Mnk2 alternative splicing inactivates its tumor suppressor activity as a modulator of the p38-MAPK stress pathway

Thesis submitted for the degree of

"Doctor of Philosophy"

By: Avi Maimon

Submitted to the senate of the Hebrew University of Jerusalem

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This work was carried out under the supervision of:

Dr. Rotem Karni
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Abstract

Alternative splicing is a fundamental post-transcriptional process that regulates the inclusion or exclusion of exons or parts of exons generating different transcripts from the same gene. Cancer cells manipulate the process of alternative splicing to down-regulate tumor suppressor and/or up-regulate tumor promoting isoforms. Missregulation of alternative splicing can be caused by enhanced expression of splicing factors such as the SR protein SRSF1, which is frequently up-regulated in several types of human cancers. It has been shown that SRSF1 acts like a proto-oncogene as its gene is amplified and it is over-expressed in many tumors and its over-expression in immortal cells induces tumor formation in nude mice.

One of the alternative splicing events regulated by SRSF1 is selection of the last exon of the MKNK2 gene which encodes the kinase Mnk2. Mnk2 alternative splicing generates two splicing isoforms with differing last exons: Mnk2a, which contains a MAPK binding domain and Mnk2b which lacks it. The kinase Mnk2 is a substrate of the MAPK pathway and phosphorylates the translation initiation factor eIF4E. We found that the Mnk2a isoform is downregulated in breast, lung and colon tumors and is tumor suppressive. In addition, we found that Mnk2a directly interacts with, phosphorylates, activates and translocates p38α MAPK into the nucleus, leading to activation of its target genes, increasing cell death and suppression of Ras-induced transformation in vitro and in vivo. In contrast, Mnk2b is pro-oncogenic and does not activate p38-MAPK while still enhancing eIF4E phosphorylation. Moreover, we generated mutants of Mnk2a that localized either to the cytoplasm or the nucleus. Mnk2aL/S, a mutant that lacks the nuclear export signal (NES) and localizes mostly in the nucleus, rendered p38α to be nuclear and to co-localize with it in the nucleus. In contrast the Mnk2aKKR mutant, which lacks the putative nuclear localization signal (NLS), is mostly cytoplasmic and co-localized with p38 primarily in the cytoplasm. Importantly, while wild type Mnk2a and the nuclear localized mutant (Mnk2aL/S) sensitized cells to apoptosis, the cytoplasmic localized mutant (Mnk2aKKR) inhibited apoptosis and did not significantly decrease colony survival in direct correlation with its inability to induce p38α target genes.

We further show in this study that oncogenic Ras suppresses the auto-inhibitory mechanism which down-regulates the expression of the splicing factor SRSF1,
leading to SRSF1 up-regulation. Thus, activated Ras elevates the expression of the splicing factor SRSF1 and induces modulation of *MKNK2* alternative splicing resulting in down-regulation of the tumor suppressive isoform Mnk2a and up-regulation of the Mnk2b isoform. Finally, we designed antisense RNA oligonucleotides that mask either the Mnk2a specific splice site or the Mnk2b specific splice site which results in a shift in *MKNK2* splicing to generate more Mnk2b or Mnk2a respectively. We were able to shift *MKNK2* splicing in cells transformed by an active Ras oncogene or by the splicing oncoprotein SRSF1. We found that elevating Mnk2a while reducing Mnk2b sensitized cells to apoptosis, reduced colony survival and inhibited transformation while inducing the opposite splicing change elevated survival and inhibited apoptosis. Thus, Mnk2a down-regulation by alternative splicing is a new tumor suppressor mechanism which is lost in breast, colon and lung cancers and is regulated by Ras.
List of abbreviations:

SRSF1: serine-arginine rich splicing factor 1.
MAPK: mitogen activated protein kinase.
MKNK2: map kinase interacting serine/threonine kinase 2.
p38-MAPK: p38 map kinase.
eIF4E: eukaryotic translation initiation factor 4e.
NLS: nuclear localization signal.
NES: nuclear export signal.
ERK: extracellular signal regulated kinase.
hnRNPA1: heterogeneous nuclear ribonucleoprotein A1.
eIF4G: eukaryotic translation initiation factor 4g.
NPC: nuclear pore complex.
JNC: c-Jun NH(2)-terminal kinase.
RRM: RNA recognition motif.
SXL: sex lethal.
NMD: non sense mediated mRNA decay.
RBP: RNA binding protein.
mTOR: mammalian target of rapamycin.
ESE: exon splicing enhancer. ESS: exon splicing silencer.
ISE: intron splicing enhancer.
ISS: intron splicing silencer.
SRPK: serine/arginine protein kinase.
EGF: epidermal growth factor.
EST: expressed sequence tag.
TIA-1: t-cell intracellular antigen-1.
Sam68: Src-associated in Mitosis 68 kDa.
PTB: polypyrimidine tract binding protein.
M KK: mitogen activated protein kinase kinase.
MEK1/2: mitogen activated protein kinase kinase 1/2.
MLK: mixed-lineage kinase.
ASK1: apoptosis signal-regulating kinase 1.
TAK1: transforming growth factor beta-activated kinase 1.
WT-1: wilms tumor suppressor 1.
JAK: janus kinase.
STAT: signal transducer and activator of transcription.
PKC: protein kinase C.
MEF: mouse embryonic fibroblast.
Clk: cdc like kinase.
Sty: serine/threonine kinase.
MK-2: MAPK activated protein kinase 2.
PTEN: phosphatase and tensin homolog.
Mcl-1: myeloid leukemia cell differentiation protein 1.
# Table of Contents

Table of Contents ................................................................................................................. 9

Introduction .......................................................................................................................... 11

Alternative splicing ............................................................................................................. 11

Alternative splicing and cancer ......................................................................................... 14

Misregulation of splicing factors in cancer .......................................................................... 15

Alternative splicing and tumor suppressor genes .............................................................. 16

Ras-MAPK pathway and splicing ....................................................................................... 17

Akt-PI3K pathway and splicing ............................................................................................ 18

SR kinases as signal transducers ......................................................................................... 19

Regulation of splicing factors by kinase pathways ............................................................. 20

MAPKs .................................................................................................................................. 21

MKNK1 (Mnk1) and MKNK2 (Mnk2) .................................................................................. 22

Experimental procedures ..................................................................................................... 33

Results ................................................................................................................................ 42

Mnk2a mRNA is down-regulated in colon, breast and lung cancers. ......................... 42

The Ras oncogene regulates alternative splicing of the MKNK2 gene. ....................... 47

Mnk2a has a tumor suppressive activity while Mnk2b is pro-oncogenic in vitro... 50

Mnk2a has a tumor suppressor activity in vivo ................................................................. 54

Mnk2a sensitizes cells to stress-induced cell death ......................................................... 61

Mnk2a but not Mnk2b enhances p38α-mediated cell death and suppression of Ras-induced transformation. ................................................................................................................... 65

