MNK1 Modulation of hnRNP A1 in Cellular Senescence

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ABSTRACT

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Heterogeneous nuclear ribonucleoprotein (hnRNP) family members are the most abundant components of messenger ribonucleoprotein complexes (mRNPs) and play regulatory roles in a variety of biogenesis of mRNA. hnRNP A1 is a member of the hnRNP A/B subfamily, is highly abundant, and is involved in pre-mRNA and mRNA metabolism such as alternative splicing, mRNA export, splice site selection, mRNA turnover, and translation. Recent studies have shown that stress stimuli such as osmotic shock or UVC irradiation induce cytoplasmic accumulation of hnRNP A1. The cytoplasmic accumulation is concomitant with an increase in its phosphorylation and that requires p38 MAPK. We have previously demonstrated that hnRNP A1 protein shows diminished expression level and altered subcellular distribution in senescent HS74 fibroblasts. In this study, we observed that phosphorylated hnRNP A1 protein levels decreased as a result of MNK1 inhibition and that reciprocal binding occurs between hnRNP A1 and MNK1. These data implicate MNK1 as the kinase in the p38 MAPK pathway that activates hnRNP A1 in IMR-90 fibroblasts. Furthermore, we demonstrate that inhibition of MNK1 activity modulates the phosphorylation and subcellular distribution of hnRNP A1 protein. These results suggest a role for MNK1 in the regulation of hnRNP A1 in senescent cells. This is the first report, to our knowledge, that shows a link between MNK1 and cellular senescence.

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Chapter 1

Introduction: Overview of the Literature

1.1 CELLULAR SENESCENCE

Cellular senescence was first described by Hayflick as a state of irreversible cell cycle arrest seen in cultured normal human fibroblasts after a limited number of cell divisions. This phenomenon, often called the Hayflick limit or replicative senescence, is not specific to human fibroblasts but is seen in many types of cells (1). Senescent cells exhibit several characteristic features: irreversible growth arrest, flattened cellular morphology, enlarged cytoplasm and nuclei, and senescence-associated heterochromatic foci (2-4) Positive staining for senescence-associated beta-galactosidase (SA-β-gal) activity is another well-known feature of senescent cells, although SA-β-gal -positive cells have also been observed in stress-induced conditions, unrelated to the state of senescence (5). Whereas quiescent cells exhibit transient and reversible growth arrest, senescent cells are arrested in the G1/G0 phase of the cell cycle and do not enter the S phase, even when they are treated with strong mitogenic stimuli such as growth factors (4,6).

Replicative Senescence and Stress-Induced Premature Senescence

There are two types of cellular senescence: replicative senescence and stress-induced premature senescence (Fig. 1). It has been proposed that replicative senescence is induced by replication-dependent telomere shortening, which is unavoidable, usually due to lack, or low, telomerase activity in normal somatic human cells. Consistent with this, overexpression of hTERT, the catalytic subunit of human telomerase reverse transcriptase, has been shown to prevent telomere shortening and to abrogate the senescence phenotype in normal untransformed cells such as human retinal pigment epithelial cells and foreskin fibroblasts (8). Conversely, inhibition of telomerase activity

inhibits proliferation and induces apoptosis of immortal transformed cells (9). Although the molecular mechanisms by which telomere shortening induces replicative senescence have not been completely elucidated, the DNA damage response triggered by genomic dysfunction appears to be crucial for replicative senescence (8,9).

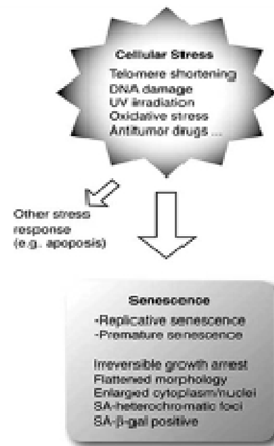


Figure 1. Cellular senescence as a stress response (7).

Cellular Senescence and Cancer

Cancer is widely known to be a disease that is triggered by the accumulation of multiple genomic mutations in cells that suffer mainly from various stressors inducing DNA damage. Apoptosis is one of the tumor-suppressive mechanisms that eliminate such damaged cells (10). Accumulating evidence suggests that cellular senescence also plays a critical role in tumor suppression. Mice deficient in p53, a major tumor suppressor with diverse functions, including induction of cellular senescence, have been shown to be susceptible to spontaneous tumorigenesis (11). Recently, it has been demonstrated that

tumorigenesis upon inactivation of another tumor suppressor, PTEN in the prostate, is suppressed through p53-dependent cellular senescence. In this model, combined inactivation of PTEN and p53 elicits early onset of invasive prostate cancer, indicating the critical role of cellular senescence in tumor suppression (12). Moreover, it has been shown that telomere dysfunction-induced cellular senescence suppresses tumor progression by a p53-dependent mechanism (13,14). These findings strongly suggest that cellular senescence, as well as apoptosis, contributes to tumor suppression (15,16).

Cellular Senescence and Aging

In addition to the beneficial role of cellular senescence in tumor suppression, possible harmful roles of cellular senescence have also been proposed. It has been reported that age-dependent accumulation of senescent cells exhibiting an increased expression of the INK4 family protein $p16^{INK4a}$ and positive SA-β-gal staining is observed in islet β-cells and forebrain progenitor cells (17,18). Accompanying these changes is a gradual decline in the integrity and function of these tissues, including islet regeneration and neurogenesis (19,20). Moreover, accumulated senescent cells often secrete several cytokines and growth factors, which may promote transformation of neighboring cells (21,22). Cellular senescence may thus facilitate the development of late-onset cancer and trigger age-related diseases (15,16) .

1.2 STRESS-ACTIVATED MAPKs IN CELLULAR SENESCENCE

The MAPK cascades are extensively studied signaling pathways that respond to stimuli from outside the cell and are evolutionarily well conserved in cells from yeasts to humans. Each cascade is typically composed of three hierarchical protein kinases:

mitogen-activated protein kinases (MAPKs), MAPK kinases (MAPKKs), and MAPKK kinases (MAPKKKs) (23,24). MAPKKKs phosphorylate and activate MAPKKs, and activated MAPKKs in turn phosphorylate and activate MAPKs (7). In mammals, there are MAPK cascades that converge on extracellular signal regulated (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAPKs (p38s) (7). Among these cascades, the ERK cascade is thought to be activated mainly by growth factors and induces cell proliferation, survival, and differentiation (7). On the other hand, JNKs and p38s were originally identified as kinases activated in response to a diverse array of cellular stressors such as proinflammatory cytokines, UV irradiation, ROS, heat and osmotic shock, and DNA damage, and are therefore called stress-activated MAPKs (7). The JNK and p38 pathways have been shown to be critically involved in the control of cellular stress responses such as cell death and survival (23,24).

The p38 MAPK Pathway in Premature Senescence

The p38 MAPK pathway has been shown to play a pivotal role in premature senescence triggered by various stressors such as overexpression of oncogenes, oxidative stress, and inappropriate culture conditions (26-28). Overexpression of oncogenic Ras has been reported to promote cell proliferation in transformed cell lines but to induce premature senescence in normal primary cells (29). Inhibition of p38 by its inhibitor SB203580 or by expression of a dominant-negative mutant of MKK3 (37; Figure 2 below) MKK6 (MAPK kinases for p38) abrogated Ras induced premature senescence, suggesting that Ras-induced premature senescence requires activation of the p38 pathway (29,30).

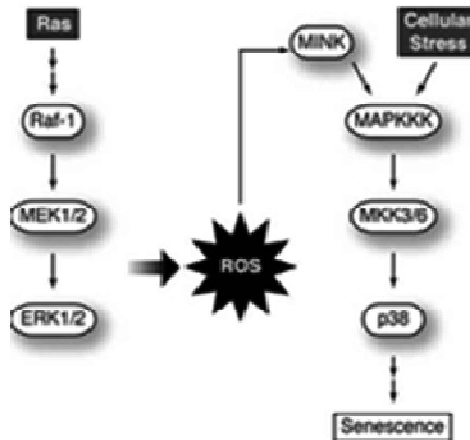


Figure 2. Involvement of p38 MAPK in senescence (7).

1.3 THE MAPK PATHWAY

MAPK Signaling Cascade

In order to perform their functions and to survive, cells need to respond to a large number of extracellular stimuli. These stimuli include mitogens, hormones, stresses, as well as changes in temperature, and osmotic pressure (31). The response of cells to these various extracellular signals is often mediated through the activation of transcription factors, which in turn, induce the necessary cellular processes. However, most extracellular signals cannot cross the plasma membrane in order to activate their

cytoplasmic or nuclear targets. Rather they use intracellular molecules to transducer the message. Many of these intracellular proteins are kinases and they amplify the original message through a cascade of phoshphorylating their target proteins, which are also kinases (7). This type of signaling is used by the mitogen-activated protein (MAP) kinase (MAPK) signaling cascades, which are evolutionarily conserved signal transduction pathways (31). Transmission of signals via these cascades is usually triggered by G-proteins, such as Ras or by activating interactions of upstream components with adaptor proteins (31).

Four MAPK cascades have been studied in detail. These are extracellular signal-regulated kinase 1 and 2 (ERK1/2; 32,33), c Jun N-terminal kinase (JNK, 34), p38 MAPK α, β, γ, and δ (35), and ERK5 (36). The four p38 MAPKs are encoded by different genes and have different tissue expression patterns, with p38α being ubiquitously expressed at significant levels in most cell types, whereas the others seem to be expressed in a more tissue-specific manner; for example, p38β in brain, p38γ in skeletal muscle and p38δ in endocrine glands (37,38). p38 MAPK family members have overlapping substrate specificities, albeit some differences have been reported, with particular substrates being better phosphorylated by p38α and p38β than p38γ and p38δ or vice versa (39).

Usually the ERK1/2 cascade plays a role in proliferation and differentiation, JNK and p38 cascades are activated mainly by cellular stresses and therefore their MAPK components are termed stress-activated protein kinases (SAPKs), and the ERK5 cascade seems to respond equally to certain stresses as well as mitogenic signals (39). However, depending on the cell lines and the stimulation, the distinct cascades may regulate

noncanonical and even opposing functions. Thus, under rare conditions, ERK1/2 may participate in the response to stress and apoptosis (40); while JNK can occasionally mediate proliferation (41). Taken together, the activation of the MAPK cascades by a large variety of stimuli indicates that these cascades are key mediators of essentially all stimulation-induced cellular processes (39). Mis-regulation of the cascades often leads to diseases such as diabetes, immune response inflammation, and cancer (42-45). Therefore, a better understanding of MAPK signaling and its regulation is warranted.

p38 MAPK

p38 MAPKs were originally identified as tyrosine phosphorylated proteins in LPS-stimulated macrophages (46,47). In mammals, four isoforms, p38 α, p38 β, p38 γ, and p38 δ, exist. The role of p38 in inflammatory and stress responses has already been well established by many studies (43,39). p38 MAPKs are activated by dual phosphorylation in the activation loop sequence Thr-Gly-Tyr (48). In response to appropriate stimuli, threonine and tyrosine residues can be phosphorylated by three dual-specificity MKKs/MAP2Ks (MAPK kinases) (49; Figure 3).

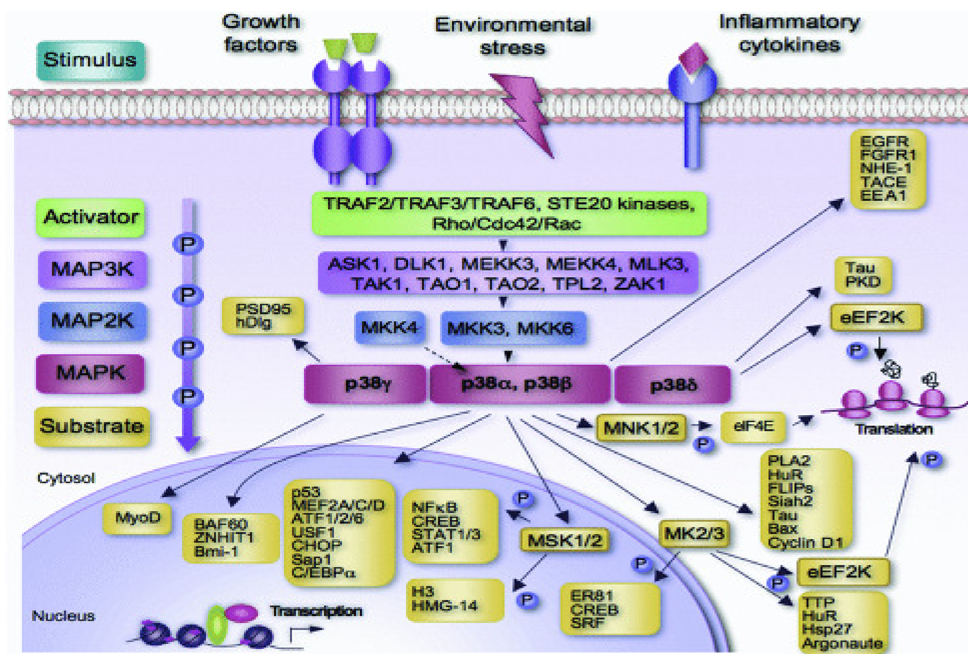


Figure 3. The Canonical p38 MAPK Pathway (50).

