also, the MNK inhibitor enhanced the LPS-induced tumour necrosis factor alpha (TNFα) and MCP-1 mRNA levels in RAW macrophages. Our findings reveal a novel role for the MNKs in immune and inflammatory responses.

Introduction

The MAP kinase interacting kinases (MNKs) are widely expressed in mammalian cells. They lie at the nexus of major intracellular signalling pathways, including the mammalian target of rapamycin complex 1 (mTOR1) and ERK pathways, which are involved in functions of both normal and cancer cells. The best-known and only fully-validated MNK substrate is eukaryotic translation initiation factor 4E (eIF4E), which plays an important role in protein synthesis [1]. In human, MNKs are encoded by the *MKNK1* and *MKNK2* genes, each of which can generate two MNK isoforms, MNK1a/b

and MNK2a/b, by alternative splicing towards the 3' end of the transcripts. MNK1a and MNK2a (which correspond to the only MNK isoforms in mice) show differences in subcellular localization and activity. MNK1a is found mostly in the cytoplasm. It has quite low activity in serum-starved cells and can be activated strongly by ERK or p38 α/β MAPK [2-4]. However, MNK2a shows high basal activity which is only enhanced slightly by activated ERK. This high activity of MNK2a probably reflects its ability to bind phosphorylated, activated ERK [5], allowing it to be continually activated and thus constitutively active. MNK1b and MNK2b lack the MAP kinase-binding sites, and show unregulated high and low activity, respectively [6].

MNKs and/or phosphorylation of eIF4E have been reported to be involved in several biological processes. Our previous study showed that deficiency or activity inhibition of MNKs blocks cancer cell migration. This inhibition also releases eIF4E from its binding to CYFIP1, a protein which plays an important role in actin remodelling [7]. A study using knock-in mice expressing non-phosphorylatable eIF4E(Ser209A) has shown that phosphorylation of eIF4E regulates the translation of IκBα, which normally serves to suppress nuclear factor-κB (NF-κB) leading to less IFN-β production [8]. This suggests that the MNKs may play a role in inflammation and antiviral host defence through eIF4E phosphorylation. Furthermore, our previous research found out that MNK2-KO mice showed less inflammation in adipose tissue under high-fat diet (HFD) compared to their wild-type littermates [9], suggesting a role for MNK2 in HFD-induced inflammation.

Lipopolysaccharide (LPS) is a major outer membrane component of Gram-negative bacteria, which plays an important role in the pathogenesis of Gram-negative bacterial infection [10] and has been used as an important active component for pathogen-induced macrophage inflammation studies. LPS stimulates toll-like receptor 4 to ignite common down-stream signalling pathways [11], such as phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt), and NF-κB, resulting in expression of pro-inflammatory mediators and inflammatory processes [12, 13]. RAW264.7 cells are a macrophage-like cell line from tumours induced in male BALB/c mice by the Abelson murine leukemia virus, and have been widely used as a primary experimental macrophage model for inflammation studies. Recently, several studies show that LPS stimulation induces morphological changes of macrophages, including an increase in cell size, production of lamellipodia and filopodia [14-17]. Cellular Src is he prototype of Src family kinases

(SFKs), which are constitutively expressed and activated in response to inflammatory stimuli in macrophages, such LPS [18]. Focal adhesion kinase (FAK) is a well-characterised substrate for the tyrosine kinase Src [19, 20]. Src has been reported to be activated by LPS, leading to FAK phosphorylation at Tyr 397 [21], which triggering macrophage adhesion and motility [22-24]. Due to the important role of macrophages in inflammation, suppression of this LPS-induced morphological change could provide a way to prevent or limit a variety of inflammatory disorders.

Here we report that inhibition of MNKs by MNK-I1 blocks LPS-induced RAW cell morphological changes, probably by regulating the actin cytoskeleton. MNK inhibition also impairs adhesion and recruitment of RAW cells induced by MCP-1. The suppression of the Src/FAK axis activity and the increased production of TNFα and MCP-1 by MNK-I1 might contribute to its inhibition on RAW264.7 cell migration.

Methods

Cell culture and treatment (RAW /inhibitor)

RAW 264.7 macrophages were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen), supplemented with 10% (v/v) fetal bovine serum (FBS) and were maintained at 37°C in humidified air with 5% (v/v) CO_2.

The MNK inhibitors used were MNK-I1, documented in patent WO 2011/104340 A1 [7]. Agents were added to the medium in DMSO vehicle at the appropriate concentration (always <1% v/v DMSO). Cells were treated with 5uM MNK-I1 for different time within each experiment. 200 ng/ml LPS were used for different time within each experiment.

Adhesion assay

96-well black assay plates (Costar) were coated with 2% gelatin (Sigma) for 1 h, or with poly-L-lysine (Invitrogen) for 10 min. RAW 264.7 cells were pretreated with MNK-I1 for 1 h, following by 200 ng/ml LPS for another hour. $5x10^4$ cells were seeded into each well (with DMSO or MNK-I1 treated) for 0.5, 1 or 2 h. Unattached cells were then removed and attached cells were washed twice with PBS. Adherent cells were

then stained with calcein AM (Life Technologies) for 45 min and measured with Nanoluc Luciferase Reader (Promega).

Migration and invasion assay

To determine whether MNK-I1 affected the LPS-induced migration of RAW 264.7 macrophages, migration assays was performed by using polycarbonate inserts (8 μm pore size, Transwell®, Beckton Dickinson) placed into a standard 24-well plate. MCP-1 was used as a chemoattractant and diluted (10 ng/ml) with serum-free medium in the bottom well.

After FBS starvation overnight, RAW264.7 cells were pretreated with 5μM MNK-I1 or DMSO for 1 h, and followed by 200 ng/ml LPS for another hour. $5x10^5$ RAW 264.7 cells in 200 μl serum free medium were seeded into the upper chamber of insert and incubated at 37°C. After 24h, the cells migrated into the bottom well were stained with calcein AM (Life Technologies), trypsinized and measured on Nanoluc Luciferase Reader (Promega).

For invasion assays, the upper chamber was coated with 2% gelatin (Sigma) at 37°C for 1 h before seeding the cells. $5x10^4$ cells were seeded. After 24 h incubation, the inserts were stained with DAPI. Five pictures were taken by microscope (Nikon DS-Qi2) and cell number was analyzed by Image J.