[9]
Mnk2a interacts with, activates and induces nuclear translocation of p38-MAPK. 65
Mnk2a co-localizes with p38-MAPK and affects its cellular localization. .......... 71
Mnk2a can phosphorylate p38-MAPK directly in vitro and in vivo. ............... 77
Mnk2a localization and kinase activity affects induction of p38-MAPK target genes and apoptosis. ................................................................. 77
Modulation of MKNK2 splicing by splice-site competitive antisense RNA oligos affects sensitivity to apoptosis and cellular transformation. ................. 81
Discussion........................................................................................................ 86
Oncogenic signals regulate alternative splicing of MKNK2 ......................... 87
The role of Mnk2 isoforms in cellular transformation .................................... 88
Mnk2a modulates the p38-MAPK stress pathway ......................................... 89
Nuclear activity of p38-MAPK ....................................................................... 93
p38-MAPK involvement in cancer .................................................................. 94
MKNK2 alternative splicing and cancer.......................................................... 95
Scheme 3: Alternative Splicing of MKNK2 is manipulated by cancer cells through the Ras - SRSF1 route................................................................. 97
References:.................................................................................................... 99
Introduction

Alternative splicing

Alternative splicing is an evolutionary conserved process which serves the purpose of enriching the repertoire of proteins emanating from a single pre-mRNA. This is accomplished by utilizing variable exon cassettes and occasionally parts of an intron [1, 2]. The ability to exclude or include exons/introns from the pre-mRNA transcript is the fundamental principle of expanding protein variability from a limited number of genes [1, 2]. Recent studies have estimated that nearly 90% of mammalian genes are alternatively spliced, affecting multiple biological processes [3, 4].

The transcribed human genome is currently thought to consist of at least ~230,000 exons and ~210,000 introns, or roughly nine exons and eight introns per gene on average [5]. Individual introns are excised from pre-mRNA, and the flanking exons joined, through two sequential transesterification reactions that are catalyzed in the nucleus by the spliceosome, a complex macromolecular machine consisting of five small RNAs and more than 100 proteins [6-8]. Formation of this complex is established by cis elements which are consensus sequences located at the ends of the introns. Location and sequence may affect definition of introns. For instance, flanking sites that differ largely from their consensus sequence are defined as weak splice sites and vice versa. Importantly, sequence diversity influences the binding affinity of cognate splicing factors involved in this process [9, 10]. A combination of weak and strong splice sites determines the mode of alternative splicing such as; inclusion of alternative cassette exons, inclusion of alternative mutually exclusive cassette exons, use of alternative 5’ splice sites or alternative 3’ splice sites, and retention of alternative introns [11]. Primary transcripts can differ as a result of the use of alternative promoters or 3’ cleavage/polyadenylation sites or they can be identical. The decision of a weak splice site is defined by both cis-regulatory sequences and trans-acting factors. Cis-regulatory sequences include exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs) and intronic splicing silencers (ISSs), depending on their sequence, location and their [11]
effect on splice site choice [9, 12]. *Trans*-acting factors function through binding to splicing enhancers and silencers and include members of the Ser/Arg-rich (SR) and heterogeneous nuclear ribonucleoprotein (hnRNP) protein families, as well as tissue-specific factors such as nPTB and PTB [13], NOVA [14] and RBFOX [15]. Some factors can induce while others suppress splice site choice. However, most of the trans-regulatory factors may act in both ways depending on the sequence and position of the target site in the pre-mRNA [16]. For example, inclusion of an alternative exon is dependent on; the intrinsic weakness of its 3′ splice site and/ or 5′ splice site; the presence of different ESEs and ESSs within the alternative exon and of ISEs and ISSs in the surrounding introns [9]. Importantly, changes in secondary structure that may expose or hide these sequences, can affect splicing decisions. In addition, nuclear concentration and post-translational modifications of cognate regulatory proteins can influence splicing choices.

Alternatively spliced mRNAs can be simultaneously expressed or their expression can be tightly regulated in a cell-type-specific manner, developmentally or in response to signaling. Protein isoforms resulting from alternative pre-mRNA splicing can have related, distinct or antagonistic functions, depending on their primary structure. Alternative splicing can serve as an on/off switch to control gene expression post-transcriptionally. For example, one mRNA isoform can encode a functional protein while another isoform of the same gene can either encode a truncated protein, or can be retained in the nucleus, or undergo nonsense-mediated mRNA decay (NMD) [17]. An example that highlights the versatility achieved by alternative splicing is the *Drosophila melanogaster Dscam* gene. Dscam is a cell surface protein which is involved in axon guidance in the developing brain, and exists as up to 38,016 alternatively spliced isoforms [18]. Homophilic interactions can be generated only between identical isoforms and consequently affect neuronal connections [19]. Another example, sex determination in Drosophila involves gender-specific alternative splicing of Sex-lethal and Transformer-2 exons with in-frame stop codons, such that inclusion of these exons in males does not generate functional proteins, whereas skipping of the same exons in females gives rise to functional proteins [20].

[12]
In general, the stronger the affinity of a trans-factor toward a cis- splice site, the higher the chances that the proximal exon will be included. However, a pair of ‘strong’ splice sites is not sufficient to define an exon; there are examples of ‘pseudo-exons’ that are flanked by predicted splice sites which are not spliced. Alternative splicing is achieved by auxiliary splicing elements which demonstrate high sequence diversity. Genome analysis has revealed cis elements such as ESSs, ESEs, ISSs and ISEs which help distinguish between authentic exons and pseudo-exons. The function of these elements depends on their availability in the pre mRNA [21, 22]. Most ESEs contain sequences with affinity for SR splicing factors. These are a family of abundant RNA-binding proteins with multiple roles in constitutive and alternative splicing, as well as additional roles in other aspects of gene expression [23, 24]. All SR proteins have closely related sequences and a modular structure with one or two copies of a RNA-recognition motif (RRM) and a C-terminal Arg/Ser-rich (RS) domain that undergoes reversible phosphorylation [25]. Their general splicing function was revealed by the requirement for at least one family member for spliceosome assembly and splicing of any pre-mRNA in vitro [26, 27]. Their involvement in alternative splicing and exon definition is evident from their pronounced concentration-dependent effects on alternative splice-site selection in vitro and in cells, and from their ability to enhance splicing via cognate ESEs [24]. Additional specific effects of particular SR proteins on mRNA export, NMD [28], and translation were recently reported [29], and in some cases reflect the ability of some SR proteins to exit the nucleus bound to mRNA, and then shuttle back to the nucleus[30]. SR proteins are ubiquitously expressed, but at levels that are characteristic for each cell type [31]. Individual SR proteins are essential at the organismal and cellular levels, and reduced expression of one family member was found to elicit genomic instability and apoptosis [32, 33]. Each of the SR proteins can compensate in splicing activity for another member of the family as a result of redundancy. Nevertheless, they also show distinct alternative activities and the lethal effects of individual SR protein knockouts have established that they possess non-overlapping roles in vivo [34-37]. In addition to the SR proteins, some ESSs and ISSs can bind a second family of splicing factors, the heterogenous nuclear ribonucleo proteins (hnRNPs). Proteins in this family contain RRM-type and hnRNP K
homology (KH)-RNA-binding domains, as well as auxiliary domains that are often involved in protein-protein interactions, and have multiple roles in pre-mRNA and mRNA metabolism [38, 39]. One of the most studied members of this family, the hnRNP A/B family, is a subset of hnRNP proteins with closely related sequences and a conserved modular structure. They can affect alternative splicing, frequently by antagonizing SR proteins, in part through the recognition of ESS elements [40]. Other functions such as mRNA trafficking, and replication and transcription of cytoplasmic RNA viruses, have also been reported [40]. A number of hnRNPs function as splicing repressors, including hnRNPA1, polypyrimidine tract binding protein (PTB/hnRNPI) and D. melanogaster gene SXL. Secondary RNA structure can also play a role in alternative splicing either by sequestering sequence elements or bringing them closer [41], thus changing the relative spacing of sequence elements. For example, proximity of sequence splice sites can enforce mutually exclusive splicing events [20].