MKK6 can phosphorylate the four p38 MAPK family members, whereas MKK3 activates p38α, p38γ and p38δ, but not p38β (64). Both MKK3 and MKK6 are highly specific for p38 MAPKs (49,51). In addition, p38α can be also phophorylated by MKK4, an activator of the JNK (c-Jun N-terminal kinase) pathway (48). The relative contribution of different MAP2Ks to p38α activation depends on the stimulus, but also on the cell type, because of variations in MAP2K expression levels among cell types (51,52). Depending on the stress stimulus, MKK3 and MKK6 also contribute to different extents to the activation of other p38 MAPK family members (53). MAP2Ks are activated by phosphorylation in two conserved serine/threonine sites of the activation loop. The kinase domain of constitutively active MKK6, with aspartate mutations replacing the two phosphorylation sites, has been crystallized recently, revealing an auto-inhibited dimer (54). Intriguingly, this mutant kinase appears to adopt an inactive conformation despite

the presence of phosphomimetic mutations (54). Several MAP3Ks (MAP2K kinases) have been shown to trigger p38 MAPK activation, including ASK1 (apoptosis signal-regulating kinase 1), DLK1 (dual-leucine-zipper-bearing kinase 1), TAK1 [TGF (transforming growth factor) β-activated kinase 1], TAO (thousand-and-one amino acid) 1 and 2, TPL2 (tumour progression loci 2), MLK3 (mixed-lineage kinase 3), MEKK (MAPK/ERK kinase kinase) 3 and MEKK4, and ZAK1 (leucine zipper and sterile-α motif kinase 1) (50). Some MAP3Ks that trigger p38 MAPK activation can also activate the JNK pathway (50). Upstream of the cascade, the regulation of MAP3Ks is more complex, involving phosphorylation by STE20 family kinases and binding of small GTP-binding proteins of the Rho family as well as ubiquitination-based mechanisms (50). The diversity of MAP3Ks and their regulatory mechanisms provide the ability to respond to many different stimuli and to integrate p38 MAPK activation with other signaling pathways (55).

Specific MAP3Ks have sometimes been linked to particular stimuli. For example, in *Drosophila* cells, MEKK1 controls the activation of p38 MAPKs by UV or peptidoglycan, whereas heat-shock-induced activation signals through both MEKK1 and ASK1, and maximal activation of p38 MAPKs by hyperosmolarity requires four MAP3Ks (56). In mammalian cells, ASK1 plays a key role in the activation of p38α by oxidative stress (57). The underlying mechanism involves ASK1 dimerization and autophosphorylation, which is facilitated by the oxidation-mediated release of ASK1-binding proteins such as thioredoxin (58). Activation of MAP3Ks such as MEKK1 or TAK1 by members of the TNF receptor family such as CD40 is thought to require assembly of multiprotein complexes at receptor intracellular domains (59). However, a

recent report has showed that complex assembly at the receptor only primes MAP3Ks for activation, whereas activation of the kinase cascade is actually delayed until the complex is released into the cytoplasm (59).

The p38 Pathway in Replicative Senescence

The p38 pathway has also been shown to be involved in replicative senescence. In human fibroblasts undergoing replicative senescence, p38 was found to be activated, and expression of hTERT appeared to abrogate such p38 activation, suggesting that p38 is activated through telomere shortening (60). In rabbit articular chondrocytes, inhibition of p38 activity by SB203580 or by expression of dominant negative MKK6 stimulated proliferation and partially delayed the onset of senescence (61). These findings suggest that activation of the p38 pathway is involved in replicative senescence induced by telomere shortening.

Constitutive activation of p38 by expression of an active mutant of MKK3 or MKK6 induced premature senescence in primary human fibroblasts (30,60). Furthermore, depletion or inactivation of Wip1 phosphatase, a p53-regulated gene product suppressing p38 activity (62), activated the p38 pathway, and caused cells to enter premature senescence (30,60,63). These findings demonstrate that the p38 pathway plays a crucial role in inducing cellular senescence. In addition to activating the p38 pathway, oncogenic Ras also activates the Raf1-MEK-ERK pathway (30). Overexpression of a constitutively active mutant of MEK1, a MAPKK for ERKs, has been shown to activate the MKK3/6-p38 pathway in human fibroblasts, resulting in premature senescence (7). Moreover, inhibition of MEK activity by its inhibitor U0126 has been found to abolish the ability of oncogenic Ras to activate MKK3 and MKK6 (7).

These findings suggest that activation of the p38 pathway in Ras-induced premature senescence is dependent on activation of the Raf1-MEK-ERK pathway. Although the precise mechanism of this functional linkage of these two signaling cascades remains unclear, several studies suggest that reactive oxygen species (ROS) play an important role (64). Overexpression of Ras has been found to induce MEK-ERK pathway-dependent accumulation of intracellular ROS required for activation of the p38 pathway (64-66). Recently, MINK, a Ste20 family kinase, has been proposed as an activator of p38 in Ras-induced cell cycle arrest (67). MINK is activated via an unknown mechanism following Ras-induced ERK activation and ROS generation, and appears to require ASK1/MAP3K5 and Tpl-2/MAP3K8 as MAPKKKs for p38 activation in this setting (67).

p16^{INK4a} and p53 have been proposed as important molecules that are regulated through the p38 pathway (27). Activated p38 up-regulates levels of p16I^{NK4a} expression through an unknown mechanism and thus inhibits CDKs, resulting in hypophosphorylation of pRB and inactivation of E2F (30,63). On the other hand, p38 phosphorylates Ser33 and Ser46 of p53, which induces p21Cip1 expression (27,68). Recently, PRAK has been identified as a downstream effector of p38 and shown to directly phosphorylate Ser37 of p53 in Ras-induced premature senescence in primary mouse fibroblasts (69).

1.4 HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEINS

Heterogeneous nuclear ribonucleoproteins (hnRNP) make up a significant subclass of known RNP (Fig. 4). They are defined by three attributes: 1) they can be co-immunoprecipitated; 2) they show RNA binding capacity; and 3) they do not belong to

other defined classes of RNP such as small nuclear RNP (70,71). hnRNPs were first described as a group of 6 chromatin associated RNA binding proteins which bound nascent polymerase II transcripts (70). They assemble on nascent RNA polymerase II transcripts to form hnRNP complexes, where they co-localize with small nuclear (sn) RNPs (70). In fact, as nascent hnRNAs emerge from the transcriptional machinery, and during their entire nuclear life, they are associated with a set of proteins collectively termed hnRNPs that are not stable components of the nuclear RNP complexes such as snRNPs (72). They bind to specific sequences on pre-mRNA which are important for pre-mRNA processing such as the 5'and 3' splice sites and the polypyrimidine stretch, (73-75).

Subsequent co-immunopurification, gene cloning and sequence analysis revealed a group of 30 abundant proteins and other less common hnRNP isoforms (77). The number of known hnRNP continues to grow and sometimes well studied proteins are later identified as hnRNP. hnRNP's in general have multiple functions which include regulation of alternative splicing, rRNA transcription, mRNA transport, polyadenylation, mRNA stability and turnover, and telomere biogenesis (78).

The most common feature of hnRNPs is the presence of two or more RNA binding domains and an auxiliary domain believed to be responsible for protein-protein, RNA-protein, and single-stranded DNA-protein interactions (Fig. 4). Most of these hnRNPs can also form homophilic interactions and heterophilic interactions with other hnRNPs (79-81). One of the most abundant hnRNPs is hnRNP A1 (70,82). hnRNP A1 has been implicated in many alternative splicing events in human and several other eukaryotes (83,84).

Characterized hnRNP complex proteins.

Major hnRNPs	RNA-binding motifs	Proposed functions
A1/A1*	2X RBD, RGG	Splicing (exonic repressor) mRNA transport Telomere biogenesis
A2/B1	2X RBD, RGG	Splicing mRNA trafficking
C1/C2	1X RBD	Transcript packaging Splicing Nuclear retention mRNA stability
D1/D2 (AUF p42, p45)	2X RBD, RGG	Transcription Recombination mRNA turnover
E1/E2		
(PCBP-1, αCP1)*	3X KH	Translational silencing
(PCBP-2, αCP2)*	3X KH	mRNA stability
F	3X RBD	Splicing (intronic enhancer) Interacts with nuclear cap-binding protein complex
G	1X RBD, RGG†	
H	3X RBD	Spicing (intronic enhancer)
H' (DSEF-1)	3X RBD	Polyadenylation
I (PTB)	4X RBD	Splicing (intronic repressor/ enhancer) Polyadenylation
K	3X KH, RGG	Transcription Translational silencing
L	4X RBD	mRNA stability mRNA export (intron-independent)
M	3X RBD	Splicing Heat-shock response
P2 (TLS/FUS)	1X RBD, RGG	Oncogenesis
R	3X RBD, RGG	
U	1X RGG	Nuclear retention
HAP/SAF-B‡	1X RBD	Scaffold attatchment factor‡ Transcription‡ Heat-shock response‡
Minor hnRNPs		
A0	2X RBD, RGG†	Splicing§
CUG-BP¶ (hNab50)	3X RBD	Splicing (intronic enhancer) Myotonic dystrophy mRNA translation**
HuR#	3X RBD	mRNA stability mRNA transport

Figure 4. Characteristics of hnRNPs (RGG:Arginine/Glycine Box) (76).

The hnRNP A/B family together constitutes 60% of the hnRNP present in a cell (85). The family includes multiple proteins and splice variants, most notably hnRNP A1, A2, B1 and B2. The hnRNP A/B family shares significant structural homology, each member has two RNP-motif RNA-binding domains and a variable glycine-rich auxiliary domain at the carboxyl terminus (70). All hnRNP A/B family proteins shuttle dynamically between the nucleus and cytoplasm (86). However, different proteins and splice variants have shown different expression profiles across cell types (87) and have been implicated in a variety of tasks depending on the expression of the specific splice variant.

In addition to their influence on splicing through recognition of specific binding sites within target RNAs, subcellular localization of hnRNP proteins has emerged as a mechanism to regulate splicing. While essentially nuclear at steady state, several hnRNP proteins (hnRNP A1/A2, K, D, E, and I) continuously shuttle between the nucleus and cytoplasm (88).

hnRNP A1

hnRNP A1 protein and its splice variants comprise the most common gene products in the hnRNP A/B family (85). HnRNP A1 regulates alternative splicing of RNA polymerase II transcripts in conjunction with the serine/arginine rich protein family (88-91). Splice variant Unwinding Protein 1 (UP1) plays a critical role along with the Telomerase Reverse Transcriptase (TERT) and the Telomerase-Associated Protein 1 (TEP1) in forming the telomerase holoenzyme responsible for telomere maintenance and promoting cell line immortalization (92-94). Several reports directly link changes in hnRNP A1 expression to the cellular response to DNA damage inducing agents, UV (95-96) and cisplatin (97). Others suggest that hnRNP A1 is affected by more general cellular stresses such as hypoxia (98), heat shock (99) and osmotic stress (96,99).

Human hnRNP A1 is a 320-amino-acid protein, of which the 196-amino-acid N-terminal domain comprises two RNA recognition motifs (RRMs) (100). The 124-amino-acid C-terminal domain is glycine rich and is believed to be responsible for cooperative binding, leading to repression of splicing (101,102). At present, there are no available structures of intact hnRNP A1, but there are high-resolution crystal structures of its N-terminal domain spanning RRM1 and RRM2, which is known as the unwinding protein 1 (UP1) (101-104).

hnRNP A1 shuttles through the action of a bidirectional transport peptide called M9 (105). While the export activity of M9 is not well understood, nuclear import of hnRNP A1 is mediated by the interaction of the M9 peptide with the import receptors transportin 1 and 2 (Trn1/2) (106-108). Import can be regulated, for example, in response to osmotic stress where activation of the p38 MKK3/6/p38 signaling pathway results in hyperphosphorylation of hnRNP A1 in a region near the M9 sequence, which disrupts the interaction with transportin and results in cytoplasmic accumulation (109-110).

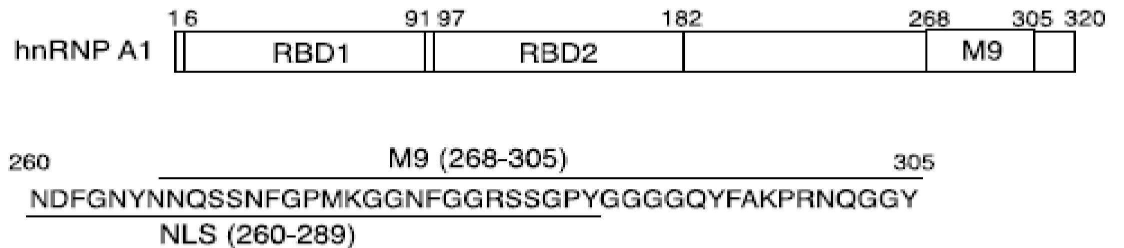


Figure 5. Structure of hnRNP A1 (111).

It has been shown that activation of the p38 $MKK_{3/6}$/p38 stress-signaling pathway in mammalian cells results in both hyperphosphorylation and cytoplasmic accumulation of hnRNP A1 and affects alternative splicing regulation (96). Allemand and colleagues mapped the stress-induced phosphorylation sites in hnRNP A1 to a stretch of serines located adjacent to the M9 motif, which mediates bidirectional transport of hnRNP A1 (109.). This phosphorylation event abrogates interactions between hnRNP A1 and its import receptor, transportin, resulting in its cytoplasmic accumulation (109).

Immunofluorescence studies have revealed cytoplasmic accumulation of hnRNP A1, induced by shortwave ultraviolet (UV-C) irradiation (180 J/m2), in 3-15% of NIH-

3T3, COS, 293 and HeLa cell lines and is detectable 2 hours following treatment and peaking at 5 hours (96). Osmotic stress in the same study resulted in similar cytoplasmic accumulation of hnRNP A1 (96). The authors showed that p38 kinase activation was both necessary and sufficient to cause cytoplasmic accumulation of hnRNP A1 (96). Other studies have reported stress granule accumulation of hnRNP A1, observed by immunofluorescence, in NIH-3T3 cells in response to heat shock, a high osmolarity environment and oxidative stress (99).