Immunofluorescence

Cover slips were coated with 4% poly-L-lysine (Sigma) at room temperature for 5 min before the RAW264.7 cells seeded. After treated with LPS for 24 h, cells were fixed with 4% paraformaldehyde for 10 min at room temperature, permeabilised with 0.1% Triton X100 in PBS for 5 min and then blocked with 5% goat serum in PBS for 30min at room temperature. The cover slips were immunostained using mouse anti tubulin antibody (Sigma) overnight followed by staining with anti-mouse Alexa Fluor® 488 (Molecular probes) and Phalloidin Rhodamine to stain for F-actin (Life Technologies) for 2 h the next day. The cells were then stained with DAPI to mark nuclei for 15 minutes and mounted on slides with Prolong Gold Antifade Mount (Life Technologies). Immunofluorescence was visualised using a confocal laser scanning microscope (Leica SP5). The results were based on three independent analyses.

Western blot

Whole cell lysates were separated by 12.5% SDS-PAGE gel and then transferred to nitrocellulose membranes (Bio-Rad). Then membrane s were then blocked in skinny milk at room temperature for 1 h and incubated with indicated primary antibodies. The primary antibodies used at 1:1000 dilution were from Cell Signalling Technology, except for pFAK (Life Technologies), pSrc and peIF4E (both Millipore). Immunoreactive bands were detected by conjugates of secondary antibodies (ThermoFisher Scientific) and scanned by Li-Cor Odyssey® imager.

Quantitative real-time PCR

Total RNA was extracted from cells using Trizol (Sigma) according to the manufacturers' protocol. Total RNA (1 μg) from each sample was used for cDNA synthesis using QuantiNova Reverse Transcription Kit (QIAGEN). The RT-qPCR was performed using cDNA as a template and Fast SYBR Green master mix (Applied Biosystems) on an Applied Biosystems detection system. Primers used for RT-qPCR were described in Table S1. The relative amount of mRNA was normalized using GAPDH.

Flow cytometry

For staining with fluorochrome-tagged antibodies, cells ($2.5x10^5$) were distributed into FACS tubes and blocked with FcR(BD) at 4°C for 5 min. Cells were washed twice with FACS washing buffer (5% FBS, 0.2% sodium azide in PBS) and followed by staining with primary antibodies or the corresponding isotype control antibody at 4°C for 30 min in the dark. Cells were then washed twice with FACS washing buffer. Cells were spun down at 400 x*g* for 5 min in 4°C, and then gently resuspend with 500 μl FACS fixing buffer (2% of 1M glucose solution, 1% formaldehyde, 0.02% sodium azide in PBS). Cell surface markers were analysed on LSR Fortessa Flow Cytometer (BD Biosciences). Further analysis was performed by Flow Jo-V.10.0.8.

Results

MNK-I1 prevents the LPS-induced morphology change of RAW 264.7 macrophages

To investigate the effects of MNKs on RAW 264.7 macrophage activity, a potent and selective MNK inhibitor, MNK-I1 [7], was used. Our previous study showed that MNK-I1 is a specific MNK inhibitor, which does not affect cell proliferation, cell death or other relevant signalling pathways in several cancer cell lines [7]. Our data show that RAW 264.7 cells start to show morphological changes at 4 h after stimulation with LPS (Fig. 1A), and the effect was almost complete by 24 h. Interestingly, MNK-I1 treatment prevented this LPS-induced change (Fig. 1A). Western blot analysis of the phosphorylation of eIF4E showed that the activity of MNKs was totally inhibited by MNK-I1 up to 24 h (Fig. 1B). These data indicate that MNK inhibition decreased activation of LPS-stimulated RAW 264.7 macrophages.