**Alternative splicing and cancer**

Like other steps in gene expression, RNA splicing shows pronounced misregulation in the context of cancer [42-45]. The transcripts of numerous genes, including oncogenes and tumor suppressors, undergo aberrant splicing, both qualitatively and quantitatively, in tumors. It is hard to assess the full extent of cancer-associated splicing misregulation because tumor-associated ESTs and cDNAs are overrepresented in the databases, compared to normal-tissue isoforms [46]. Tumor cells may express mRNA isoforms that are normally characteristic of a different tissue or that are never present in normal cells. Many examples of oncogenic splicing isoforms that contribute to various aspects of malignancy have been described [44]. Though it has long been known that cancers show aberrant splicing patterns, it was previously impossible to know whether these events played a causal role in cancer or were simply an effect of cancer. However, advances in systematic research studies of the cancer genome using massively parallel sequencing (MPS), [47] have shed light on this question [48].
Exome sequencing studies of chronic myelogenous leukemia (CML) have shown that the spliceosome gene SF3B1 is mutated in 10 to 15% of the cases, and other splicing factor genes, such as SRSF2 and U2AF2, are also mutated at lower frequencies [49, 50]. Similar studies of myelodysplastic syndrome (MDS), have even more striking results: 45 to 85% of the cases harbor mutations in a spliceosome gene, with SF3B1 and U2AF1 being the most common and other genes (such as SF3A1, ZRSR2, SRSF2, and U2AF2) occurring at lower frequencies [51, 52]. Spliceosomal genes have also been found significantly mutated in solid tumors most notably, U2AF1, SF3B1, U2AF2, and PRPF40B mutations in lung adenocarcinomas [53]. Mapping the mutation profiles from tumors uncovers a specific mutually exclusive pattern, with mutations appearing in only a single splicing factor per tumor sample, which suggests that these mutations play similar roles and are thus functionally redundant with respect to causing cancer [54]. Evidence that implicates a specific mutation to aberrant cancer related splicing events have been shown in several studies. U2AF1 mutations have been linked to increased progression from MDS to AML, and SRSF2 mutations correlate with the so called chronic myelomonocytic leukemia (CML) subtype of MDS. In CLL, SF3B1 mutations correlate with more rapid disease progression and lower overall survival [50, 55]. U2AF1 mutations were associated with poor progression-free survival in lung adenocarcinoma [53].

**Misregulation of splicing factors in cancer**

Many disease-associated alternative splicing events have been found to result from mutations in conserved splicing signals or cis acting regulatory elements [9]. However, little is known about the contribution of trans-acting splicing regulators to specific disease processes. Similar to the activities of other ‘master’ regulators in key biological pathways, it has long been suspected that the activity of SR proteins in alternative splicing is fundamental in modulating diverse cellular functions, and defects in these splicing factors can certainly produce a disease phenotype [56]. As global regulators of splicing, SR proteins could potentially account for the extensive misregulation of splicing associated with cancer. SR proteins show abnormal expression in a variety of tumors [57-60], and an undefined number of pre-mRNAs is
expected to have altered splicing in response to these concentration changes. Indeed, slight over-expression of SRSF1 is sufficient to transform immortal rodent fibroblasts, which can then form tumors in nude mice. Thus, these genes have properties of oncogenes [57]. In a similar way, it was shown that the splicing factor hnRNPA2/B1 is overexpressed and is a driving oncogene in glioblastoma and modulates the splicing of some tumor suppressors and oncogenes [61]. Recent analysis of gene copy number has shown that the SR protein SRSF6 is amplified, overexpressed and acts as a proto-oncogene in colon and lung tumors [62]. Upon transducing immortal lung epithelial cells with SRSF6, these cells exhibited higher proliferation, increased chemotherapy resistance and enhanced tumorigenesis [62]. Recently, it was found that SRSF1 and SRSF9 are required elements in the regulation of β-catenin biosynthesis, and by this contribute to tumorigenesis [63]. Furthermore, like SRSF1 which is known to act as an oncogene, SRSF9 is also found frequently overexpressed in tumors and shows characteristics which resemble an oncogene. Other studies suggest that SRSF3 (SRp20) is involved in cell proliferation and senescence and acts as a proto-oncogene [64, 65]. In addition, SRSF3 can regulate TP53 alternative splicing and downregulation of SRSF3 increases the p53β isoform which induces cellular senescence [66].

**Alternative splicing and tumor suppressor genes**

Alternative splicing of the Fas pre-mRNA is an example of an oncogenic mechanism by which tumor cells can escape elimination by the immune system. The Fas protein (also known as CD95) is a widely expressed cell surface receptor. Binding of Fas to Fas ligand (FasL), which is expressed on cytotoxic T cells, can induce cell death [67]. Fas pre-mRNA is regulated by alternative splicing to produce a number of shorter products. Among them is an isoform that is missing 63-nucleotides of exon 6 (ΔE6) due to skipping of exon 6. As a consequence of this skipping event the entire transmembrane domain is deleted [68, 69]. The protein produced by the ΔE6 Fas isoform is soluble and capable of inhibiting Fas-mediated cell death, presumably by binding to FasL and preventing the interaction of FasL with membrane-bound Fas. Elevated production of soluble Fas (sFas) has been observed in a wide range of
cancers, as determined by Fas serum concentrations, which show a strong correlation with tumor staging [70]. Several RNA Binding Proteins (RBPs) have been shown to be involved in promoting the production of full-length Fas mRNA. T-cell intracellular antigen-1 (TIA-1) and TIA-1-related protein (TIAR), two closely related RNA recognition motif (RRM)-containing proteins involved in apoptosis, bind to U-rich sequences downstream from Fas E6 and promote its inclusion in the mRNA [71]. The regulation of Fas alternative splicing has been extensively investigated. One important observation was that RNA binding protein 5 (RBM5) binds to U2AF65 and prevents inclusion of exon 6 to Fas mRNA. RBM5 is a putative tumor suppressor that contains a large number of motifs, including a pair of RRMs, two Zn finger motifs, an RS domain, and an octamer repeat (OCRE) motif, a newly identified domain recently implicated in splicing regulation [72]. Overexpression of RBM5 increases the production of Casp-2L isoform and induces apoptosis. Deleting or mutating the RBM5-binding site in the Casp-2 pre-mRNA decreases Casp-2L production and increases Casp-2S production [73].