Properties of hnRNP A1

As a consequence of enhanced protein stability, hnRNP A1 expression is induced by p210 BCR/ABL in a dose- and kinase-dependent manner (110). The *bcr/abl* fusion gene, formed by rearrangement of the breakpoint cluster region (bcr) on chromosome 22 with the c-abl proto-oncogene on chromosome 9, is present in virtually all CML patients (112). In fact, hnRNP A1 levels are higher in chronic myelogenous leukemia-blast crisis (CML-BC) than chronic myelogenous leukemia - chronic phase (CML-CP) progenitors (110). The use of a nucleus-localized dominant negative mutant hnRNP A1 deficient in shuttling activity indicated that the mRNA export activity of hnRNP A1 is required for cytokine-independent proliferation, survival, and tumorigenesis of acute phase CML blasts and BCR/ABL-expressing myeloid progenitor cell lines (110). Thus, hnRNP A1 controls the nuclear export of mRNAs encoding factors important for the leukemic phenotype of CML-BC progenitors. Indeed, the antiapoptotic factor BCL-X_L and SET, the physiologic inhibitor of protein phosphatase 2A (PP2A), are among the cytoplasmic mRNAs whose export and translation are a consequence of hnRNP A1 shuttling activity (110).

Several observations suggest that hnRNPs are important for telomere biology. First, hnRNP A1, hnRNP D and hnRNPs C1/C2 are capable of interacting with the human telomerase holoenzyme (113,114). Second, hnRNPs A1, A2-B1, D and E and hnRNP homologous proteins from other organisms can associate with the single stranded telomeric repeat sequence *in vivo* (115-117). Third, hnRNPs including those found to associate with telomerase and telomeres are integral components of the nuclear matrix (118-122). The nuclear matrix is a putative attachment site for telomeres making it possible that hnRNPs and telomeres are in close proximity if not directly associated (123,124). Fourth, short telomeres in hnRNP A1 deficient mouse CB3 cells are elongated after reconstituting hnRNP A1 expression (125). This effect of hnRNP A1 on telomere length is not due to A1-induced alternative splicing, since expressing the proteolytic fragment of hnRNP A1, UP1, that does not influence alternative splicing, also increases telomere length in the hnRNP A1 deficient mouse cells (114).

It has been shown that osmotic shock or UVC irradiation induce cytoplasmic accumulation of hnRNP A1 (96,99). The cytoplasmic accumulation is concomitant with an increase in its phosphorylation and requires p38 MAPK (99). We have previously demonstrated that hnRNP A1 protein levels show diminished expression and altered subcellular distribution in senescent HS74 fibroblasts (126,127). These findings raise the possibilities that there is a relationship between hnRNP A1 and p38 MAPK proteins and suggest that hnRNP A1 may play a significant role in cellular senescence under the control of p38 MAPK pathway. However, the precise molecular mechanisms by which this pathway might regulate hnRNP A1 have yet to be identified.

We have previously shown that hnRNP A1 and p38 MAPK interact *in vivo* and that the p38 MAPK pathway regulates the expression level and subcellular distribution of hnRNP A1 (128). Inhibition of p38 MAPK increased the level of hnRNP A1 protein expression in young and G0-arrested IMR-90 cells suggesting a p38 MAPK-dependent regulation (128). Our findings of decreased protein expression and increased phosphorylation of hnRNP A1 during senescence indicate that the p38 MAPK pathway might regulate the stability of hnRNP A1 protein via phosphorylation (128). We have also shown that the phosphorylation level of hnRNP A1 was elevated in senescent cells (128).

In the experimental goals outlined in this study, we investigate the molecular mechanisms responsible for the regulation of hnRNP A1 downstream of p38 MAPK. Furthermore, evidence for involvement of MNK1 as a putative modulator of hnRNP A1 protein levels will be examined. We hypothesize that MNK1 regulates the phosphorylation and the subcellular distribution of hnRNP A1 and that MNK1 may play a role in the induction of senescence.

The research findings of the current project are important because they strive to shed light on diseases that affect the aging United States population. These disorders include, but are not limited to, Alzheimer's' disease, Parkinson's disease, diabetes, heart disease, cataract, and cancer. More research is warranted to elucidate the molecular, genetic, and environmental mechanisms that regulate how the aging process predisposes individuals to the diseases above.

1.5 Protein Kinases and hnRNP A1

Protein kinases are key regulators of cell function that constitute one of the largest and most functionally diverse gene families. Protein phosphorylation was discovered as a regulatory mechanism by Krebs and colleagues in the late 1950s through their discovery of phosphorylase kinase (129). The eukaryotic protein kinases comprise a large superfamily of homologous proteins. They are related by virtue of their kinase domains (also known as catalytic domains), which consist of approximately 250-300 amino acid residues (130). The kinase domains that define these proteins contain 12 conserved subdomains that fold into a common catalytic core structure (130). There are two main subdivisions within the superfamily: the protein-serine/threonine kinases and the protein-tyrosine kinases (130). These enzymes use the γ-phosphate of ATP or GTP to generate phosphate monoesters using protein alcohol groups (Ser and Thr) and/or protein phenolic groups (Tyr) as phosphate acceptors (130). By adding phosphate groups to substrate proteins, they direct the activity, localization and overall function of many proteins, and serve to orchestrate the activity of almost all cellular processes (130). Kinases are particularly prominent in signal transduction and co-ordination of complex functions such as the cell cycle. The diversity of essential functions mediated by kinases is shown by the conservation of some 50 distinct kinase families between yeast, invertebrate and mammalian genomes (131). Of the 518 human protein kinases, 478 belong to a single superfamily whose catalytic domains are related in sequence (131). This corresponds to nearly 2% of the entire genome and demonstrates the extraordinary importance of this family for regulating biological events (132). A further 40 'atypical' kinases have no sequence similarity to typical kinases, but are known or predicted to have

enzymatic activity, and some are predicted to have a similar structural fold to typical kinases (131). Human protein kinases are arranged in groups containing a variety of family members. For example, the AGC group contains PKA and PKC; the CMGC group contains MAPK and CDK; CK1 contains casein kinase; group TK are the tyrosine kinases; group STE contains homologs of yeast sterile kinases; and TKL are the tyrosine kinase-like proteins (133).

Phosphorylation is a well documented post-translational modification with regulatory functions that affect the properties of a wide range of many hnRNP core proteins (134,135). hnRNP A1 protein has been shown to serve as a substrate for the catalytic subunits of cAMP-dependent protein kinas A (PKA), casein kinase II (CKII), protein kinase C (PKC), and AKT (136-138). Initially, research demonstrated that hnRNP A1 was phosphorylated *in vitro* by an endogenous unidentified protein kinase associated with a 40 S hnRNP complex (135). There are a few reports on the regulation of hnRNP A1 by phosphorylation *in vivo*. One explanation for this is that it is difficult to detect. The data suggests that phosphorylation of hnRNP A1 occurs on a serine residue, Ser 199, in the glycine-rich C-terminal domain of hnRNP (136,137). This region of hnRNP A1 protein has been shown to support the cooperative binding of the protein to single-stranded DNA and RNA and to be required for the annealing reaction (86). To explain such activity it has been suggested that the protein-protein binding capacity of this domain (139) might be responsible for an increase of the local concentration of Al-coated strands that can in turn lead to the rapid renaturation of complementary sequences (140,141). Phosphorylation may alter the carboxyl terminus of the protein in such a way that it interferes with the capacity of protein-protein interaction of A1 (136). It is

conceivable that phosphorylation of A I might have a modulatory effect in pre-mRNA processing, for example, by facilitating and/or destabilizing RNA -RNA base pairing (136).

Phosphorylation of hnRNP A1 by the above kinases affects the function of the protein. Cobianchi and colleagues demonstrated that phosphorylation of hnRNP A1 by PKA resulted in the suppression of the ability of protein A1 to promote strand annealing *in vitro*, without any detectable effect on its nucleic acid binding capacity (136). AKT negatively regulates hnRNP A1-mediated internal ribosome entry site (IRES) activity via phosphorylation at Ser^{199} (138). Furthermore, AKT was shown to directly regulate the ability of hnRNP A1 to promote cyclin D1 and c-myc IRES activity via phosphorylation (138). This data suggests that serine 199 phosphorylation of hnRNP A1 inactivates its IRES trans-acting factor function for the cyclin D1 and c-myc IRESs (138). It is not known whether the p38 MAPK pathway regulates hnRNP A1-mediated cyclin D1 and c-myc IRES activity. It is known, however, that inhibiting hnRNP A1 expression sensitizes quiescent AKT-containing cells to rapamycin (138). This data implicates the hnRNP A1-cyclin D1 or c-myc IRES interactions as potential targets for intervention and suggest that the serine 199 phosphorylation state of hnRNP A1 may be an effective indicator of tumor cell responses to mTOR inhibitors (138). A recent report used a phosphomimetic mutant of hnRNP A1 (S199E) that is capable of binding both the cyclin D1 and c-MYC IRES RNAs *in vitro* but lacks nucleic acid annealing activity, resulting in inhibition of IRES function in dicistronic mRNA reporter assays (142). Utilizing cells in which AKT is conditionally active, it was demonstrated that overexpression of this mutant renders quiescent AKT-containing cells sensitive to rapamycin *in vitro* and in xenografts (142).

Furthermore, it was shown that activated AKT is strongly correlated with elevated Ser(P)(199)-hnRNP A1 levels in a panel of 22 glioblastomas (142). These data demonstrate that the phosphorylation status of hnRNP A1 serine 199 regulates the AKT-dependent sensitivity of cells to rapamycin and functionally links IRES-transacting factor annealing activity to cellular responses to mTOR complex 1 inhibition (142).

PKC phosphorylates hnRNP A1 at three sites: two in the N-terminal domain (spanning residues 2-196) and one in the C-terminal domain (spanning residues 197-320) (137). Amino acid sequencing revealed that these sites were Ser95, Ser192, and Ser199; phosphorylation at Ser192 was more abundant than at Ser95 and Ser199 (137). Phosphorylation by PKC inhibited the strand annealing activity of A1 (137). A conformational change in the C-terminal domain of A1 was observed upon PKC phosphorylation, and this was associated with a decrease in A1's affinity for single-stranded polynucleotides (137). These results are consistent with a role for the phosphorylation of A1 in regulating its strand annealing activity *in vivo*.

Chapter 2 Materials and Methods

2.1 Cells and culture conditions

Experiments were carried out with young, pre-senescent, and senescent IMR-90 fibroblasts. This cell strain was derived from fetal lung fibroblasts and cells were cultured at 37°C in DMEM-HAM media supplemented with 10% fetal bovine serum (FBS) and 1% penicilllin/streptomycin (Mediatech, Gibco-Life Technologies, Manassas, VA). Cells were kept in a 37°C, 5% CO_2 incubator. Media was changed twice weekly and cells were serially passaged at a ratio of 1:4 for IMR-90 cell until terminal passage was reached (129). IMR-90 fibroblasts undergo up to 70 population doublings before reaching a senescent state. Population doublings were calculated as follows: it was determined that each 1:4 split represents two population doublings for previous experiments in which cell numbers were determined at each passage (143).

2.2 Cell lysis and protein quantification:

Each culture was rinsed 3 times in ice cold 1X phosphate buffered saline (PBS;137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 x $7H_2O$, 1.4 mM KH_2PO_4,), pH 7.4, and 0.75 mL of cold 1% Empigen BB lysis buffer (Sigma, St. Louis, MO) in 1X PBS containing 1mM EDTA, 0.1mM DTT, 10 mM NaF, and 0.1mL of Phosphatase Inhibitor Cocktail I (Sigma) was added to each 100mm culture dish. Cells were scraped and lysed on ice by sonication three times for 5 sec each. Lysates were collected by centrifugation at 10,000 rpm at 4ºC for 5 min. Supernatants were then transferred into fresh 1.5 mL Eppendorf tubes. Protein concentration was quantified in triplicate using the RC DC Protein Assay (Bio-Rad, Hercules, CA).

2.3 Western blot analysis:

Western blot analysis was performed using standard western procedures (144). Cell lysates were prepared in 1X SDS-lysis buffer [62.5 mM Tris-HCl (pH 6.8) at 25°C, 2% SDS, 10% glycerol, 50 mM DTT, 0.01% w/v Bromophenol Blue] and sonicated 3 times for 5 sec and stored at -80°C. . Protein concentration was quantified in triplicate using the RC DC Protein Assay (Bio-Rad, Hercules, CA). Cell lysates were suspended in protein sample loading buffer (145), separated by 10% SDS-PAGE, and electrophoresed at 160 volts until the dye front was at the end of the gel. Proteins were transferred onto PVDF membranes in transfer buffer (10X Tris-Glycine and methanol). Nonspecific binding was blocked by incubating the membranes in blocker (1X PBS, 0.1% Tween-20) and 5% nonfat dry milk at room temperature for 1 h. Membranes were incubated in the appropriate dilution of primary antibodies in 3% bovine serum albumin (BSA), 0.1% Tween-20 in 1X PBS overnight at 4°C. Blots were washed three times for 5-10 min per wash in wash buffer (0.1% Tween-20 in 1X PBS) followed by incubation in appropriately diluted horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (GE Healthcare, Piscataway, NJ) for 1 h at room temperature. The blots were washed three times in 1X PBS-Tween-20 for 10 min each in wash buffer (0.1% Tween-20 in 1X PBS). The SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL) kit was used for antigen detection. For a positive control for p38 MAPK expression, lysates were treated with 5 µM Anisomycin (Sigma), a well-known activator of p38 MAPK for 30 min (146).

The primary antibodies used were the following: 4B10 for hnRNP A1, generously provided by Dr. Serafin Pinol-Roma; anti-phospho-serine antibody (Zymed, San

Francisco, CA), for anti-phospho-hnRNP A1; anti-β-actin antibody (Millipore, Billerica, MA); anti-GAPDH antibody (CHEMICON, Temecula, CA); anti-phospho-eIF4E (Cell Signaling, Danvers, MA); anti-eIF4E (Cell Signaling); anti-phospho-MNK1 (Cell Signaling); anti-MNK1 (Cell Signaling), anti-phospho-p38 MAPK (Cell Signaling); anti-p38 MAPK (Cell Signaling); anti-hsp27 (Cell Signaling); anti-phospho-hsp27 (Cell Signaling); anti-hsp25 (Thermo Fisher Scientific), anti-phospho-hsp25 (Millipore, Billerica, MA), anti-hnRNP A0 (Cell Signaling), anti-phospho-hnRNP A0 (Millipore), anti-MAPKAPK2 (Cell Signaling); and anti-phospho-MAPKAPK2 (Cell Signaling).