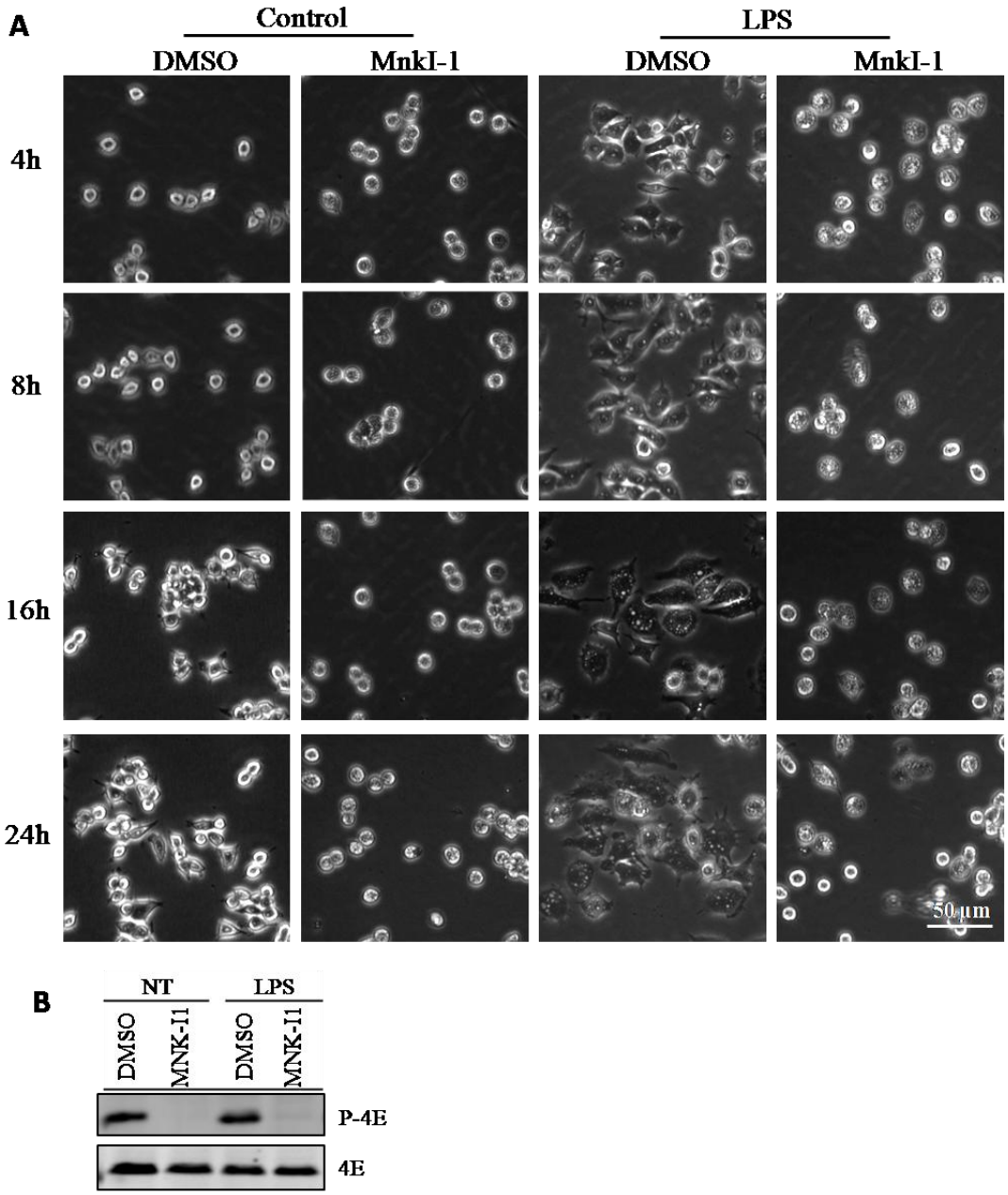

Figure 1 MNK-I1 prevents the LPS-induced morphology change of RAW 264.7 macrophages RAW cells were pretreated with 5 μM MNK-I1 for 1 h, and then treated with 200 ng/ml LPS. **(A)** Photos were taken after 4, 8, 16 and 24 h by light microscope. **(B)** Cells were lysed after 24 h and protein level was analysed by western blot with indicated antibodies. DMSO was used as a negative control for MNK-I1.

To further investigate the effects of MNK-I1 on morphological changes, we performed immunofluorescence staining of F-actin and tubulin in RAW cells. More than 80% of RAW cells showed that the LPS-stimulated morphological changes inhibited by MNK-I1, demonstrating that MNK-I1 inhibited the formation of lamellipodia and filopodia induced by LPS (Fig. 2).

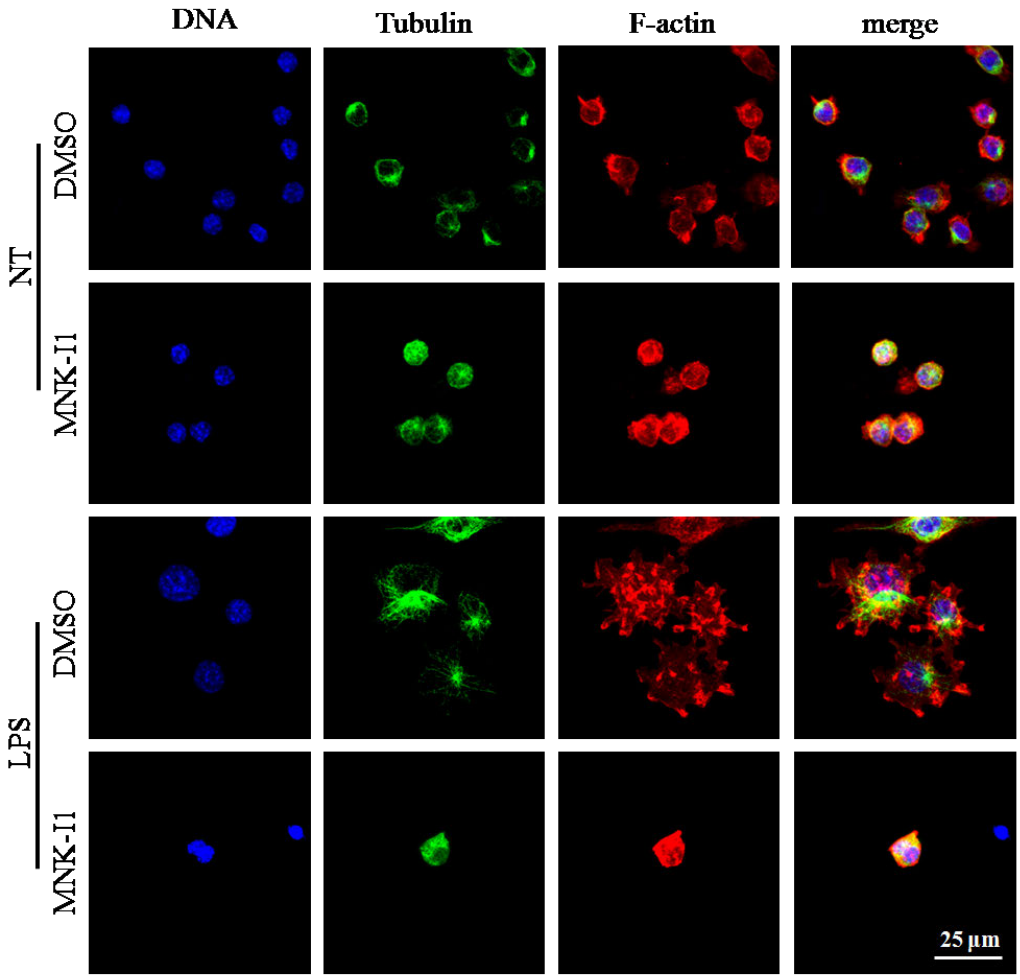

Figure 2 MNK-I1 inhibits the LPS-induced cytoskeleton remodelling of RAW 264.7 macrophages RAW cells were pretreated with 5 µM MNK-I1 for 1 h, and then treated with 200 ng/ml LPS. DMSO was used as a negative control for MNK-I1. After 24 h, cells were then fixed and stained with indicated antibodies. (Blue for DAPI, Green for tubulin and Red for F-actin)

MNK-I1 blocks the recruitment of RAW 264.7 cells to MCP-1

To further study the morphological changes of RAW cells, we tested cell surface expression of CD206 and CD86 by FACS, which are M1 and M2 macrophage markers respectively. Neither MNK-I1 nor LPS affected CD206 and CD86 levels on the cell surface (Suppl Fig. 1), suggesting this LPS-induced cell morphological change is not related to macrophage polarization.