Another tumor suppressor which is regulated by alternative splicing is KLF6. It has been shown that the oncogene Ras, through the PI3K-Akt pathway, and phosphorylation of the splicing factor SRSF1, regulates alternative splicing of the tumor suppressor KLF6 in colorectal cancer [74]. A recent study showed that mutation in the WT1 (Wilms tumor) tumor suppressor gene induced up-regulation of the splicing factor kinase SRPK1 which in turn phosphorylates and activates SRSF1 leading to increased angiogenesis and malignant behavior of Wilms' tumor cells in Denys Drash Syndrome [75].

**Ras-MAPK pathway and splicing**

Alternative splicing is tightly coupled with transcription for co-transcriptional RNA processing as well as post-splicing steps for mRNA transport and stability control [76, 77]. It is assumed that alternative splicing is subject to regulation by a variety of cellular signaling events. However, compared to numerous signal-induced gene expression events that are regulated at the transcriptional and translational levels, little is known about how specific signals are transduced to regulate alternative splicing in
the nucleus [78, 79]. It is clear that signal transduction pathways initiated by growth factors can influence alternative splicing. Expression of the cell-surface molecule CD44 is an excellent example of signal-dependent regulation of alternative splicing. CD44 class I trans-membrane glycoproteins mediate the response of cells to their cellular microenvironment. CD44 proteins participate in many cellular processes, which include the regulation of growth, survival, differentiation and motility [80]. CD44 transcripts are subject to alternative splicing, which affects predominantly the extracellular, membrane-proximal stem structure of CD44 proteins [81, 82]. After mitogenic stimulation, CD44 can form multimeric complexes that include receptor tyrosine kinases and other protein partners that maintain the optimal signaling capacity and enhance cell growth, survival, motility or other functions. Several lines of evidence indicate that inclusion of CD44 exon 5 (CD44v5) is induced by signaling through the Ras-ERK pathway. An initial study used transient transfection with a CD44v5- minigene construct to identify both positively and negatively acting RNA sequences that modulate exon 5 (v5) inclusion. It was shown that several stimuli, including activated Ras, could induce v5 inclusion [83]. How Ras signaling activates v5 inclusion is currently unknown. Matter et al. [81] have provided evidence that the nuclear RNA-binding protein known as Src Associated in Mitosis 68 kD (SAM68) activates v5 inclusion in response to phosphorylation by ERK. Transfection studies showed that excess SAM68 enhanced v5 inclusion, but only in response to Ras signaling. Mutation of several Ser/Thr residues in SAM68 (putative ERK targets) resulted in reduced levels of v5 inclusion. Finally, the reduction of SAM68 levels in vivo blocked the inclusion of v5 RNA following the activation of signaling. In addition, phorbol esters or cytokines activate Ras to regulate CD45 splicing during T cell development [84].

**Akt-PI3K pathway and splicing**

Another important pathway, the Akt pathway, appears to modulate the function of the SR family of splicing factors that act on exonic splicing enhancers [85]. For example, serine phosphorylation of the SR protein SRSF5, via a phosphatidylinositol 3-kinase (PI 3-kinase) signaling pathway, has been shown to regulate alternative splicing of protein kinase C (PKC) bII mRNA [86]. Moreover, activated Akt has been further
implicated in directly phosphorylating SR proteins [87] or indirectly regulating splicing through SR protein specific kinases, such as SRPK2 [88] or Clk/Sty [89]. It has been shown that Akt activates SRPKs in EGF-treated cells by employing an unusual allosteric mechanism in the absence of any consensus motif in SRPK [90]. Akt activation induces a series of rearrangements with molecular chaperones and other regulatory factors to regulate the cellular distribution of the splicing kinases [91]. Interestingly, EGF signaling was capable of inducing massive reprogramming in alternative splicing. Blocking individual branches in the EGF signaling pathway demonstrated that Akt plays a major role, while other branches, such as the JAK/STAT and ERK pathways, make minor contributions to EGF-induced splicing [90].

**SR kinases as signal transducers**

Most SR proteins are subjected to extensive phosphorylation on Ser residues within their RS domain, and their phosphorylation status affects protein-protein interactions [92, 93] and regulates protein activity. The mammalian genome encodes two SRPK kinases families, one including SRPK1 and 2, and an additional gene which encodes Clk/Sty. All of these have been identified as SR-protein kinases [94], with SRPK1 being ubiquitously expressed in most cell types and tissues and SRPK2 expression being restricted mostly to neurons [95]. Most SRPK molecules are localized in the cytoplasm until the cell is stimulated by an extracellular signal [96]. This mechanism is thought to involve chaperones that localize in the cytoplasm. Upon receiving a mitogenic signal SRPK is translocated into the nucleus and can regulate the phosphorylation state of SR proteins, thus regulate splicing activity [91]. Therefore, SRPKs appear to act as signal transducers for regulating splicing in mammalian cells. These kinases have dual specificity and phosphorylate Tyr as well as Ser/Thr residues [97, 98]. The Clk/Sty kinases can phosphorylate SR proteins and have been shown also to co-localize with them in the nucleus [99]. In vitro assays have shown that Clk/Sty can influence the splicing activity of SR proteins by altering their phosphorylation status [100], and changes in CLK/STY autophosphorylation can modify its activity and specificity [101]. Although these properties indicate important
roles for these kinases in cell signaling, supportive evidence is limited. Recently, a role for the Clk/Sty kinase in the regulation of VEGF splicing has been demonstrated. It was shown that induction of the anti-angiogenic isoform of VEGF by TGFβ1 was inhibited by TGF003, a specific kinase inhibitor of Clk/Sty and also by SB203820, a kinase inhibitor of p38 MAPK [102].

**Regulation of splicing factors by kinase pathways**

Upstream signals may affect subcellular localization of hnRNPA1 and thus affect alternative splicing of its target genes. The ability of hnRNPA1 to shuttle to and from the nucleus may be important for nuclear export of mRNAs [103], translation and posttranslational processes. Additionally, a mechanism which controls hnRNPA1 localization can have a role in regulating its splicing activity. The first study to demonstrate that stress pathways such as the MAPK kinase 3 and 6 (MKK3/6)-p38-MAPK induce clearance of hnRNPA1 from the nucleus and in this way modulate its splicing activity was reported by the Cáceres group [104]. In this study, exposure to osmotic shock stimulus caused accumulation of hnRNPA1 (but not other members of the family or SR proteins) in the cytoplasm. Importantly, co transfection with a constitutive active form of MKK3/6 and p38α was sufficient to promote the above phenotype. In contrast, using a dominant negative form of MKK3/6 prevented accumulation of hnRNPA1 in the cytoplasm. Finally, in order to examine if activation of the MKK3/6-p38α pathway regulates alternative splicing, cells were transfected with the E1A adenovirus gene reporter and evaluated for alterations in distal/proximal splice site selection. In cells treated by osmotic stress the use of the 5' distal splice site was suppressed, meaning that, clearance of hnRNPA1 from the nucleus lead to SR protein domination which favors an increase in proximal 5' splice site selection. The authors of this study conclude that protein kinases involved in the phosphorylation of SR and/or hNRNP A/B may have a role in modulation of alternative splicing following upstream signals. The study of Blaustein et. al. 2005, presents a different model in which Clk and/or SRPK are responsible for SR activation [87]. In this paper they demonstrate that over expression of Clk1/2/3 or SRPK1/2 changed splice site choice following activation of PI3-Akt, by reducing the inclusion of an alternative
exon in the fibronectin transcript. Importantly, active Akt enhanced the translation of the fibronectin gene from a reporter plasmid [90]. The proposed model involves a direct phosphorylation of the SRSF1 protein by Akt, followed by formation of an ESE-mRNA-SR complex which serves as a better template for translation [87]. Further analysis is required to determine where in the cell the interaction between Akt and SRSF1 occurs and its role in the translation process. The contradicting actions of Akt and Clk (and/or SRPK) may imply a concentration dependent mechanism, by which several kinases exist to control the activity of splicing in the cell.