2.4 Immunodetection of phosphorylated hnRNP A1

Cells were prepared under conditions that disrupt the hnRNP core complex: these were lysed in 0.5ml 1% Empigen BB detergent with added phosphatase inhibitors, and sonicated for 5 sec, 3 times each. Samples were cleared at 14,000*g* for 10 min at 4ºC and incubated with 4B10- Protein-A-sepharose beads at 4ºC for 1 h. The immune complex was collected and extensively washed with lysis buffer. Beads were drained of excess buffer with a loading tip and resuspended in 2X sample loading buffer containing dithiothreitol. Immunoprecipitation reactions were subjected to 12.5% SDS-PAGE, transferred to PVDF membranes, and blocked for 1 h in 3% BSA in 1X PBS-0.5% Tween-20. Membranes were washed in wash buffer (0.5%T-20 1XPBS) 3 times for 10 min followed by incubation in 1µg/mL anti-phosphoserine antibody (Zymed) for 1 h at room temperature. The membranes were washed 3 times for 10 min and incubated in a 1:2000 dilution of 2° HRP-anti-rabbit antibody. Membranes were washed 3 times 10 minutes and bands visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific).

2.5 Immunocytochemistry:

IMR-90 cells were fixed with 4% paraformaldehyde for 20 min at room temperature and washed in 1X PBS 3 times. Cells were permeablized with 0.2% TrintonX-100 for 10 min at room temperature. Slides were washed three times with 1X PBS for 5 min and blocked using 10% FBS/PBS at room temperature for 40 min. Slides were treated with monoclonal antibodies diluted in 10% FBS/PBS (blocking buffer) and incubated overnight at 4°C: for hnRNP A1, 4B10 (Kindly donated by Dr. Pinol-Roma; 1:1000), MNK1 (1:200; Cell Signaling), and MAPKAPK2 (1:200; Cell Signaling). Slides were washed 3 times in 1X PBS for 5 min on a shaker at room temperature. Slides were then incubated for 1 h at room temperature with either Alexa Fluor 594 or Alexa Fluor 488 secondary antibodies (Invitrogen, Carlsbad, CA). Slides were washed 3 times with 1X PBS for 5 min at room temperature and mounted in ProLong Gold Antifade Reagent with DAPI (Invitrogen, Carlsbad, CA). Images were captured using a Zeiss Axioplan fluorescence microscope at a magnification of 400 X.

2.6 Inhibition of kinase activities:

Inhibition of p38 MAPK kinase activity

In order to observe the effects of p38 MAP kinase inhibition on total hnRNP A1 levels in young and senescent IMR-90 fibroblasts, cells were treated with 10 μM SB203580 (Santa Cruz Biotechnology, Santa Cruz, CA) or dimethyl sulfoxide (DMSO) equivalent for 8 days at 37°C. Lysates were collected in Empigen buffer. The levels of hnRNP A1 in SB203580- or DMSO-treated IMR-90 cells were assayed by immunobloting of normalized whole cell lysates prepared from young and senescent cells treated with 10 μM SB203580 for 1 h.

Inhibition of MNK1 kinase activity

Young and senescent IMR-90 cells were treated with varying concentrations of CGP 57380 (Sigma, St. Louis, MO; 40 μM were used for the immunocytochemistry studies) or an equal volume of DMSO 3 or 8 days at 37°C. Lysates were collected in Empigen buffer.

Inhibition of MAPKAPK2 kinase activity

Young and senescent IMR-90 cells were treated with varying concentrations of MK2a (EMD Chemicals, Gibbstown, NJ), a specific inhibitor of MAPKAPK2a or an equal volume of DMSO 3 or 8 days at 37°C. Lysates were collected in Empigen buffer.

In separate studies, young and senescent IMR-90 cells were treated with varying concentrations of KKKALNRQLGVAA (EMD Chemicals), a 13-residue inhibitor of hsp25 kinase (MAPKAPK2) or an equal volume of DMSO 3 or 8 days at 37°C. Lysates were collected in Empigen buffer.

2.7 Inhibition of *in vivo* p38 MAP kinase activity

In order to observe the effects of *in vivo* p38 MAP kinase inhibition on total hnRNP A1 levels in IMR-90 fibroblasts were treated with 2.5 μM SB203580 or DMSO equivalent for 1 h at 37°C. Lysates were collected in the appropriate lysis buffer for whole cell and co-immunoprecipitation analysis. The levels of hnRNP A1 in SB203580 or DMSO-treated IMR-90 cells were assayed by immunoblotting of normalized whole cell lysates prepared from cells treated with 2.5 μM SB203580 for 1 h. Cells prepared under the same experimental conditions were immunoprecipitated with immobilized phospho-p38 MAP kinase (Thr180/Tyr182) beads, subjected to 12% SDS-PAGE and

immunoblotted with the 4B10 antibody.

2.8 Immunoprecipitation:

Whole cell lysates were immunoprecipitated for hnRNP A1 or MNK1 using 4B10monoclonal and MNK1 antibodies, respectively, via the Dynabeads® Protein A kit (Invitrogen). Dynabeads were resuspended by pipetting. 50 µL of Dynabeads was transferred to1.5 mL tubes, placed on a magnet, and the supernatant was removed. The Dynabeads were resuspended in 200 µL of antibody binding and wash buffer and pre-incubated with 5 uL of 4B10 antibody or 10 uL of MNK1 antibody for 30 min at 4ºC. Complexes were then incubated with 500 µg of lysates for 3 h at 4ºC. Immunoprecipitates were centrifuged at 14,000 x*g* for 5 min, washed 5 times with 200 µL of wash buffer per wash, then resuspended in 1X sample buffer (62.5 mM Tris-HCl, pH 6.8 at 25°C, 2% w/v SDS, 10% glycerol, 50 µM dithiothreitol, 0.01% bromophenol blue), and immediately subjected to 12 % SDS-PAGE.

2.9 siRNA inhibition for MNK1 and MAPKAPK2

siRNA MNK1 oligonucleotides were purchased from Santa Cruz Biotechnology and MAPKAPK2 oligonucleotides were purchased Thermo Fisher Scientific or Lonza. IMR-90 cells were seeded at a density of 2.5 x 10^5 cells in a 60 mm plate. After 24 h of incubation at 37° C, when the cells reached approximately 60% confluence, transfection of cells with RNA oligos for MNK1 was performed using oligofectamine (Invitrogen) according to the manufacturer's instructions. Briefly, 20-80 pmol siRNA was diluted into 100 µL siRNA Transfection Medium. 2-8 µL of siRNA Transfection Reagent was diluted into 100 µl siRNA Transfection Medium. Cells treated without siRNA oligonucleotides

were used as a control. Western analysis using either MNK1 or MAPKAPK2 antibodies were used to confirm knockdown of the proteins.

Chapter 3. The Role of p38 MAP Kinase Pathway in hnRNP A1 Protein Regulation

3.1 Introduction

Heterogeneous nuclear ribonucleoprotein (hnRNP) family members are the most abundant components of messenger ribonucleoprotein complexes (mRNPs) and play regulatory roles in a variety of biogenesis of mRNA (76,77). Over 24 major proteins designated A1-U (34 kDa-120 kDa) have been identified in hnRNP complexes and the proteins found in the core hnRNP complex are hnRNP's A, B, and C in the 30-43 kDa range (70). hnRNP A1 is a member of the hnRNP A/B subfamily, is highly abundant, and is involved in pre-mRNA and mRNA metabolism such as alternative splicing, mRNA export, splice site selection, mRNA turnover, and translation (70,90,91,147-151,86). In addition, hnRNP A1 has nucleocytoplasmic shuttling activity (86) and this is thought to be required for cell proliferation, differentiation, and survival of normal and transformed cells (110).

Recent studies have shown that stress stimuli such as osmotic shock or UVC irradiation induce cytoplasmic accumulation of hnRNP A1 (96,99). The cytoplasmic accumulation is concomitant with an increase in its phosphorylation that requires p38 MAPK (96). We have previously demonstrated that hnRNP A1 protein shows diminished expression level and altered subcellular distribution in senescent HS74 fibroblasts (126,127). Furthermore, we demonstrated that hnRNP A1 forms a complex with phosho-p38 MAPK *in vivo* and its expression level and subcellular distribution are regulated by the p38 MAPK pathway in IM-90 human fibroblasts (derived from fetal lung) (128). These findings raise the possibilities that there is a relationship between hnRNP A1 and p38 MAPK proteins and suggest that hnRNP A1 plays a significant role in cellular

senescence under the control of p38 MAPK. However, the precise molecular mechanisms by which the p38 MAPK pathway might regulate hnRNP A1 have yet to be identified.

3.2 Results

A. Effect of MAPKAPK2 inhibitors

The relationship between hnRNP A1 and p38 MAPK mentioned above indicates that a member of the p38 MAPK pathway may regulate hnRNP A1 protein levels via phosphorylation. hnRNP A1 does not appear to be a direct substrate for p38 MAPK (96). Thus, we wanted to identify putative kinases in the p38 MAPK pathway that might be responsible for phosphorylating hnRNP A1 in IMR-90 human fibroblasts. As for candidate kinases, we selected two downstream targets of p38MAPK, MAPKAPK2 and MNK1.

To assess this, we initially used inhibitors for MAPKAPK2 and MNK1 and determined the levels of protein expression of hnRNP A1 and known direct substrates of these kinases via immunoblot analysis. We first examined the effect of MAPKAPK2 inhibition on the protein expression levels of proteins that are downstream of p38 MAPK by using MK2a, a specific inhibitor of MAPKAPK2a. MK2a is a reversible *p*-amidophenolic compound that selectively inhibits the phosphorylation of MK2a (mitogen-activated protein kinase-activated protein kinase 2a; K_i^{app} = 330 nM) by p38α in a non-ATP-competitive manner. It does not block the kinase activity of p38α towards the other two known p38 substrates, MBP and ATF-2 (EMD Chemicals Web site). IMR-90 cells were incubated in the presence or absence of MK2a for 30 min, 3 h, or 24 h at varying concentrations. We examined phospho-MAPKAPK2 and MAPKAPK2 protein levels by western blot analysis and found that the phospho-MAPKAPK2 protein levels

were relatively unchanged after 30 min of treatment (Figure 6A), reduced after 3 h treatment (Figure 6B), and increased after 24 h treatment (Figure 6C) compared to total MAPKAPK2 levels Therefore, our results are inconclusive in regards to the effect of MAPKAPK2 inhibition on phospho-MAPKAPK2 protein levels.

We next examined the effect of MAPKAPK2 inhibition on the phosphorylation levels of hsp27, a known substrate for MAPKAPK2 in cells after 30min, 3 h or 24h of treatment. Phospho-hsp27 protein levels were relatively unchanged compared to total hsp27 levels after 30 min and 3 h of treatment (Figures 7A and 7B). However, phospho-hsp27 protein levels were decreased at the highest concentration of MK2a, 20 μM, after 24 h of treatment (Figure 7C). These data indicate that the affect of MK2a on MAPKAPK2 kinase activity was inconsistent in IMR-90 cells.

We next examined the effects of a different inhibitor of MAPKAPK2, an hsp25 inhibitor, on MAPKAPK2 kinase activity. This inhibitor is a 13-residue, cell-permeable peptide that acts as a potent and selective inhibitor of mammalian heat-shock protein (Hsp25) kinase [also called mitogen-activated protein kinase-activated protein kinase-2 (MAPKAP kinase-2)]. Inhibition is competitive with respect to the substrate peptide (K_i = 8.1 μM) and non-competitive with respect to ATP (K_i = 134 μM) (EMD Chemicals Web site).

IMR-90 cells were treated with the hsp25 inhibitor. Cells were incubated for various times followed by the measurement of the protein expression levels of phospho-hnRNP AO and hnRNP AO via immunoblot analyses. We detected no reduction in phospho-hnRNP A0 levels compared to total hnRNP A0 levels after 3 h, 24 h, and 8 days of treatment (Figures 8A, 8B, and 8D). We were only able to detect a decrease in

phospho-A0 protein levels after 2 days of treatment (Figure 8C). These results suggest that the hsp25 inhibitor is an insufficient inhibitor because it produced variable results, depending on the timing of the analysis. Since we achieved variable results with both MAPKAPK2 inhibitors, we decided to look at a more direct method of inhibiting MAPKAPK2 kinase activity, such as using siRNA.

B. Effect of MAPKAPK2 downregulation on hnRNP A1 protein levels

In these studies, we did not detect any effects by the two MAPKAPK2 inhibitors on MAPKAPK2 kinase activity in IMR-90 cells. Therefore, to ascertain if MAPKAPK2 was still a candidate kinase that may phosphorylate hnRNP A1, we next performed siRNA knockdown analysis of MAPKAPK2. We transfected IMR-90 cells with various concentrations of MAPKAPK2 oligonucleotides and transfection reagents from different vendors followed by incubation for various days (Figures. 9 and 10). We observed dramatic reductions in MAPKAPK2 protein levels when we transfected 100 μM and 80 μM of MAPKAPK2 oligonucleotides and used the reagent from Lonza and Qiagen, respectively (Figure. 10), indicating that we were able to successfully downregulate the expression of the target gene.

We next wanted to examine the levels of hnRNP A1 protein in IMR-90 cells transfected with MAPKAPK2 siRNAs. hnRNP A1 protein levels were relatively unchanged in siRNA transfected cells (Figure 11). This result suggests that MAPKAPK2 does not modulate endogenous hnRNP A1 protein levels in IMR-90 cells.