We suspected that the morphological changes (spreading) of LPS-stimulated RAW cells could be involved in the migration ability of RAW cells. To study this, we used

monocyte chemoattractant protein 1 (MCP-1) to recruit RAW cells. MCP1, also called CCL2, is one of the key chemokines which recruit monocytes, memory T cells, and dendritic cells to the sites of inflammation produced by either tissue injury or infection [25, 26]. Our results show that LPS stimulation induced RAW264.7 cell invasion towards MCP-1 (Fig. 3B) but not their migration (Fig. 3A), while MNK-I1 significantly blocks both abilities (Fig. 3A, B). The inhibition of RAW cell invasion by MNK-I1 was much more marked than its effect on cell migration. The differing effects of LPS stimulation on migration and invasion suggests that LPS may promote other functions, such as the adherent behaviour of RAW cells. MNK-I1 did not affect the levels of C-C chemokine receptor type 2 (CCR2), a major receptor for MCP-1 (Suppl Fig. 2). Thus, the inhibition by MNK-I1 of the effects of MCP-1 is not due to downregulation of its receptor.

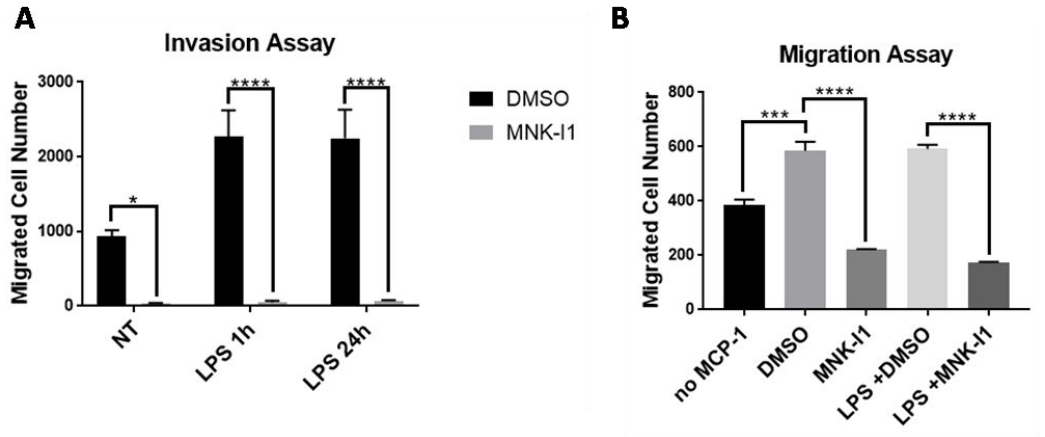

Figure 3 MNK-I1 blocks the recruitment of RAW 264.7 to MCP-1
RAW cells were pretreated with 5 μM MNK-I1 for 1 h and then with 200 ng/ml LPS for another hour. (A) For the invasion assay, inserts of transwell were coated with gelatin. $5x10^4$ pretreated cells were seeded on the top of gelatin and incubated at 37 °C. After 24 h, invasive cells on the inserts were stained with DAPI. Cell numbers were counted by taking photos and analyzied with image J. (B) $5x10^5$ cells were seeded into uncoated transwell for migration assay. After incubate for 24 h, migrated cells in the bottom wells were then stained with calcein AM and measured by a fluorescence reader. Data are shown as mean ± S.E.M. from three replicates. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

MNK-I1 inhibits the adhesion of RAW 264.7 cells to gelatin and poly-L-lysine

To assess whether MNK inhibition also blocks LPS-induced cell adhesion, we used gelatin and poly-L-lysine as matrices (ECM) to assess the adhesive abilities of RAW cells. The adhesion assay shows that MNK-I1 also inhibits the adhesion of RAW cells

to either gelatin or poly-L-lysine (Fig. 4A). RAW cells attach better to poly-L-lysine better than to gelatin after 2 h. To investigate if MNK-I1 can also block cell adhesion induced by LPS, we tested adhesion of RAW cells at different times. The data show that LPS induced adhesion of RAW cells to both gelatin and poly-L-lysine by 30 min, while at later times (60 and 90 min), LPS only showed increasing number of attached cells to gelatin, but not to poly-L-lysine. Inhibition of MNK activity by MNK-I1 inhibits adhesion induced by LPS at 30 min, while after 60 min, both basal and LPS-induced cell adhesion were blocked by MNK-I1 (Fig. 4A, B). These data suggest that inhibition of MNKs by MNK-I1 inhibits RAW cell adhesion to gelatin and poly-L-lysine. The number of cells adhering to poly-L-lysine fell after LPS stimulation for 90 min (Fig. 4B), showing that LPS-induced adhesion of RAW cell is transient