**MAPKs**

Mitogen-activated protein kinases (MAPKs) are serine-threonine kinases that function in transducing extracellular signals such as growth factors, stresses, and cytokines into cellular responses involved in cell proliferation, differentiation, survival, death, and transformation [20, 105]. The mammalian MAPK family consists of extracellular signal-regulated kinase (ERK), p38, and c-Jun NH2-terminal kinase (JNK). Each of these enzymes exists in several isoforms: ERK1 to ERK8; p38-α, -β, -γ, and -δ; and JNK1 to JNK3 [20]. The MAPK signaling axis comprises of at least three components: a MAPK kinase kinase (MAP3K), a MAPK kinase (MAP2K), and a MAPK. Activation of the MAPK cascade occurs through a series of binary interactions between the kinase components or by formation of a signaling complex containing multiple kinases that is guided by a scaffold protein. Such scaffold proteins mediate the activation of MAPK signaling pathways consisting of specific kinase components [23]. The JNK and p38 signaling pathways are activated by proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β or in response to cellular stresses such as genotoxic, osmotic, hypoxic, or oxidative stress. The JNK pathway consists of JNK, a MAP2K such as SEK1 (also known as MKK4) or MKK7, and a MAP3K such as ASK1, MEKK1, mixed-lineage kinase (MLK), or transforming growth factor-β-activated kinase 1 (TAK1) [30]. In the p38 pathway, several MAP2Ks such as MKK3 and MKK6 activate p38 and are themselves activated by the same MAP3Ks (such as ASK1 and TAK1) that function in the JNK pathway. In the ERK pathway, ERK1 or ERK2 is activated by MEK1/2, which in turn is activated by a Raf isoform such as A-Raf, B-Raf, or Raf-1 (also [21]
known as C-Raf). The kinase Raf-1 is activated by the small GTPase Ras, whose activation is mediated by the receptor tyrosine kinase (RTK)-Grb2-SOS signaling axis [20]. Both Mnk1 and 2 are targeted by both the ERK and p38 MAPK pathways. Subsequently, activated MAPKs phosphorylate various substrate proteins including transcription factors such as Elk-1, c-Jun, ATF2, p53 and Mns [20, 106]. Consistent with their critical roles in cellular activities, the MAPK signaling pathways have been implicated in the pathogenesis of many human diseases. For example, oxidative stress is considered to be a risk factor in the development of Alzheimer disease (AD) [107]. Reactive oxygen species (ROS) can trigger a cellular response which involves the activation of JNK and p38 in AD [108, 109]. Consistent activation of MAPK signaling is thought to contribute to AD pathogenesis through various mechanisms including induction of neuronal apoptosis [110, 111]. Cancer-associated mutations have been found in the MAPK signaling pathways, attributed mostly to Ras and B-Raf proteins, both of which participate in the ERK signaling pathway [20, 112]. Mutations in K-Ras occur frequently in many human cancers including those of the lung and colon [113]. Transgenic mice expressing an activated form (G12D) of K-Ras manifest MEK dependent hyper-proliferation of colonic epithelial cells, whereas expression of the corresponding activated form (G12D) of N-Ras did not have such an effect, suggesting that K-Ras (G12D), but not N-Ras (G12D), activates the MEK-ERK signaling pathway and thereby promotes the proliferation of colonic epithelial cells [114, 115].

**MKNK1 (Mnk1) and MKNK2 (Mnk2)**

Mnk1 (MAP kinase-interacting kinase 1) and Mnk2 are threonine/serine protein kinases which were originally discovered in a screen for substrates or binding partners of ERK [116, 117]. Both Mns are capable of phosphorylating the translation initiation factor eIF4E [116, 118, 119]. Mnk1 is responsible for inducible phosphorylation of eIF4E following extracellular signals that are transduced through MAPKs. Mnk2 has high basal activity which is essential for constitutive phosphorylation of eIF4E when MAPK activities are low. eIF4E is an important factor involved in the initiation of cap dependent translation [120, 121]. Formation of the eIF4F complex depends on the scaffold protein eIF4G, RNA helicase eIF4A [122, [22]
and eIF4E, which is essential for bringing the capped-mRNA. Among eIF4G functions is recruitment of the 40S ribosomal subunit to the mRNA via its interaction with eIF3 and binding of eIF4B, a protein that aids the RNA-helicase function of eIF4A, thus facilitating the translation of mRNAs that contain structured 5'-UTRs. The fact that eIF4E is the only component which can interact with the 5' cap mRNA makes it a limiting factor in the cap-dependent translation initiation. Therefore, eIF4E availability is crucial for de novo synthesis of proteins. Cells control eIF4E activity through eIF4E-binding proteins (4E-BPs) which compete with eIF4G on a binding site located in eIF4E. Activation of mTOR complex1 (mTORC1) results in the phosphorylation of 4E-BPs and their release from eIF4E, allowing eIF4E to form eIF4F complexes. The contribution of mTOR complex1 (mTORC1) to cancer transformation is mainly dependent on its regulatory role on the eIF4F complex. For instance, over-expression of eIF4E can confer resistance to the mTORC1 inhibitor rapamycin [124]. mTORC1 regulates the eIF4F complex assembly that is critical for the translation of mRNAs associated with cell growth, survival, angiogenesis and transformation. Inactivation of 4E-BP and the subsequent dissociation of 4E-BP from eIF4E, enables eIF4E to interact with the scaffold protein eIF4G permitting assembly of the eIF4F complex for the translation of structured mRNAs (Scheme 1) [125, 126]. Separate from its role in translation which operates through eIF4F complexes residing in the cytoplasm, eIF4E can also be found in the nucleus where it facilitates the export of a set of mRNAs from the nucleus to the cytoplasm [127]. eIF4E is specifically attracted to capped mRNAs that contain a 50-nucleotide structural element in their 3' UTR, known as an eIF4E sensitivity element (4E-SE) [128, 129]. RNAs targeted for eIF4E export include c-Myc, Hdm2, NBS1, ODC, and Cyclin D1 [128, 129]. A fraction of these targets also contain highly structured 5' UTRs, making them also translation targets of eIF4E. Coordinate actions through shuttling mRNAs and targeting them for translation, serve as a regulatory mechanism by which eIF4E could promote and/or sustain tumor formation (Scheme 1). A recent elegant study further extended the oncogenic activity of eIF4E in relation to its ability to alter the composition of the nuclear pore complex (NPC) [130]. Over-expression of eIF4E down-regulates a potent inhibitor of eIF4E-dependent mRNA export, RanBP2, while
Scheme 1: eIF4E regulation by the MAPKs and mTOR.
Signaling by the Ras MAPK pathway involves a phosphorylation cascade which results in the activation of Mnk1 and 2. Both Mnks can bind to eIF4G which serves as a scaffold protein to facilitate phosphorylation of eIF4E on serine 209. In parallel, activation of mTOR liberates eIF4E from its negative regulator 4EBP which is now free to undergo phosphorylation by Mnk. eIF4E enhances translation of mRNAs possessing complex 5'-cap structures in their 5' UTR, which are involved in survival and proliferation. In the nucleus, eIF4E increases the export of mRNAs containing 4E-SE elements in their 3' UTR to the cytoplasm.
up-regulating RanBP1 which enhances export by promoting faster release and/or recycling of export complexes. RanBP2 may promote the disruption between 4E-SE targets with the eIF4E nuclear ribonuclear particles (RNPs) that associate with proximity to the NPC. Notably, a mutant of eIF4E, S53A, exhibits defects in mRNA export, transformation but not in translation. RNA immunoprecipitation (RIP) experiments reveal that the S53A mutant does not bind mRNAs in the nucleus but does bind mRNAs, including VEGF, in the cytoplasm with similar efficiency to wild-type eIF4E. In summary, the oncogenic activity of eIF4E is in part mediated by remodeling the NPC, therefore enabling efficient export of some essential mRNAs and this mechanism is presumably dependent on phosphorylation of serine 209.