C. Effect of MAPKAPK2 downregulation on hnRNP A1 phosphorylation

To determine whether or not hnRNP A1 might be a substrate for MAPKAPK2,

we performed MAPKAPK2 knockdown by siRNA tranfection and determined phospho-hnRNP levels followed by immunoprecipitation with the 4B10 hnRNP A1 antibody. The membranes were probed with a phosphoserine antibody to detect phospho-hnRNP A1. We detected a band representing phospho-hnRNP A1 in IMR-90 cells transfected with MAPKAPK2 siRNA oligonucleotides (Figure 12A). This band did not decrease with increasing concentration of MAPKAPK2 siRNA oligonucleotides. This result suggests that MAPKAPK2 does not phosphorylate hnRNP A1. Immunoprecipitation of hnRNP A1 was confirmed by reprobing these membranes with the 4B10 hnRNP A1 monoclonal antibody (Figure 12B).

D. Effect of MNK1 inhibition on hnRNP A1 expression

We next wanted to investigate the possibility that another downstream kinase in the p38 MAPK pathway, MNK1, may regulate hnRNP A1 expression. We have previously demonstrated that inhibitor for p38 MAPK kinase activity upregulated hnRNP A1 protein levels in young IMR-90 cells (128). To examine the possibility that MNK1 regulates hnRNP A1 protein levels, we blocked MNK1 kinase activity using the following inhibitor: 4-Amino-5-(4-fluoroanilino)-pyrazolo[3,4-d]pyrimidine (CGP 57380). Young and senescent IMR-90 cells were treated for 24 h or three days in the presence or absence of CGP 57380 followed by western analysis for the detection of phosphorylation levels of eIF4E, which is a downstream target of MNK1. The data demonstrate that 40 μM CGP 57380 for three days blocked kinase activity, as indicated by the disappearance of the 25 kDa band in young cells that corresponds to phospho-eIF4E (Figure 13B). Likewise, CGP 57380 at 20 μM and 40 μM blocked MNK1 kinase

activity in senescent cells (Figure 13C). Our results indicate that MNK1 kinase is active in both young and senescent fibroblasts.

We subsequently determined whether or not inhibition of MNK1 kinase activity affected the expression levels of hnRNP A1 protein. We blocked MNK1 kinase activity using CGP 57380 in young and senescent cells and conducted western blot analyses with the 4B10 monoclonal hnRNPA1 antibody. The levels of endogenous hnRNP A1 in young were not altered by CGP 57380 compared to DMSO treated cells (Figures 14A-C). These data suggest that MNK1 inhibition does not change hnRNP A1 protein levels in young cells. However we did detect a decrease in hnRNP A1 protein levels at higher CGP 57380 doses in senescent cells. MNK1 may not alter the expression levels of hnRNP A1 in young cells, but it may regulate hnRNP A1 protein levels in senescent cells via a p38 MAPK independent pathway.

We next investigated the possibility that inhibition of MNK1 affects hnRNP A1 protein levels after activation of the p38 MAPK pathway. To examine this possibility, IMR90 cells were treated in the presence or absence of 5 μM anisomycin, a well-known activator of p38 MAPK (132) for 30 min and MNK1 kinase activity was blocked for 1 h using CGP 57380 (Figure 15). CGP 57380 inhibition did not alter hnRNP A1 protein levels in the absence (Figure 15A) nor in the presence (Figure 15B) of anisomycin. These results suggest that MNK1 activity may not alter hnRNP A1 levels under activation of the p38 MAPK pathway. To obtain more reliable results, we will need control data to compare hnRNP A1 levels resulting from plus or minus anisomycin treatment alone versus a longer duration (3 days) of CGP 57380 treatment.

E. Effect of MNK1 inhibition on hnRNP A1 phosphorylation

Because hnRNP A1 was observed to be phosphorylated by the p38 MAPK pathway (109), we determined whether there is a physical interaction between phosho-hnRNP A1 and MNK1 by co-immunoprecipitation assays. Young IMR-90 cells were treated in the presence or absence of varying concentrations of CGP 57380 for 3 days. The cell lysates were then immunoprecipitated with the hnRNP A1 specific monoclonal antibody, 4B10. Subsequently, membranes were probed with a phospho-serine antibody. A 34 kDa band was detected, representing phoshohnRNP A1 (Figure 16). The phosphorylation level of hnRNP A1 decreased with increasing concentrations of CGP 57380 (Figure 16A), indicating that MNK1 kinase activity is required for phosphorylation of hnRNPA1 *in vivo*. Immunoprecipitation of hnRNP A1 by 4B10 antibody was confirmed by reprobing these membranes with antibodies specific for hnRNP A1 (Figure 16B).

F. Interaction of MNK1 with hnRNP A1

We next investigated whether or not there is a physical interaction between hnRNP A1 and MNK1 using co-immunoprecipitation studies in the presence or absence of CGP 57380. Cell lysates were immunoprecipitated with 4B10 antibody and then the membranes were probed with MNK1 antibody. MNK1 co-immunoprecipitated with hnRNP A1 as indicated by a 50 kDa band in the complex, Figure 17, DMSO (lane 3) and 40 µM (lane 5). Taken together, the data from Figure 16 suggest that phosphorylation of hnRNP A1 is not required for its interaction with MNK1.

G. Inhibition of MNK1 expression

To assess the effect of downregulation of MNK1 expression on hnRNP A1, we performed siRNA knockdown analysis of MNK1. We transfected IMR-90 cells with different MNK1 siRNA oligonucleotides followed by incubation for time points. We then determined the protein levels of MNK1 by western blot analysis (Figure 18). MNK1 protein levels were not reduced in any condition we examined. Further studies are needed to determine the experimental condition that effectively down regulates MNK1 by siRNA inhibition.

We have found that in contrast to MAPKAPK2, MNK1 is capable of phosphorylating hnRNP A1 in fibroblasts. These results show the specificity in the p38 MAP Kinase signaling pathway in the phosphorylation of hnRNP A1 *in vivo.*

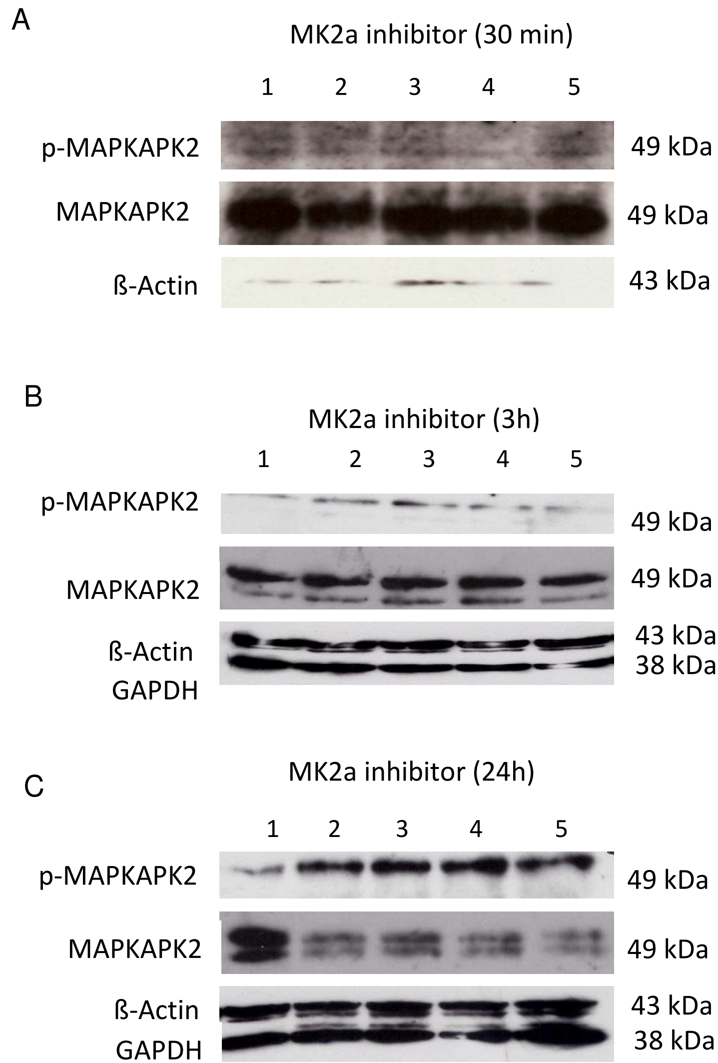


Figure 6. Effect of MAPKAPK2 Inhibition on p-MAPKAPK2 and MAPKAPK2 Protein Expression Levels. IMR-90 fibroblasts were incubated with either DMSO or the indicated concentrations of MK2a for 30 min (A), 3 h (B) or 24 h (C). Lane 1 (DMSO-treated cells); Lanes 2-5 represent MK2a inhibitor-treated cells at the following concentrations: 5, 10, 15, and 20 µM, respectively. Whole cell lysates were prepared using SDS lysis buffer and protein concentrations were normalized. 60 µg of lysates were simultaneously subjected to 10% SDS-PAGE and immunoblotted with either phosphoMAPKAPK2 and MAPKAPK2 specific antibodies. The blots were then stripped and reprobed for total actin and GAPDH levels. The blots are representative of at least three independent experiments.

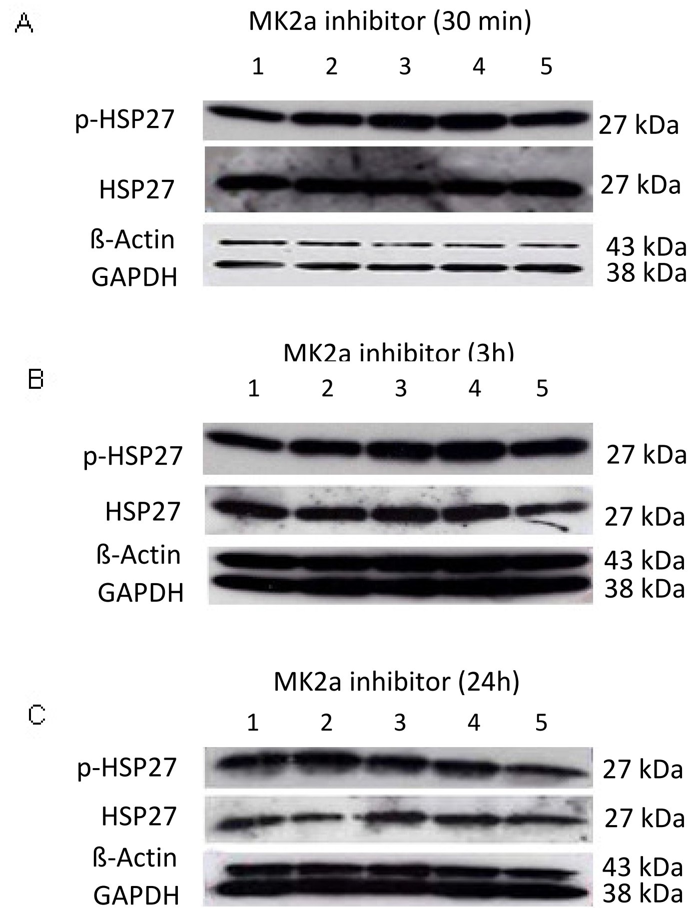


Figure 7. Effect of MAPKAPK2 Inhibition on p-hsp27 and hsp27 Protein Expression Levels. IMR-90 fibroblasts were incubated with either DMSO or the indicated concentrations of MK2a for 30 min (A), 3h (B) or 24 h (C). Lane 1 (DMSO-treated cells); Lanes 2-5 represent MK2a inhibitor-treated cells at the following concentrations: 5, 10, 15, and 20 μM, respectively. Whole cell lysates were prepared using SDS lysis buffer and protein concentrations were normalized. 60 μg of lysates were simultaneously subjected to 10% SDS-PAGE and immunoblotted with either phospho-hsp27 or hsp27 specific antibodies. The blots were then stripped and reprobed for total actin and GAPDH levels. The blots are representative of at least three independent experiments.

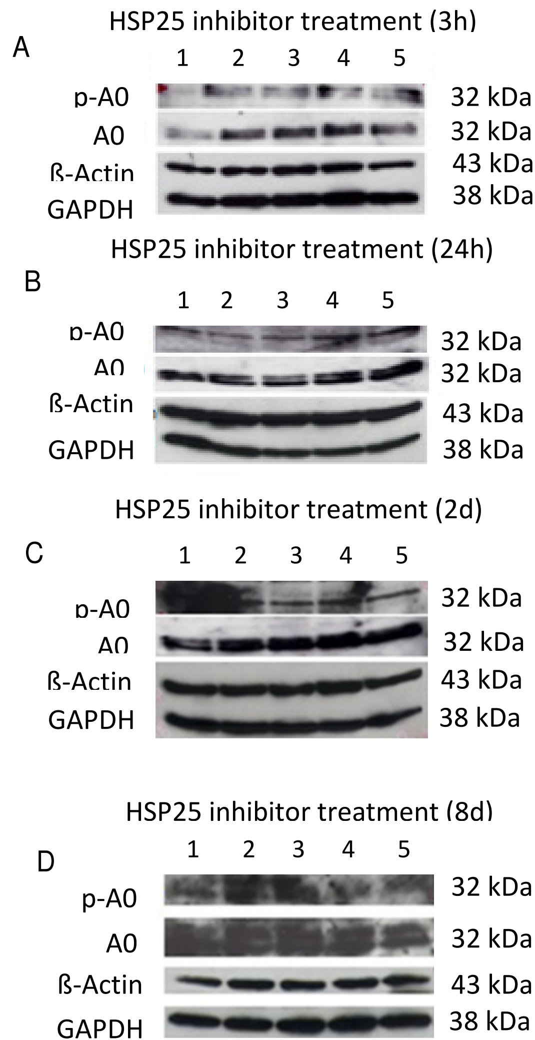


Figure 8. Effect of hsp25 Inhibitor on p-hnRNP A0 and hnRNP A0 Protein Expression Levels. IMR-90 fibroblasts were incubated with either DMSO or the indicated concentrations of hsp25 inhibitor for 3h (A), 24h (B), 2 days (C) or 8 days (D). Lane 1 (DMSO-treated cells); Lanes 2-5 represent MK2a inhibitor-treated cells at the following concentrations: 5, 10, 15, and 20 µM, respectively. Whole cell lysates were prepared using SDS lysis buffer and protein concentrations were normalized. 60 µg of lysates were simultaneously subjected to 10% SDS-PAGE and immunoblotted with either phospho-hnRNP A0 or hnRNP A0 specific antibodies. The blots were then stripped and reprobed for total actin and GAPDH levels. The blots are representative of at least three independent experiments.