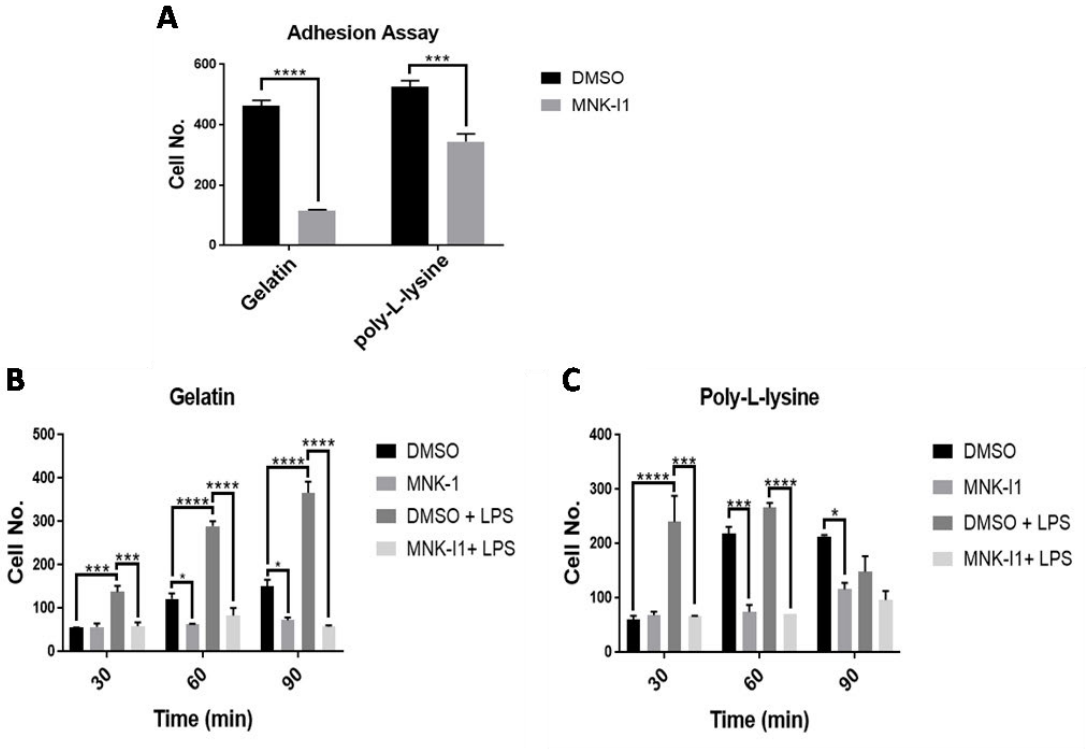

Figure 4 MNK-I1 inhibits the adhesion of RAW 264.7 cells to gelatin and poly-L-lysine
(A) RAW cells were pretreated with 5 μM MNK-I1 for 1 h. Then put $5x10^4$ cells into a gelatin or poly-L-lysine coated plates to attach for 2 h, then wash with PBS twice. Cell number was analysed by Calcein AM staining and fluorescence reader. RAW cells were pretreated with 5 μM MNK-I1 and followed with 200 ng/ml LPS for 1 h. Cells were seeded into **(B)** gelatin or **(C)** poly-L-lysine coated plates for 30, 60, 90 min. Cells were then stained with calcein AM and counted by fluorescence reader. Data are shown as mean ± S.E.M. from three replicates. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

MNK-I1 suppresses LPS-induced activation of the Src/FAK axis in RAW 264.7 cells

To further investigate the mechanism of the MNK-I1 effects on RAW cells, we examined pathways involved in cell adhesion. MNK-I1 did not affect mRNA expression for matrix metalloproteinases, such as MMP2, MMP10 or MMP12, which could have been involved in the effects of MNK-I1 on cell invasion (Suppl Fig. 2). Inhibition of MNKs in RAW264.7 cells did not change cell surface markers involved in cell adhesion and migration, such as CD11b or CD206 (Suppl Fig. 1). The Src/FAK axis has been demonstrated to play a critical role in LPS-mediated macrophage mobilization [19, 23, 27]. LPS markedly and rapidly increased phosphorylation of FAK at Tyr397 (by 30 min), and this effect was suppressed by MNK-I1 (Fig. 5A). Then we tested the effects on FAK phosphorylation at longer times. After LPS stimulation for 4 h and 8 h, MNK-I1 inhibited FAK phosphorylation in LPS-stimulated RAW cells (Fig. 5B). Given this, the attenuation of Src/FAK axis activation in LPS-stimulation RAW264.7 cells might contribute to MNK-I1-inhibited cell adhesion and migration.

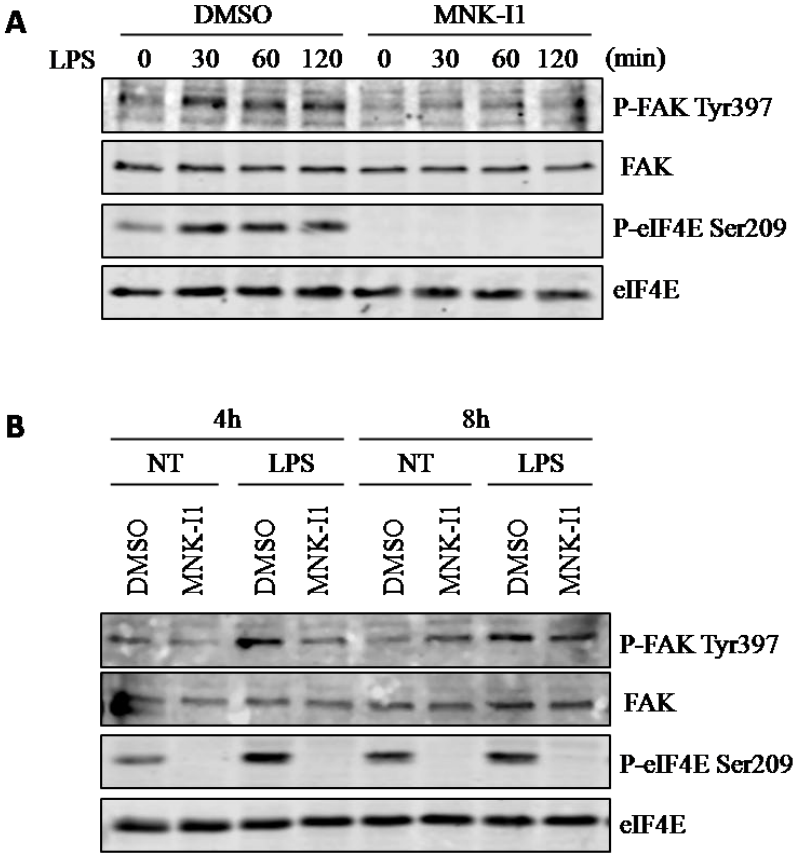

Figure 5 MNK-I1 suppressed LPS-induced Src/FAK axis activation in RAW 264.7 cells
RAW cells were pretreated with 5 μM MNK-I1 or DMSO as control for 1 h, and then treated with 200 ng/ml LPS. Cells were lysed after **(A)** 30, 60, 120 min or **(B)** 4, 8, 24 h incubation. Total proteins and their phosphorylation level were analysed by western blot with indicated antibodies.

MNK-I1 increases expression of pro-inflammatory markers in LPS-stimulated RAW 264.7 cells.