Aberrations in the PI3K/PTEN/Akt/mTOR pathway are frequently found in human tumors, and those tumors sometimes display insensitivity and resistance to cancer therapy [124, 131]. Following extracellular signaling by growth factors, mitogens and/or cytokines, PI3K initiates a cascade of events which result in the activation of Phosphoinositide-Dependent Protein kinase 1 (PDK1). PDK1 induces Akt phosphorylation which in turn inactivates the tumor suppressor complex comprising of TSC1 and 2 (tuberous sclerosis complex 1/2), resulting in the activation of mTORC1 (target of rapamycin complex 1) by Rheb-GTP [132, 133]. PTEN is a critical tumor suppressor gene and is often mutated or silenced in human cancers [134, 135]. Its loss of function results in the activation of Akt and increased downstream mTORC1 signaling. Considering the importance of the PI3K/Akt/mTOR pathway in regulating mRNA translation of genes that encode for pro-oncogenic proteins and activated mTORC1 signaling in a high proportion of cancers, these kinases have been actively pursued as oncology drug targets [136, 137]. However, it has recently become clear that the mTOR pathway participates in a complicated feedback loop that can impair activation of Akt [124, 138]. It has been shown that prolonged treatment of cancer cells or patients with mTOR inhibitors causes elevated PI3K activity that leads to phosphorylation of Akt and eIF4E, and promotes cancer cell survival [139]. The fact that eIF4E is a downstream modulator for Akt and mTOR, makes it capable of executing their oncogenic actions in tumorigenesis and drug resistance. Therefore, Akt signaling via eIF4E is an important mechanism of oncogenesis and drug resistance in vivo [124]. For these reasons, strategies aimed
against Akt and mTOR, or directly inhibiting eIF4E activity, have been proposed as treatments for cancer [140, 141]. Another signaling pathway that controls eIF4E is the Ras/Raf/MAPK signaling cascade. Among many targets, activation of the MAPks ERK1/2 and p38, leads to phosphorylation of MAP kinase-interacting kinase 1 (Mnk1) and Mnk2 (Scheme 1). The ERK pathway is misregulated in many cancers, for example activating mutations in Ras (found in 20% of tumor cells) or loss of function of the Ras GTPase-activator protein NF1. As a consequence of Mnk1/2 activation, eIF4E is specifically phosphorylated on serine 209 in the eIF4F complex, by virtue of eIF4G which serves as a bridge between eIF4E and the Mnks, to ensure proper phosphorylation [142, 143]. The biological significance of eIF4E phosphorylation and its effect on translation is not completely understood. Biophysical studies indicate that phosphorylation of eIF4E actually decreases its affinity for the cap of mRNA, which plays a role in facilitating scanning or permitting the transfer of eIF4E from mRNAs that are already undergoing translation to other mRNAs whose translation is subsequently promoted [143]. Several studies have attempted to make a direct connection between phosphorylation of eIF4E and the tumorigenesis process. Over-expression of eIF4E-ST209/210AA, a double mutant in which serine 209 and threonine 210 were replaced with alanine precluding its phosphorylation on these sites, substantially reduced the ability of cells to form colonies in the anchorage-independent growth assay as compared to cells with wild type eIF4E [144]. In a similar experiment, substitution of serine 209 to alanine or aspartic acid (which mimics the phosphorylated form of eIF4E) (S209A, and S209D respectively) also abrogated the ability of cells to develop colonies, suggesting that phosphorylation by Mnk1/2 is essential for eIF4E oncogenic activity. Using a mouse model in which lymphomas are generated from Eμ-Myc expression, transgenic hematopoietic stem cells (HSCs) transfected with wild-type eIF4E greatly enhanced Myc-mediated lymphomagenesis compared to control HSCs. In contrast, mice reconstituted with cells carrying the S209A mutant were defective in tumor development, whereas reconstitution with cells expressing the phosphomimetic S209D mutant displayed accelerated tumor onset comparable with wild-type eIF4E [145]. In addition, HSC cells expressing constitutive active Mnk1T332D (substitution of threonine to aspartic acid which mimics phosphorylation) but not kinase dead
Mnk1T2A2 (substitution of two threonine located in the catalytic site to alanine which preclude activation) accelerated lymphoma development in a similar manner to eIF4E alone. Active Mnk1T332D and eIF4E-expressing lymphomas showed low levels of apoptosis compared to control tumors. This was attributed to the ability of eIF4E or Mnk1 to increase the expression of the anti-apoptotic protein Mcl-1. Notably, Mnk1-mediated phosphorylation of eIF4E on Ser209 correlated with the level of Mcl-1 expression [145]. In support of the above findings, primary MEF cells from knock-in (KI) mice which contain the eIF4E mutant S209A, increased foci formation in comparison to WT MEFs when infected with a combination of retroviruses expressing RASV12 and c-MYC or E1A [146]. Similarly, co expression of RASV12 and c-MYC in MEF cells described above reduced by almost 10 fold the number of colonies when seeded into soft agar compared to WT MEFs. Given that deletion of the tumor suppressor gene Pten is expected to result in increased eIF4E phosphorylation, crossing the knock-in mice with Pten conditional knockout mice (those mice develop prostate cancer) presumably will produce mice with defects in tumor development. Indeed, lesions from prostate lobes displayed lower incidence of high grade prostatic intraepithelial neoplasia (PIN) in eIF4E-KI/PtenFlox/Flox mice than eIF4E-WT/PtenFlox/Flox mice. Taken together, these studies indicate that eIF4E phosphorylation plays an important role in Pten loss-induced tumorigenesis. Moreover, cells derive from eIF4E-KI/PtenFlox/Flox mice which contain the unphosphorylated form of eIF4E (S209A) are resistant to Ras-induced transformation. This data highlights the role of oncogenes in the Mnk-eIF4E route as critical factors in tumorigenesis.