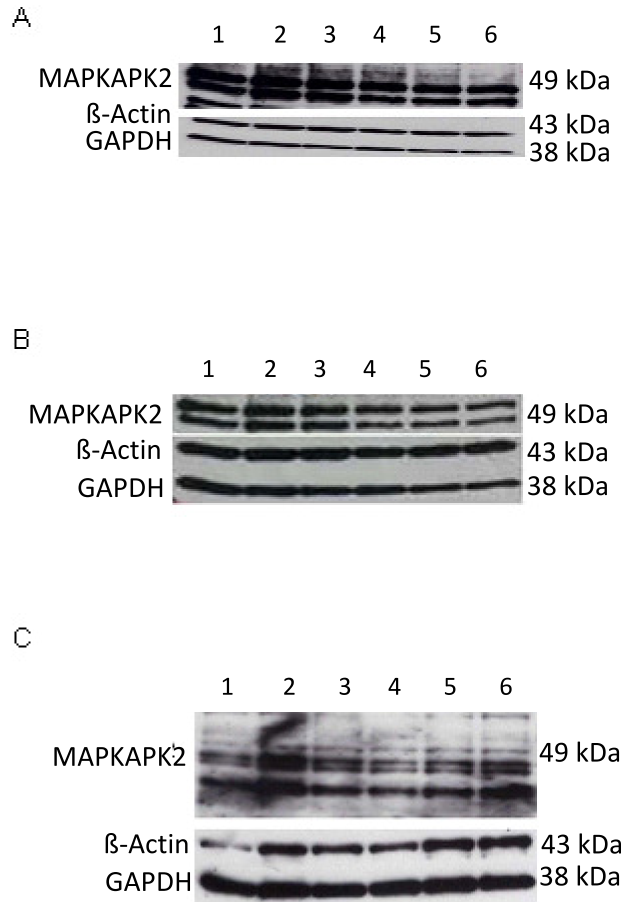


Figure 9. siRNA Inhibition of MAPKAPK2 Expression in IMR-90 Fibroblasts. IMR-90 cells were transfected with siRNA against MAPKAPK2 from Thermo Fisher Scientific or Santa Cruz overnight and incubated in fresh regular media for various days. Control cells were treated with oligofectamine in the absence of siRNA. The cells were harvested followed by isolation of protein for western blot analysis. 30 µg of each total cell lysate was loaded onto 10% SDS-PAGE gels and then immunoblotted with MAPKAPK2 specific antibodies. The blots were then stripped and reprobed for total actin and GAPDH levels. Panel A: lane 1 (untreated), lane 2 (-siRNA), lane 3 (scrambled), lane 4 (50 nM), lane 5 (100 nM), and lane 6 (100 nM new and old). Panel B: lane 1 (untreated), lane 2 (-siRNA), lane 3 (scrambled), lane 4 (50 nM), lane 5 (100 nM), and lane 6 (100 nM new and old). Panel C: lane 1 (untreated), lane 2 (-siRNA), lane 3 (scrambled), lane 4 (100 nM), lane 5 (200 nM), and lane 6 (300 nM). The blots are representative of at least three independent experiments.

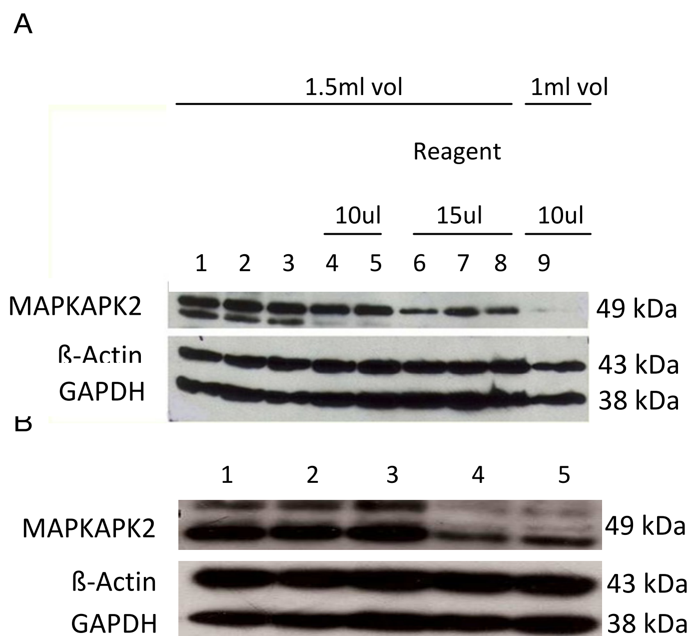


Figure 10. siRNA Inhibition of MAPKAPK2 Expression in IMR-90 Fibroblasts. IMR-90 cells were transfected with siRNA against MAPKAPK2 from Thermo Fisher Scientific overnight and incubated in fresh regular media for 3 days. Control cells were treated with oligofectamine in the absence of siRNA. The cells were harvested followed by isolation of protein for western blot analysis. 30 µg of each total cell lysate was loaded onto 10% SDS-PAGE gels and then immunoblotted with MAPKAPK2 specific antibodies. The blots were then stripped and reprobed for total actin and GAPDH levels. Lane 1 (-oligo/-siRNA), lane 2 (+oligo/-siRNA), lane 3 (scrambled), lane 4 (100 nM), lane 5 (200 nM), lane 6 (100 nM), land 7 (200 nM), lane 8 (400 nM), and lane 9 (400 nM). The blots are representative of at least three independent experiments.

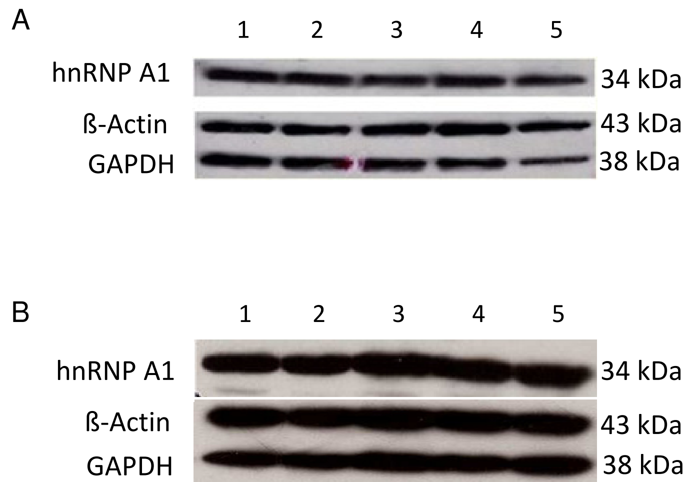


Figure 11. Effect of siRNA Inhibition of MAPKAPK2 on hnRNP A1 Expression in IMR-90 Fibroblasts. IMR-90 cells were transfected with siRNA against MAPKAPK2 and incubated in fresh regular media for three days. Control cells were treated with oligofectamine in the absence of siRNA. The cells were harvested followed by isolation of protein for western blot analysis. 5 µg of each total cell lysate was loaded onto 10% SDS-PAGE gels and then immunoblotted with the 4B10 monoclonal antibody. The blots were then stripped and reprobed for total actin and GAPDH levels. Panel A: lane 1 (untreated), lane 2 (-siRNA), lane 3 (scrambled), lane 4 (100 nM), and lane 5 (200 nM). Panel B: lane 1 (untreated), lane 2 (-siRNA), lane 3 (scrambled), lane 4 (80 nM), and lane 5 (160 nM). The blots are representative of at least three independent experiments.

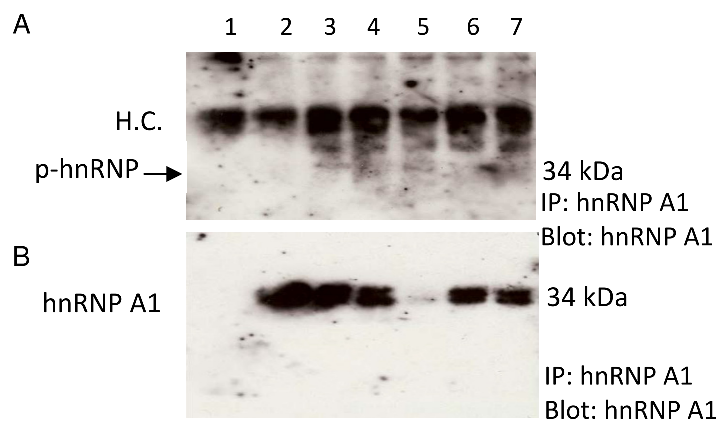
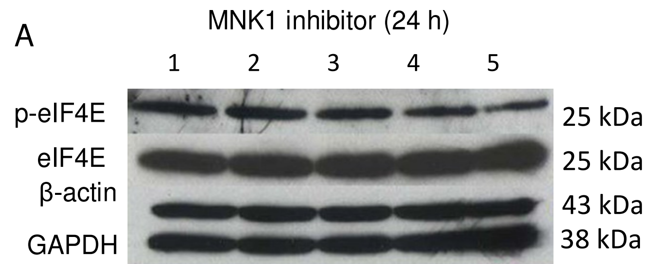
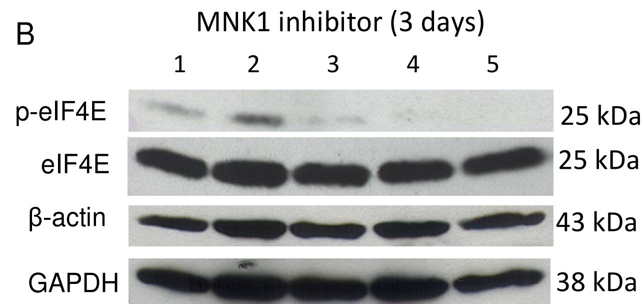


Figure 12. Effect of siRNA Inhibition of MAPKAPK2 on hnRNP A1 phosphorylation in IMR90 Fibroblasts. IMR-90 cells were transfected with siRNA against MAPKAPK2 and incubated in fresh regular media for three days. Control cells were treated with oligofectamine in the absence of siRNA. The cells were harvested followed by isolation of protein for western blot analysis. Cell lysates were immunoprecipitated with MAPKAPK2 antibody beads, subjected to 12% SDS-PAGE followed by immunoblotting with phosphoserine antibody to detect phosphohnRNP A1 (A). Mock IP was included to establish the molecular weight of the antibody heavy and light chain, 55 and 25 kDa, respectively. This reaction contained antibody and beads only. Blots were stripped and reprobed using 4B10-hnRNP A1 antibody to determine the total protein levels of hnRNP A1 (B). Lane 1 (mock), lane 2 (untreated), lane 3 (-siRNA), lane 4 (scrambled), lane 5 (100 μM), lane 6 (200 μM), and lane 7 (400 μM). The blots are representative of at least three independent experiments.

Young





Sen

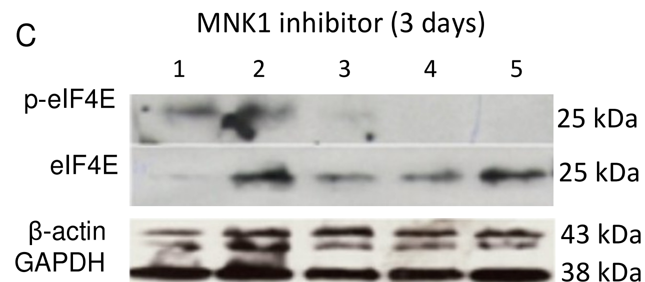
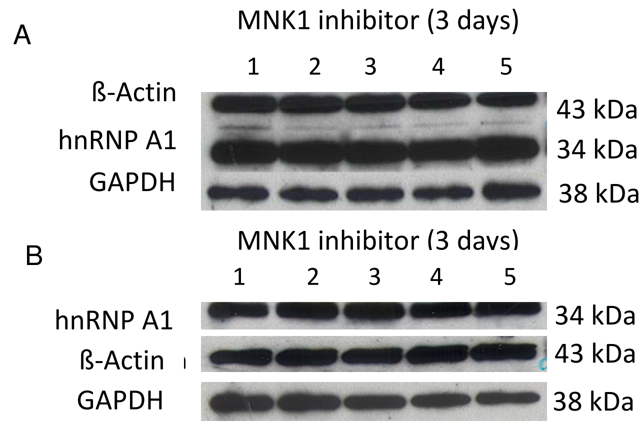


Figure 13. Effect of MNK1 Inhibition on p-eIF4E levels. Young (A and B) and Senescent (C) IMR-90 fibroblasts were incubated with either DMSO (lane 1) or 5 µM, 10 µM, 20 µM, and 40 µM of the MNK1 inhibitor, CGP 57380, lanes 2-5, respectively for 24 h or three days. Whole cell lysates were prepared using SDS lysis buffer and protein concentrations were normalized. 60 µg of lysates were simultaneously subjected to 10% SDS-PAGE and immunoblotted with P-eIF4E and eIF4E specific antibodies. The blots were then stripped and reprobed for total actin levels. The blots are representative of at least three independent experiments.

Young



Sen

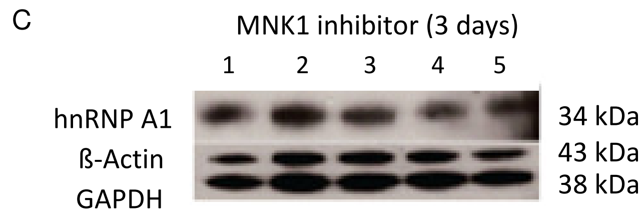


Figure 14. Effect of MNK1 Inhibition on hnRNP A1 levels. Young (A and B) and Senescent (C) IMR-90 fibroblasts were incubated with either DMSO (lane 1) or 5 µM, 10 µM, 20 µM, and 40 µM of the MNK1 inhibitor, CGP 57380, lanes 2-5, respectively for 24 h or three days. Whole cell lysates were prepared using SDS lysis buffer and protein concentrations were normalized. 5 µg of lysates were simultaneously subjected to 10% SDS-PAGE and immunoblotted with hnRNP A1 specific 4B10 antibody. The blots were then stripped and reprobed for total actin levels. The blots are representative of at least three independent experiments.