To study how MNK-I1 affects LPS-induced RAW cell activation, we examined selected cytokines and receptors. Our data show that MNK-I1 caused increased TNFα mRNA expression in RAW264.7 cells (Fig. 6A), which does not match data for the CGP57380 compound used as an MNK inhibitor in several other studies [28, 29]. This could reflect non-specific effects of CGP57380 on other protein kinases and signalling pathways which it inhibits [30]. To test this, we used CGP57380 in bone marrow-derived macrophages (BMDMs) from MNK double-knockout (DKO) mice. The results showed that within cells lacking both of the MNKs, CGP compound still blocked LPS-induced production of TNFα protein (Suppl Fig. 3). Importantly, this emphasizes the

fact that CGP57380 affects cellular components other than the MNKs in macrophages. Interestingly, MNK-I1 accelerated the induction of the MCP-1 mRNA (Fig. 6B). Together, these data suggest that MNK-I1 may promote the inflammatory response to LPS.

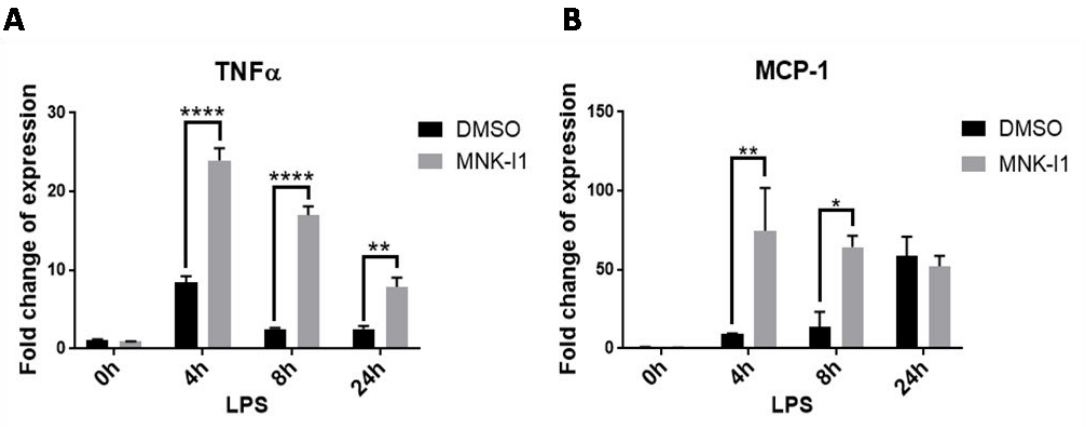

Figure 6 MNK-I1 increased pro-inflammatory markers in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were pretreated with 5 μM MNK-I1 for 1 h and then with 200 ng/ml LPS. Cells were then harvest with Trizol after 4, 8, 24 h. **(A)** TNFα and **(B)** MCP-1 mRNA expression were analysed by qPCR. The relative amount of mRNA was normalized to GAPDH. Data are shown as mean ± S.E.M. from three replicates. * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$.

Discussion

Activated macrophages play an important role in inflammatory response and relevant diseases. The morphological changes of macrophages are the hallmarks of the macrophage inflammatory response. The formation of lamellipodia and filopdia is essential for attachment to the extracellular matrix, which is required for cell adhesion and migration to inflammatory sites. In this study, we showed that MNK-I1, a potent and specific MNK inhibitor, blocks the LPS-induced morphological changes of RAW264.7 macrophages, including F-actin remodelling and lamellipodia and filopdia formation. Importantly, our results demonstrated that MNK-I1 significantly prevented LPS-induced adhesion and migration of RAW264.7 macrophages. LPS causes the activation of FAK presumably via stimulation of Src [22, 31]. FAK phosphorylation at Tyr346 promotes the formation of the Src/FAK complex, which plays a critical role in cell spreading and migration [20]. During the further investigation on mechanism, MNK-I1 showed the effective inhibition on LPS-induced phosphorylation of FAK. The

important role of Src/FAK axis in macrophage movement suggests that the attenuation of Src/FAK axis activation by MNK-I1 might contribute to the inhibition of RAW264.7 cell adhesion and migration.

In contrast, we found that MNK-I1 increased the levels of the mRNAs for TNFα and MCP-1 in LPS-induced RAW264.7 macrophages, which suggests that MNK inhibition accelerates the inflammatory response to LPS stimulation. Previous studies showed the opposite results when using a different compound, CGP57380, as an MNK inhibitor [28, 29]. Our data reveal that CGP57380 non-specifically inhibits LPS-induced TNFα mRNA level in MNK-knockout BMDMs, suggesting that CGP57380 also affects on other pathways, and is not specific for the MNKs. MNK-I1 is a more effective and specific inhibitor, which shows no effect on cell proliferation, cell death or relevant signalling pathways [7]. A study using knock-in mice expressing non-phosphorylatable eIF4E(Ser209A) has shown that abolishing eIF4E phosphorylation inhibited the translation of *Nfkbia* mRNA, which encodes the NF-κB repressor, IκBα [8]. This resulted in the enhanced activity of NF-κB and the increased production of interferon-β (IFN-β) [8]. Since the MNKs are the only physiological kinases for eIF4E, these data suggest that MNKs are involved in regulation of NF-κB activity. Given this idea, we suggest that inhibition of MNK activity, by suppressing the phosphorylation of eIF4E, promotes NF-κB signalling, followed by the induced production of cytokines response to LPS-stimulation inflammation.

Our previous research showed that (compared to WT mice) MNK2-deficient mice gain less weight, and show better insulin sensitivity and significantly decreased inflammation in adipose tissue when fed high-fat diet (HFD) [9]. However, no difference in proinflammatory marker (such as TNFα and IL-6) was detected between LPS-treated WT and MNK2-KO BMDMs [9]. Together of these results, we suggest that inhibition of MNKs promotes LPS-induced inflammation, but suppresses the whole body inflammatory response by blocking adhesion of macrophages and their migration to inflammatory tissues.