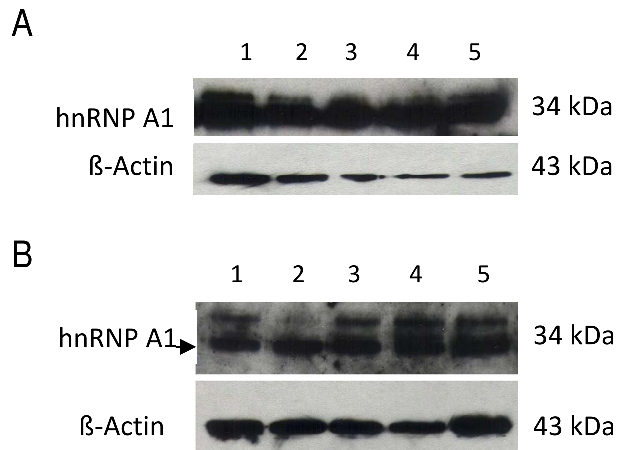


Figure 15. Effect of MNK1 Inhibition on hnRNP A1 Protein Levels in the Presence or Absence of Anisomycin. IMR-90 fibroblasts were incubated with either DMSO (lane 1) or 5 µM, 10 µM, 20 µM, and 40 µM of the MNK1 inhibitor, CGP 57380, lanes 2-5, respectively for 1 h in the absence (A) or presence (B) of 5 µM of Anisomycin. Whole cell lysates were prepared using SDS lysis buffer and protein concentrations were normalized. 10 µg of lysates were simultaneously subjected to 10% SDS-PAGE and immunoblotted with hnRNP A1 specific antibodies. The blots were then stripped and reprobed for total actin levels. The blots are representative of at least three independent experiments.

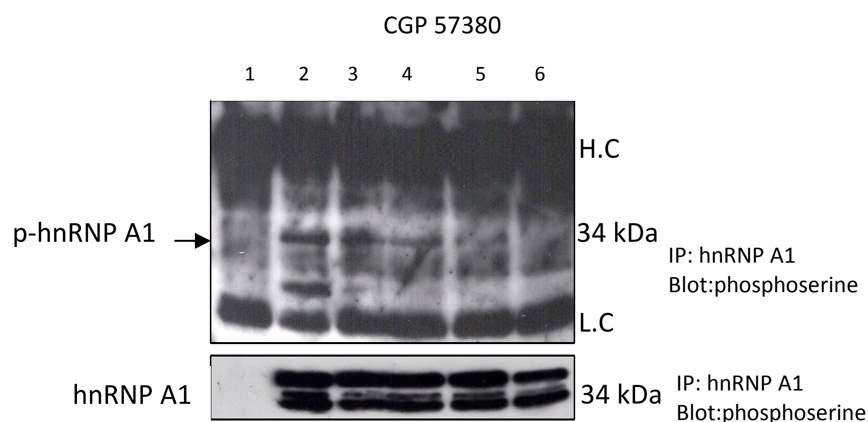


Figure 16. Effect of MNK1 inhibition on phosphorylated hnRNP A1 levels. Young IMR-90 cells were treated in the absence, lane 1 (mock) and lane 2 (DMSO), or presence of varying concentrations, lanes 3-6 of CGP 57380: 5 µM, 10 µM, 20 µM, and 40 µM. Cell lysates were immunoprecipitated with the 4B10 hnRNP A1 antibody immobilized beads, subjected to 12% SDS-PAGE followed by immunoblotting with phosphoserine antibody to detect phospho-hnRNP A1. Mock IP reactions without lysates were included to establish the positions of the antibody heavy and light chains. Blots were stripped and reprobed with the 4B10 monoclolnal antibody to detect total hnRNP A1 (lower panel). The blots are representative of at least three independent experiments.

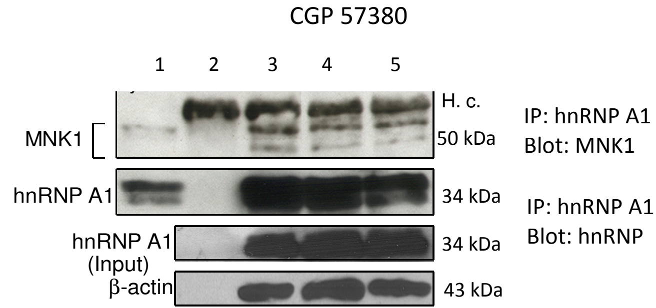


Figure 17. MNK1 forms a complex with hnRNP A1 *in vivo*. Total lysates from young IMR-90 cells were treated in the absence, lane 1 (lysates), lane 2 (mock), and lane 3 (DMSO), or presence of varying concentrations of CGP 57380: 20 μM (lane 4) and 40 μM (lane 5), respectively. Lysates were immunoprecipitated using the hnRNP A1 antibody, subjected to 12% SDS-PAGE, and probed with a MNK1 specific antibody. Blots were stripped and reprobed with the hnRNP A1 monoclonal antibody (A, lower panel) to detect total hnRNP A1. The blots are representative of at least three independent experiments.

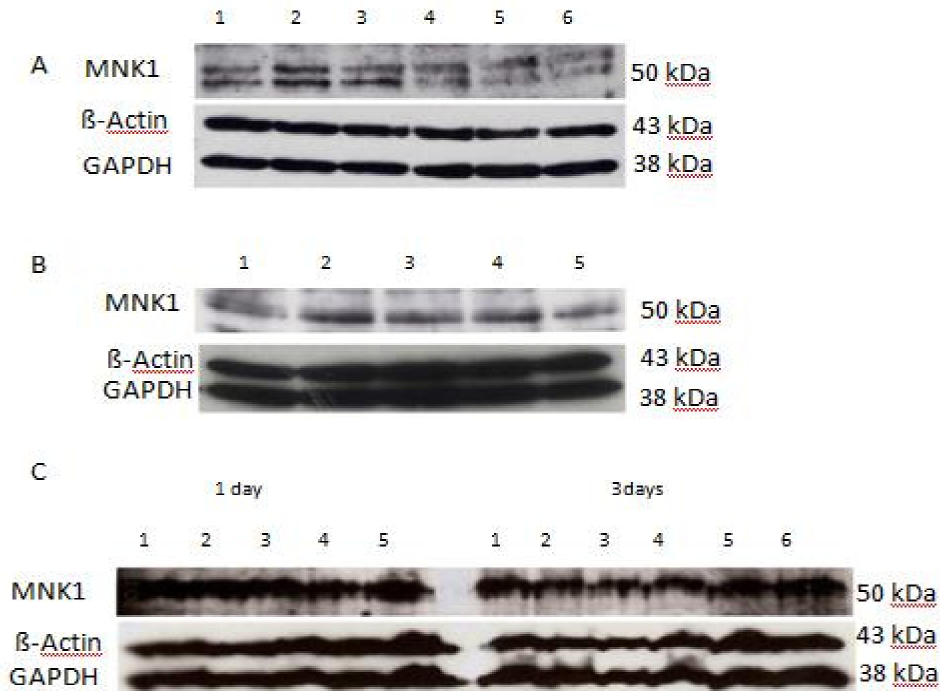


Figure 18. Effect of siRNA Inhibition of MNK1 in IMR-90 Fibroblasts. IMR-90 cells were transfected with siRNA against MNK1 and incubated in fresh regular media for various days. Control cells were treated with oligofectamine in the absence of siRNA. The cells were harvested followed by isolation of protein for western blot analysis. 60 µg of each total cell lysate was loaded onto 10% SDS-PAGE gels and then immunoblotted with the MNK1 specific antibody. The blots were then stripped and reprobed for total actin and GAPDH levels. Panel A: lane 1 (untreated), lane 2 (-siRNA), lane 3 (scrambled), lane 4 (100 nM), lane 5 (200 nM), and lane 6 (300 nM). Panel B: lane 1 (untreated), lane 2 (-siRNA), lane 3 (scrambled), lane 4 (50 µM), and lane 5 (100 µM). Panel C, left side: lane 1 (scrambled), lane 2 (8 siRNAs), lane 3 (9 siRNAs), lane 4 (10 siRNAs), and lane 5 (11 siRNAs); right side: lane 1 (scrambled), lane 2 (8 siRNAs), lane 3 (9 siRNAs), lane 4 (10 siRNAs), lane 5 (11 siRNAs), and lane 6 (Mixture of siRNAs from lanes 2-5). The blots are representative of at least three independent experiments.

3.3 Discussion/Summary

Although hnRNP A1 accumulates predominantly in the nucleus, it shuttles continuously between the nucleus and the cytoplasm. Some but not all serine-arginine (SR) proteins also undergo nucleo-cytoplasmic shuttling, which is affected by phosphorylation of their serine/arginine (RS)–rich domain (96). The signaling mechanisms that control the subcellular localization of these proteins are unknown. It has been demonstrated that exposure of NIH-3T3 and SV-40 transformed green monkey kidney (COS) cells to stress stimuli such as osmotic shock or UVC irradiation, but not to mitogenic activators such as PDGF or EGF, results in a marked cytoplasmic accumulation of hnRNP A1, concomitant with an increase in its phosphorylation (96). These effects are mediated by the MKK3/6-p38 pathway, and moreover, p38 activation is necessary and sufficient for the induction of hnRNP A1 cytoplasmic accumulation (96). hnRNP A1 has been shown to be phosphorylated by several kinases, including AKT (138), the catalytic subunit of cAMP-dependent protein kinase (PKA) and casein kinase II (CKII) (136), and PKC (137).

As previously mentioned in section 1.5, the phosphorylation status of hnRNP A1 at serine 199 affects its function in the following processes: strand annealing activity (136,137) and cyclin D1 and c-myc internal ribosome entry site function (138). We have shown previously that the phosphorylation level of hnRNP A1 was elevated in senescent cells (128). This may be responsible for a role for hnRNP A1 in establishing the senescent morphology of cells. We hypothesize that the phosphorylation of hnRNP A1

by the p38 MAPK pathway is critical for its subcellular distribution (discussed in Chapter 4).

In regards to the stochiometry of phosphorylated hnRNP A1 protein, we have previously observed that the majority of A1 protein is phosphorylated relative to total hnRNP A1 protein levels in senescent cells compared to young cells (128). This data suggests that the phosphorylation status of hnRNP A1 affects the protein's subcellular localization and cytoplasmic accumulation in senescent cells (128; and Chapter 4 below).

Effect of MAPKAPK2 Kinase Inhibition on Protein Expression

In this study, we attempted to identify putative kinases in the p38 MAPK pathway that might modulate the protein expression levels of hnRNP A1, presumably by phosphorylation. To this end, we examined the effects of inhibition of the kinase activity of MAPKAPK2 and MNK1 on hnRNP A1 protein levels. We initially measured the relative expression levels of proteins that are downstream of p38 MAPK as a function of MAPKAPK2 inhibition using the MK2a inhibitor. MK2a is a p38α inhibitor that is noncompetitive with ATP and specifically blocks the activation of MAPKAPK2 by p38α *in vitro* (152). MAPKAPK2 is a substrate of both p38α and p38β (153). Our results show that phosphorylated MAPKAPK2 protein levels did not decrease. Phosphorylation levels of hsp27 protein, an indicator of MAPKAPK2 kinase activity, showed no decrease as well. These results suggest that MK2a treatment is unable to block the activation of MAPKAPK2 by p38α in IMR-90 cells and/or that blocking both p38α and p38β is necessary to exhibit complete MAPKAPK2 inactivation.

Effect of Inhibition of MAPKAPK2 Expression on Protein Levels

Our studies indicate that downregulation of MAPKAPK2 by siRNA inhibition did

not inhibit phosphorylation of hnRNP A1 protein. This finding suggests that MAPKAPK2 does not phosphorylate hnRNP A1 in IMR-90 fibroblasts and is consistent with reports from others that hnRNP A1 is not a direct substrate of MAPKAPK2 (96, 154).

We observed that phosphorylated hnRNP A1 protein levels decreased in CGP 57380 treated cells. This data is consistent with the findings from Buxadé et al. showing that MNK1 phosphorylates hnRNP A1 *in vitro* and *in vivo* (154). Further support for the notion that MNK1 is the presumptive kinase that phosphorylates hnRNP A1 comes from our observations that reciprocal binding occurs between hnRNP A1 and MNK1. These studies implicate MNK1 as the kinase in the p38 MAPK pathway that activates hnRNP A1 in IMR-90 fibroblasts. An examination of the molecular mechanisms by which MNK1 regulates hnRNP A1 is warranted and these studies are discussed in Chapter 4.

Chapter 4. MNK1 Regulation of the Subcellular Distribution of hnRNP A1 in Normal Human Fibroblasts.

4.1 Introduction

It has been reported that mitogen-activated protein kinase-interacting kinase 1 (MNK1) might posses a MAPK-binding domain that allows it to bind to ERK and p38 MAPK followed by phosphorylation through these two kinases, but not JNK (155). MNK1 phosphorylation of eukaryotic initiation factor-4E (eIF4E), which is a translation initiation factor that binds to the 5' cap structure of eukaryotic cytosolic mRNAs, has been well studied (156). Recently, AU-rich element (ARE) binding proteins, including hnRNP A1, have been identified as MNK1 substrates *in vitro* (157).

We have previously shown that there is diminished expression and altered subcellular distribution of hnRNP A1 protein in senescent human diploid fibroblast cells, which terminated their cell growth and reached cell cycle arrest (126,127). We have recently demonstrated that hnRNP A1 and p38 MAPK interact *in vivo* and that the p38 MAPK pathway regulates the expression level and subcellular distribution of hnRNP A1 (128). However, the molecular mechanisms responsible for the regulation of hnRNP A1 downstream of p38 MAPK in human diploid fibroblasts have yet to be identified.