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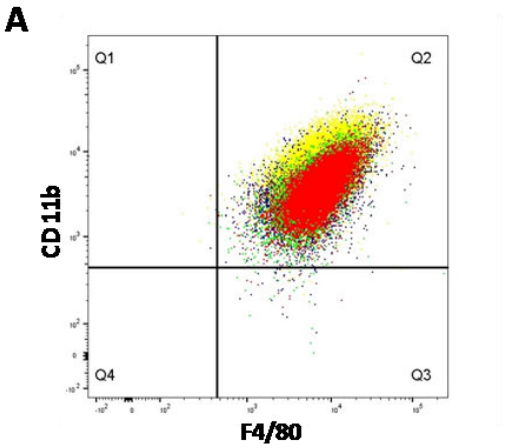
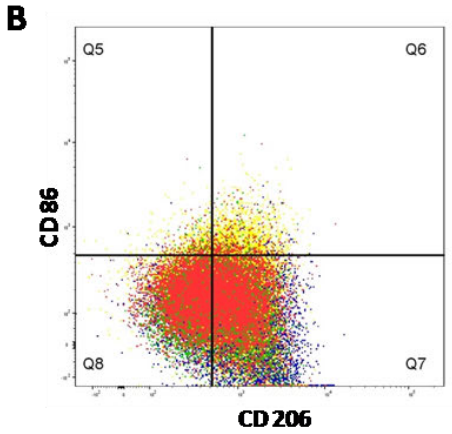
Supplementary Information

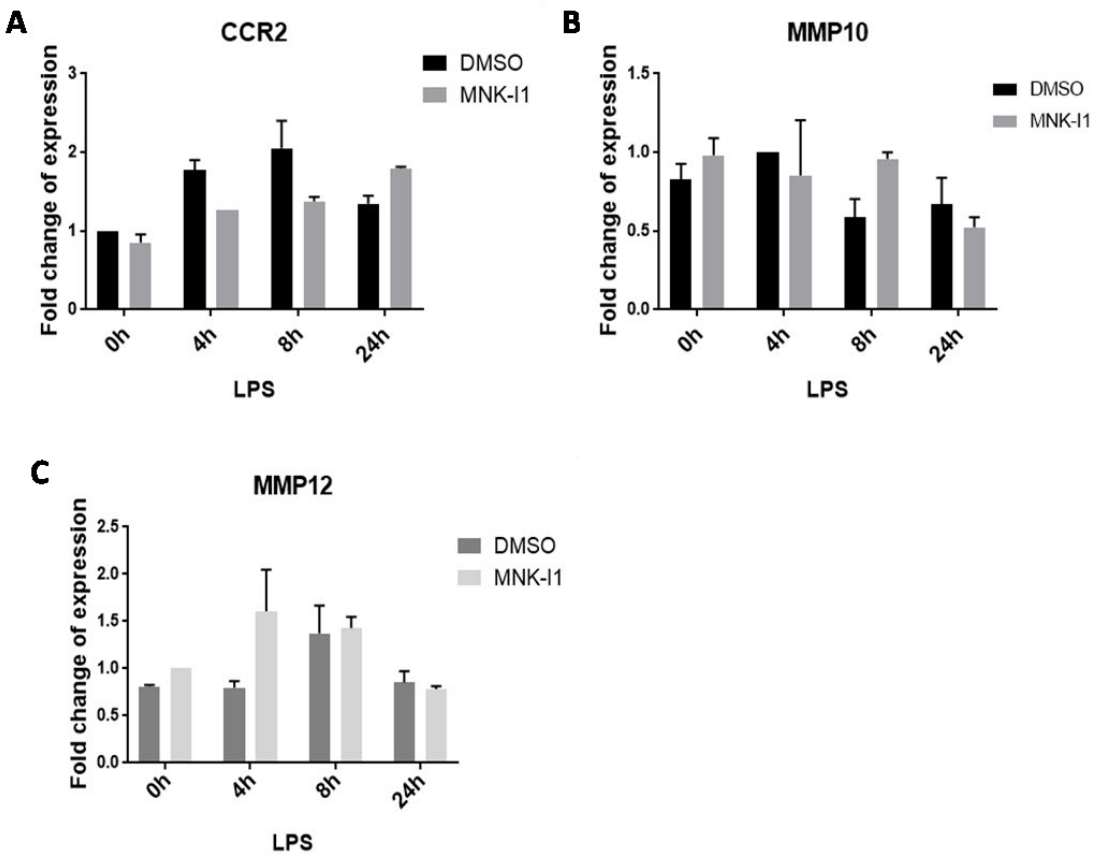


	Sample	Freq. of Parent Q2
	DMSO	99.9
	MNK-I1	99.7
	LPS DMSO	99.9
	LPS MNK-I1	99.8



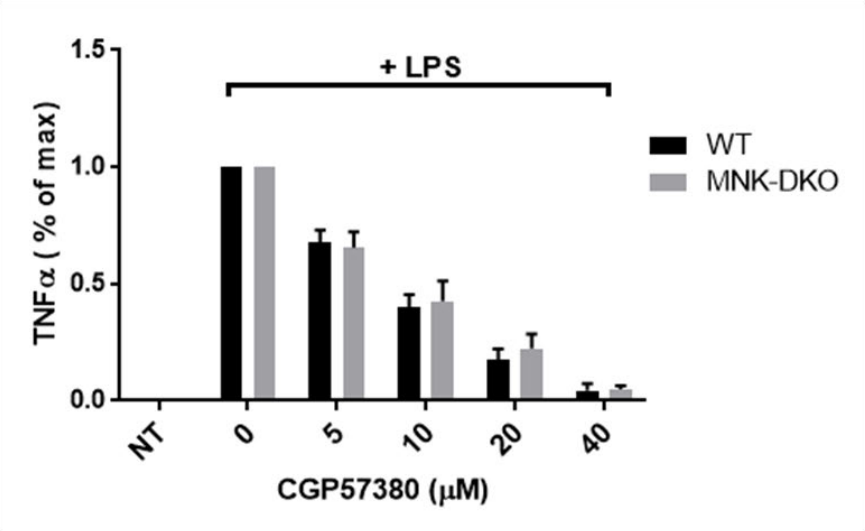
	Sample	Freq. of Parent			
		Q8	Q7	Q6	Q5
	DMSO	35.7	60.3	3.15	0.89
	MNK-I1	44.7	54.2	0.66	0.43
	LPS DMSO	20.9	61.6	14.1	3.32
	LPS MNK-I1	24.8	73.7	1.21	0.27

Suppl Figure 1. MNK-I1 did not affect the polarization of RAW 264.7 cells. RAW 264.7 cells were treated with 5 μM MNK-I1 for 1 h and followed with 200 ng/ml LPS for 24 h. Cell surface markers were analysed by FACS.



Suppl Figure 2 MNK-I1 did not affect mRNA expression of CCR2, MMP10 or MMP12 in RAW 264.7 cells.

RAW 264.7 cells were pretreated with 5 μM MNK-I1 for 1 h and then with 200 ng/ml LPS. Cells were then harvest with Trizol after 4, 8, 24 h. MRNA expression of **(A)** CCR2 and **(B)** MMP10, and **(C)** MMP12 in RAW 264.7 cells were analysed by qPCR. The relative amount of mRNA was normalized using GAPDH. Data are shown as mean ± S.E.M. from three replicates



Suppl Figure 3 CGP57380 showed similar inhibition on LPS-induced production of TNFα protein in WT and MNK-DKO BMDMs.
BMDMs were treated with 5, 10, 20, 40 μM CGP57380 for 1 h and followed with 200 ng/ml LPS for 4h. Protein levels of TNFα from WT and MNK-DKO BMDMs were measured by ELISA (n = 6–9). Data are mean ± SEM (two-way ANOVA followed by Tukey's post test).

Table.1 Sequence of Real time PCR Primers

Target		Sequence (from 5’ to 3’)
Mouse TNFα	Forward	TCTCAGCCTCTTCTCATTCCTGCT
	Reverse	AGAACTGATGAGAGGGAGGCCATT
Mouse MHC II	Forward	GACGCTCAACTTGTCCCAAAAC
	Reverse	GCAGCCGTGAACTTGTTGAAC
Mouse CCR2	Forward	ATTCTCCACACCCTGTTTCG
	Reverse	GATTCCTGGAAGGTGGTCAA
Mouse MCP-1	Forward	GCATCCACGTGTTGGCTCA
	Reverse	CTCCAGCCTACTCATTGGGATCA
Mouse MMP10	Forward	CCTGATGTTGGTGGCTTCAGT
	Reverse	CTGGTGTATAATTCACAATCCTGTAGGT
Mouse MMP12	Forward	TTGGATTATTGGAATGCTGC
	Reverse	GCACATTTTGATGAGGCAGA
Mouse GAPDH	Forward	GGTCCTCAGTGTAGCCCAAG
	Reverse	AATGTGTCCGTCGTGGATCT

Conclusions

The MAP kinase-interacting kinases (MNKs) are widely expressed in mammalian cells. They lie at the nexus of major intracellular signalling pathways, including the mammalian target of rapamycin complex 1 (mTOR1) and ERK pathways, which are involved in functions of both normal and cancer cells. The best-known and only fully-validated MNK substrate is eukaryotic translation initiation factor 4E (eIF4E), which plays an important role in protein synthesis [66]. In this thesis, novel roles of MNKs were identified and explored; these are potentially important in several diseases.

Metastasis is one of the hallmarks of an aggressive tumour, which causes most of cancer-associated mortality. Our studies revealed that MNKs are involved in cell migration. Importantly, we introduced a new MNK inhibitor, MNK-I1, which – unlike previously reported compounds - is both efficient and specific for inhibiting the MNKs. Migration was decreased in MNK-KO fibroblasts, and was also blocked by MNK-I1 in cancer cell lines. The protein level, but not the mRNA level, of a mesenchymal marker, vimentin was blocked by MNK-I1, suggesting that MNKs mediate its expression at the level of translation of its mRNA. The cytoplasmic FMRP-interacting protein 1 (CYFIP1) has been identified as an eIF4E binding partner, which is involved in mRNA translation or actin remodelling by forming different complex in cells. We also demonstrated that MNK inhibition increases the association of CYFIP1 and eIF4E, and this increase was also observed in MNK-KO MEF cells. This effect of the MNKs on CYFIP1-eIF4E binding potentially provides a molecular mechanism for the regulation of cell migration by the MNKs. Since the MNKs are not essential for animal viability, blocking MNKs by a selective inhibitor may be a potential approach to prevent tumour metastasis. Further work is needed to examine whether the MNKs do play a role in tumour metastasis *in vivo*.

During the further study of the role of the MNKs in cancer cell migration in Chapter 3, we described NDRG1 as a novel target for regulation by MNK1, which is not affected by MNK2. NDRG1 has been reported as a tumour suppressor, which play roles in several cellular processes related to oncogenesis and tumour metastasis [207]. NDRG1 undergoes phosphorylation at multiple sites including well-conserved ones which are targets for the protein kinase called serum and glucocorticoid-regulated kinase 1 (SGK1), as well as GSK3 at different sites [208]. We found that SGK1 inhibition blocks cell migration induced by NDRG1 knock down. This suggests that SGK1 promotes cell migration through other substrates. We demonstrated MNK1 is an SGK1

substrate, at least *in vitro*, and identify the SGK1 phosphorylation site as Ser 353. MNK1 is clearly also involved in promoting NDRG1 phosphorylation at Thr346/356/366, since NDRG1 phosphorylation decreases in MNK1-KO MEFs and MNK inhibitor treated breast cancer cells. However, the mechanism is not clear since MNK1 does not phosphorylate NDRG1 directly. The inhibition of cell migration by MNKs does not require NDRG1. Here, we showed the novel pathway that MNK1 is involved i.e., the phosphorylation of NDRG1. This also provides the first example of a protein which is regulated by one MNK but not the other.

Studies in Chapter 4 showed that MNKs might also be involved in inflammatory response to LPS stimulation. The MNK inhibitor, MNK-I1, blocked the LPS-induced morphological change of RAW 264.7 macrophages, and their ability to adhere and to migrate towards MCP-1. Inhibition of the MNKs also prevents the LPS-stimulated remodelling of cytoskeletal proteins, such as tubulin and F-actin. Inhibition of MNKs decreased the activity of Src/FAK axis, which plays an important role in focal adhesion and actin remodelling. Migration of macrophages into tissue is an essential step in the host response to infection. The MNK inhibitor also enhanced LPS-induced TNFα and MCP-1 mRNA levels in RAW macrophages, suggesting that inhibition of MNKs might induce the inflammatory response to LPS. Our findings reveal a novel role for the MNKs in immune and inflammatory responses. Together, the data suggest that although MNK inhibition induces the inflammatory response to LPS, it probably also blocks macrophage migration to the other inflammatory sites. The overall effect of the MNKs on inflammatory responses still needs further investigation.

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Errata sheet

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