In this study, we demonstrate that inhibition of MNK1 activity modulates the phosphorylation and subcellular distribution of hnRNP A1 protein. These results suggest a role for MNK1 in the regulation of hnRNP A1 in senescent cells. This study reports the first link between MNK1 and cellular senescence.

4.2 Results

A. MNK1 is required for the subcellular distribution of hnRNP A1

We have previously demonstrated that activation of p38 MAPK is required for the cytoplasmic accumulation of hnRNP A1 in senescent cells (128). It has been previously shown that MNK1/2 mediated stress-induced phosphorylation and cytoplasmic accumulation of hnRNP A1 in HeLa cells (99). In Chapter 3, we found that MNK formed a complex with hnRNP A1 and modulated its phosphorylation in young cells (Figures 16 and 17). These findings raised the possibility that MNK1 might potentially regulate the subcellular distribution of hnRNP A1 downstream of p38 MAPK in senescent cells.

To asses this, we treated young and senescent IMR-90 cells with CGP 57380 or an equivalent amount of DMSO, then determined the localization pattern of hnRNP A1 by immunocytochemistry. CGP 57380 is a selective inhibitor of MNK1 (IC_{50} = 2.2 μM) with no inhibitory activity against p38 MAPK, JNK1, ERK1/2, PKC, or Src-like kinases (158). Control young cells displayed a predominant nuclear localization of the protein (Figure 19b). Control senescent cells showed predominantly cytoplasmic accumulation (Figure 19j), as previously reported by us (127). Young CGP 57380 treated cells did not show any alteration in their nucleo-specific localization pattern (Figure 19f). In contrast, CGP 57380 treatment inhibited the cytoplasmic accumulation of hnRNP A1 in senescent cells, as the majority of the protein was localized in the nucleus (Figure 19n). These results indicate that the accumulation of hnRNP A1 in the cytoplasm of senescent cells requires the kinase activity of MNK1. We subsequently determined the localization

pattern of MNK1 before and after inhibition with CGP 57380. Control young cells showed a predominant nuclear localization of the protein (Figure 19c) and control senescent cells showed predominant cytoplasmic accumulation (Figure 19k). The localization patterns were similar to that of hnRNP A1, suggesting that MNK1 and hnRNP A1 may physically interact with each other in both young and senescent cells, further supporting our co-immunoprecipitation results. Another substrate for p38 MAPK is MAPKAPK2, which is known to phosphorylate hnRNP A0, which has structural similarity to other hnRNPs (159). siRNA inhibition of MAPKAPK2 did not result in changes in either location or protein levels of hnRNP A1 (data not shown). CGP5 380 inhibition did not induce any change in MNK1 subcellular distribution in young (Figure 19g) and senescent (Figure 19o) cells, indicating that the kinase activity of MNK1 is not required for its subcellular distribution.

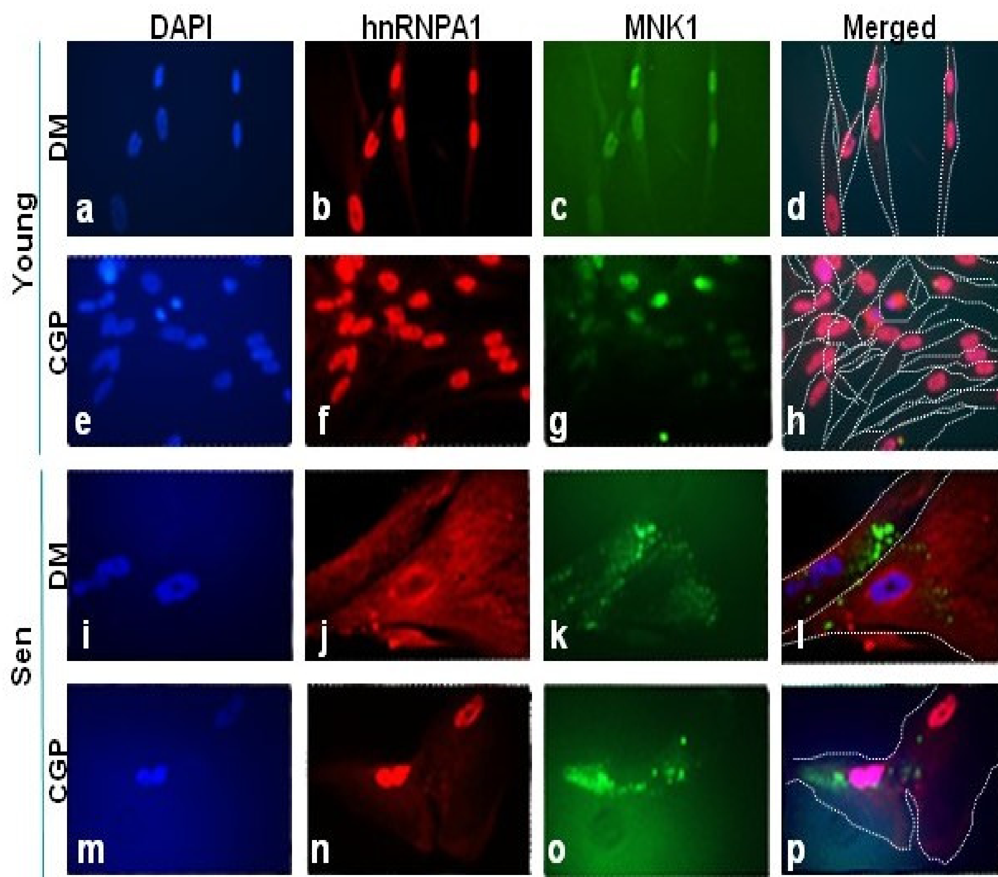


Figure 19. Effect of MNK1 inhibition on the subcellular distribution of hnRNP A1 and MNK1. Young and senescent IMR-90 cells were treated with 40 µM of CGP 57380 (panels e-h and m-p) or an equal volume of DMSO (panels a-d and i-l) for 3 days and then subjected to immunocytochemistry for hnRNP A1 and MNK1. DAPI was used to stain nuclei. Magnification: 400X in each field. Shown are representative images of three independent experiments. The scale bar represents 20 µm. Identical fields were taken for DAPI, hnRNP A1, and MNK1 fluorescent signals. The corresponding merged images (Merged) are shown on the right (panels d, h, l, and p).

4.3 Discussion/Summary

Phosphorylation and the p38 MAPK Pathway

Phosphorylation through the p38 MAPK pathway cascades are important for the regulation of numerous biological processes, such as inflammation, infection, and environmental stresses (39,43). The mitogen-activated protein kinase (MAPK) pathways control crucial cellular processes such as proliferation, differentiation, cell survival, apoptosis, gene regulation, and motility (160,161). The typical mammalian MAPK pathways include the well characterized MEK1-2/ERK1-2, JNK1-3, MEK5/ERK5, and p38MAPK pathways, while the atypical MAPK pathways include the less studied ERK3, ERK4, and ERK7 (161-163). The classical MAPK pathways consist of a three partite module in which a MAPK kinase kinase phosphorylates and activates downstream MAPK kinases, which in turn phosphorylate and activate MAPK. MAPK can then phosphorylate non-protein kinase substrates or yet other protein kinases. The latter are referred to as mitogen-activated protein kinase-activating protein kinases (MAPKAPK) (160,161). MAPKs phosphorylate a diverse set of well-characterized substrates, including transcription factors, translational regulators, MAPK-activated protein kinases (MAPKAP kinases), phosphatases, and other classes of proteins, thereby regulating metabolism, cellular morphology, cell cycle progression, and gene expression in response to a variety of extracellular stresses and molecular signals (164).

In mammalian model systems, protein phosphorylation is a global response to stress, which can be triggered by a variety of stimuli, including infection, ultraviolet irradiation, inflammation, and osmotic, and other stressors. For example, phosphorylation of the HIV-1 accessory protein Vpr by PKA induces cell cycle arrest in T-cells (165). It has been demonstrated that the expression of the basic zipper transcriptional regulator

ATF5 is induced in response to many different stresses, including endoplasmic reticulum stress, arsenite exposure, and proteasome inhibition, by a mechanism requiring eIF2 phosphorylation (166). Klotho, an antiaging hormone present in the kidney, was recently shown to serve as an anti-inflammatory modulator, negatively regulating the production of NF-κB-linked inflammatory proteins via a mechanism that involves phosphorylation of Ser(536) in the transactivation domain of RelA (167). Protein phosphorylation is also a response to osmotic stress (168,169). After UV irradiation, p53 is phosphorylated specifically at murine residue Ser389 (170).

The phosphorylation response to stressors has been shown to increase with age. It has been demonstrated that growth factors increase locomotor activity in Parkinson's disease and aging models as well as increase dopamine bioavailability and ser31 tyrosine hydroxylase phosphorylation in the substantia nigra (171). Furthermore, blockage of the p38 MAPK signaling pathway has been shown to promote skin aging (172). Thus, protein phosphorylation is up-regulated as a function of age as a response to age-dependant stress stimuli. Therefore, further research is warranted to elucidate the mechanisms by which signaling pathways regulate gene expression during aging.

The role of p38 in inflammatory and stress responses has already been well established by many studies (39,43). A member of the heterogeneous nuclear ribonucleoprotein (hnRNP) family, hnRNP A1 is known to function in the biogenesis of mRNA. We previously showed age-dependent changes in the expression level and subcellular distribution of hnRNP A1 (128). However, the molecular mechanisms that regulate these changes have not been elucidated. Additionally, we have demonstrated that the p38 MAPK pathway regulates hnRNP A1 protein levels. The p38 MAPK pathway

has been shown to play an essential role in induction of senescence and our studies suggest that there is an interplay between this pathway and hnRNP A1. Previous studies have demonstrated that hnRNP A1 are elevated in tumor cells and human cancers (173,174). Considering the fact that cellular senescence acts as a tumor suppression mechanism *in vivo*, hnRNP A1 might be a putative target for cancer therapy.

Phosphorylation and cytoplasmic localization of A1 by MNK1

p38 MAPK plays a causative role in cellular senescence. We have previously shown that p38 MAPK is required for the subcellular distribution of heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) in senescent cells and regulates the protein level in young cells (128). In this study, we investigated molecular mechanisms responsible for the regulation of hnRNP A1 downstream of p38 MAPK during senescence. We found that MNK1 modulates the subcellular distribution of hnRNP A1. This result suggests that p38 MAPK regulates hnRNP A1 localization in MNK1-dependent manner. We suspect that phosphorylation of hnRNP A1 by MNK1 is critical for its subcellular distribution. This hypothesis is supported by the findings of others that MNK1 phosphorylates three serine residues of hnRNP A1 at its C-terminal peptide, F-peptide (154). Phosphorylation of the F-peptide results in hnRNP A1 cytoplasmic accumulation by reducing its interaction with the nuclear receptor, transportin (109).

The roles of MNK1 in cellular senescence

MNKs have been reported to promote protein synthesis, cell cycle progression, and proliferation in tumor cells through phosphorylation of its substrate, eIF4E (175), which is a central regulator for cap-dependent translation initiation. On the other hand, we found an age-dependent increase in MNK1 and phospho-MNK1 expression and

eIF4E phosphorylation in senescent cells (Figure 20). Overexpression of eIF4E increases cellular senescence in mice as measured by β-galactosidase staining (176). Senescence is delayed and lifespan is extended in *Caenorhabditis elegans* mutants that are defective in eIF4E (177). The MNK1-eIF4E pathway in senescent cells appears to have an opposite regulating role in protein synthesis from that in tumor cells. It is has been shown that protein synthesis is elevated in carcinogenesis, whereas the rate of general protein synthesis is diminished during aging (178). Moreover, negative effects of MNK1/2 activity and phosphorylation of eIF4E on cap-dependent translation have been demonstrated by others (158,179,180). Therefore, we suggest a putative role for MNK1 in senescence. Possible mechanisms that can explain the age-dependent effects by MNK1 is that the increase in the phosphorylation of eIF4E by MNK1 somehow decreases cap-dependent translation followed by a decline in *de novo* synthesis of the proteins required for longevity. It would be intriguing to assess whether the rate of protein synthesis during senescence is affected by inhibition of MNK1 activity. One study showed that knockdown of MNK1/2 was essential for the phosphorylation of eIF4E in embryonic fibroblasts and adult tissue, but not for general protein synthesis, cap-dependent translation and cell growth in development (181). It seems that MNKs do not play a crucial role in the early development of normal mice. However, the role of MNK has not been studied in senescent mice.

We have previously demonstrated the downregulation of hnRNP A1 and/or A2 by siRNA transfection contributes to a partial senescence-like morphology (128). hnRNP A1 is known to involve a variety of RNA metabolism including alternative splicing and mRNA transport and stability. Our findings suggest that the MNK1 induced changes in

localization of hnRNP A1 contribute to the genetic features of senescence through altered regulation of its target mRNAs. In general, genes required for stress and immune/inflammatory responses are up-regulated during senescence (182). Some may be potentially regulated by MNK1/hnRNP A1 pathway. To support this idea, it has been shown that MNK1 induces the synthesis of tumor necrosis factor (TNF) α via hnRNP A1 phosphorylation in Jurkat cells (138). Activation of MNK1 by stress stimuli causes phosphorylation and cytoplasmic accumulation of hnRNP A1 in stress granules (99) whereas stress responsive mRNAs are translated. Further analysis of RNA metabolism regulated by the MNK1-hnRNP A1 pathway could reveal potential molecular mechanisms, that establish the senescent phenotype.

This is the first report, to our knowledge, that shows a link between MNK1 and cellular senescence. Our findings imply that the MNK pathway could provide a novel contributing factor to study for age-related disorders, including Alzheimer's disease, heart disease, and diabetes. The continued study of the role of MNK pathways in hnRNP A1 functionality will extend our knowledge of signaling pathways beyond that of tumorigenesis and inflammatory responses. Further studies are warranted to elucidate the precise molecular mechanisms by which MNK1 regulates cellular senescence through hnRNP A1 and eIF4E.

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