As Mnk-eIF4E interaction serves to execute oncogenic signals emanating from the PI3K-Akt pathway, the impact of Mnk deletion in tumorigenesis was investigated. Results from PTEN conditional knockout mice, where both Mnk1 and Mnk2 were specifically deleted in T cells (Mnk1/2-DKO tPTEN−/− mice) showed that Mnk1/2-DKO tPTEN−/− mice exhibited attenuated tumor growth compared to the parental tPTEN−/− mice [147]. Moreover, lymphoma tissues from Mnk1/2-DKO tPTEN−/− mice appeared smaller in size than those of control mice. Analysis of lymphoma tissues from Mnk1/2-DKO tPTEN−/− mice revealed a complete reduction in the phosphorylation status of eIF4E, which was greatly enhanced in lymphomas from
tPTEN−/− mice. These results highlight the kinase property of Mnk1 and Mnk2 as critical transducers in the tumorigenesis process. Pharmacological inhibition of Mnk1/2 was reported to suppress transformation in vitro [144, 148]. In addition high protein levels of Mnk1 have been found in human glioma tumors [149]. Collectively, these data prompted the examination of the effects of abolishment of Mnk1 activity in cancer glioma cells. When U87MG Mnk1 knockdown cells were injected into nude mice, they showed substantially reduced levels of phosphorylated eIF4E and markedly decreased tumor formation compared to control tumor cells [147]. Complete abrogation of eIF4E phosphorylation could affect cellular translation and consequently have harmful biological defects. Surprisingly, while Mnk activity is necessary for eIF4E-mediated oncogenic transformation, it is dispensable for normal development even without activation of eIF4E [150]. Furthermore, protein synthesis which was determined by measuring the incorporation of 35S methionine and 35S cysteine was not affected in MEF cells from Mnk1/2 DKO mice in comparison to wild type MEF cells [150].

**MKNK splicing isoforms**

Four human Mnk isoforms (Mnk1a, 2a, 1b and 2b) and two mouse Mnk isoforms (Mnk1 and 2) have been reported [116, 117, 151, 152]. Several kinases involved in signal transduction have been reported to be alternatively spliced in response to elevated levels of SR protein [57]. One of these is Mnk2. *MKNK2*, the gene coding for the kinase Mnk2, can produce two isoforms (Mnk2a and Mnk2b) following alternative splicing at the 3′ prime splice site at exon 13. SRSF1 over-expression affects *MKNK2* alternative splicing resulting in increased Mnk2b expression, decreased Mnk2a expression and enhanced eIF4E phosphorylation (on serine 209) without activation of the upstream kinases in the MAPK pathway [57]. In addition to *MKNK2* there are other examples of alternatively spliced isoforms whose products are necessary for cancer transformation [153, 154].

As a consequence of 3′ prime alternative splicing in exon 13 of human *MKNK1* and *MKNK2*, exon 13a selection creates longer versions referred to as Mnk1a and Mnk2a which possess a MAPK-binding motif. Whereas selection of exon 13b produces shorter isoforms referred to Mnk1b and Mnk2b which contain a truncated form. In the
corresponding murine proteins no short forms have yet been identified [155]. Another recent study showed that resistance of pancreatic cancer cells to Gemcitabine is mediated by SRSF1 up-regulation and a switch in Mnk2 alternative splicing, which enhances eIF4E phosphorylation implicating this alternative splicing event with chemotherapy resistance [156]. Sequence alignment analysis reveals that all four isoforms have a nuclear localization signal (NLS) and an eIF4G-binding site in their N-terminal regions which, respectively, allow the kinases to enter the nucleus and to phosphorylate eIF4E efficiently. The central catalytic domains of the pairs of isoforms Mnk1a/b and Mnk2a/b are identical and closely homologous between Mnk1 and Mnk2 proteins [157]. The main structural differences lie within the C-terminal domain (Fig. 1). The C-terminal regions of Mnk1a and Mnk2a contain a MAPK-binding site, and thus can be phosphorylated and activated by ERK and p38 MAPK [116, 142]. The short isoforms, Mnk1b and 2b, however, lack this domain and are poor substrates for ERK or p38 MAPK [151, 152, 157, 158]. At least two threonine residues (Thr209 and Thr214 in human Mnks indicated in Fig. 3A) in this region are phosphorylated by MAPKs, and their replacement with alanine results in inactive kinases [116, 159]. The threonine residues in Mnks correspond to the residues in MK2/3 (MAPK-activated protein kinases), which can also be phosphorylated by p38, suggesting a similar activation mechanism [160]. While the activity of Mnk1a is tightly regulated by ERK and p38 MAP kinase, Mnk2a shows high basal activity. Mnk1b and Mnk2b show, respectively, quite high and low activity, which appears to be unregulated, likely reflecting their lack of binding sites for ERK/p38 MAPK [118]. Several studies have identified different functions for Mnk2 and Mnk1 proteins in their role in translation. The scaffold protein eIF4G can be regulated by a variety of stimuli given its multiple phosphorylation sites [161, 162]. Importantly, Ser1108 phosphorylation of eIF4G has been reported to facilitate the translation process (Scheme 2) [163]. Insulin and IGF1 can increase Ser1108 phosphorylation in a rapamycin-sensitive manner, indicating crosstalk between this phosphorylation event and the IGF1 to mTOR pathway [164]. Furthermore, enhanced protein synthesis in rat skeletal muscle and heart after feeding is associated with Ser1108 phosphorylation and assembly of the active eIF4E-eIF4G complex [165, 166]. In contrast, Ser1108 phosphorylation is diminished under conditions that negatively affect protein
Scheme 2: Mnk2a is a negative regulator of translation.
In skeletal muscle tissue stimulation by insulin growth factor-1 (IGF-1) or insulin mediate mTOR activation which in turn phosphorylates its downstream substrate S6K. S6K induces phosphorylation of eIF4G, indirectly, which enhances cap translation executed by eIF4E. When cells are either treated with rapamycin or starved for growth factors mTOR becomes inactivated and eIF4G phosphorylation is inhibited by various molecular mechanisms. Mnk2a was shown to interact with mTOR and inhibit S6K phosphorylation independently of its kinase activity. In addition, Mnk2a induces the activation of serine rich protein kinase (SRPK) which functions as a negative regulator of eIF4G.
synthesis, such as starvation and sepsis [167]. Collectively, these results suggested that eIF4G phosphorylation at Ser1108 is a molecular marker associated with enhanced protein translation. Knockdown of Mnk2, but not Mnk1, increased eIF4G Ser1108 phosphorylation in cultured myotubes, and overcame rapamycin’s inhibitory effect on this phosphorylation event [168]. Moreover, phosphorylation of Ser1108 in muscle tissue was increased in mice lacking Mnk2, but not those lacking Mnk1, and this increased phosphorylation was maintained in Mnk2-null animals under atrophy conditions and upon starvation [168]. Conversely, overexpression of Mnk2 decreased eIF4G Ser1108 phosphorylation. A recent study revealed that serine-arginine–rich protein kinases linked increased Mnk2 activity to decreased eIF4G phosphorylation. In addition, it was found that MNK2 is capable of interacting with mTOR and suppressed phosphorylation of a downstream target, the ribosomal kinase p70S6K (70-kD ribosomal protein S6 kinase), through a mechanism independent of the kinase activity (Scheme 2) [168]. In conclusion, this study showed that Mnk2 plays a unique role, not shared by its closest homolog Mnk1, in regulating protein translation through its negative effect on eIF4G Ser1108 phosphorylation and p70S6K activation. An earlier paper [169] showed similar negative effects on translation by Mnk2, strengthening these conclusions.
Hypothesis and study aims:

The notion that the Mnk2a isoform may interact with p38-MAPK, together with its ability to suppress translation led us to hypothesize that Mnk2a is a regulator of the p38 stress pathway and functions upstream rather than downstream to it. The main goal of this work will be to emphasize alternative splicing as a novel oncogenic process which is manipulated by cancer cells. Specifically, the work investigates whether alternative splicing of the MKNK2 gene plays a role in the oncogenic process by down-regulating a tumor suppressor isoform while up-regulating a tumor promoting isoform. To this end we decided to elucidate and characterize from a biological and biochemical view the role of Mnk2 splicing isoforms in cancer development.

Specific Aims of this study:

1. To characterize the oncogenic and biochemical properties of Mnk2 splicing isoforms and their role in cancer.
2. To identify oncogenic signals involved in the regulation of MKNK2 splicing and the role of Mnk2a as a modulator of the p38-MAPK stress pathway.
**Experimental procedures**

**Plasmids**

Mnk2a and Mnk2b cDNAs were amplified by RT-PCR from HeLa cell RNA extracts using primers coding for an N-terminal T7 tag and subcloned into the EcoRI site of the pCDNA3.1 and pBABE plasmids. Mnk2a-kinase dead (Mnk2aKD), Mnk2aL/S and Mnk2aKKR were generated by site directed mutagenesis. Mnk2aKD: lysine 113 (K113) was replaced by alanine. Mnk2aL/S: leucines 281/285 were replaced by serines. Mnk2aKKR: lysines 60/61 and arginines 62 were replaced by alanines. Mutagenesis primers are described in Table S1. pWZL-HA-p38α(D176A+F327S) was generated by sub-cloning of HA-p38α(D176A+F327S) from pCDNA3.1 [170-172] into the EcoRI site of pWZL-hygro. shMnk2a-1 and shMnk2a-2 were constructed in the MLP vector [173]. shRNA sequences appear in Table S1.

**Cells**

HEK293, MCF-7, MDA-MB-231, Panc-1, Patu 8988T, MEF MKK3/6 wild type and MKK3/6−/− cells were grown in DMEM supplemented with 10% (v/v) FBS, penicillin and streptomycin. Human breast cells: MCF-10A, were grown in DMEM/F12 supplemented with 5% (v/v) horse serum (HS, Biological Industries, Israel), 20 ng/ml epidermal growth factor (EGF) (Sigma), 10 μg/ml insulin (Biological Industries, Israel), 0.5 μg/ml hydrocortisone (Sigma), 100 ng/ml cholera toxin (Sigma), penicillin and streptomycin. HMLE cells were grown in MEBM/DMEM/F12 supplemented as described above. HMT-3522-S1 cells were grown in DMEM/F12 supplemented with 250 ng/ml insulin, 10 μg/ml transferrin, 5 μg/ml prolactin, 10 ng/ml EGF, 10−10 M 17β-estradiol, 10−8 M sodium selenite, 0.5 μg/ml hydrocortione. HMEC cells were derived from normal breast tissue and were purchased from Cell Lonza and were grown in human mammary epithelial cells serum-free medium (ECACC). MDA-MB-468 cells were grown in Leibovitz-F12 medium supplemented with 10% (v/v) FBS, penicillin and streptomycin. SUM159 cells were grown in Ham's F12 medium with 5% calf serum, 5 μg/ml insulin and 1 μg/ml hydrocortisone. Patu 8902 cells were grown in DMEM supplemented with 10% (v/v) FBS, 2.5% HS, penicillin and streptomycin. AXPC3 and BXPC3 cells were grown in RPMI medium supplemented
with 10% (v/v) FBS, penicillin and streptomycin. All cell lines were grown at 37 °C and 5% carbon dioxide. To generate stable cell pools, NIH 3T3 and MCF-10A cells were infected with pBABE-puro retroviral vectors [174] expressing T7-tagged human Mnk2 isoform cDNA. Medium was replaced 24 hours after infection, and 24 hours later, infected cells were selected for by the addition of puromycin (2 µg/ml) or hygromycin (200 µg/ml) for 72-96 hours. In the case of double infection with pWZL-hygro-Ras [174] or pWZL-hygro-p38α, cells were treated with hygromycin for 72 hours after selection with puromycin. In the case of infection with MLP-puro-shRNAs vectors, MCF-10A cell transductants were selected for with puromycin (2 µg/ml) for 96 hours.

**Immunoblotting**

Cells were lysed in Laemmli buffer and analyzed for total protein concentration as described previously [57]. 30 µg of total protein from each cell lysate was separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were blocked, probed with antibodies and detected using enhanced chemiluminescence. Primary antibodies were phospho-eIF4E Ser209 (1:1000), eIF4E (1:1000), phospho-p38 (Thr180/Tyr182) (1:1000), phospho-MNK (T197/T202) (1:1000), phospho-MK2 (1:1,000), MK2 (1:1,000), (Cell Signaling Technology). MNK2 (1:1000, Santa Cruz), β-catenin (1:2000, Sigma); SRp55 (mAb 8-1-28 culture supernatant); T7 tag (1:5000, Novagen); p38 (1:1000, Santa Cruz). Secondary antibodies were HRP-conjugated goat anti-mouse, goat anti-rabbit or donkey anti-goat IgG (H+L) (1:10,000, Jackson Laboratories).

**Cytoplasmic/Nuclear Fractionation**

Cellular fractionation was performed using the NE-PER (#78833) fractionation kit of Pierce according to the manufacturer’s instructions.

**Anchorage-independent growth**

Colony formation in soft agar was assayed as described previously [57]. Plates were incubated at 37°C and 5% CO2. After 10–18 days, colonies were counted from ten different fields in each of two wells for each transductant pool and the average
number of colonies per well was calculated. Colonies containing ~70 cells per colony were used as the cutoff for colony formation. Colonies were stained and photographed under a light microscope at 100× magnification. Although NIH3T3 and MCF-10A cells had a limited ability to form colonies, we determined that these cells were not able to form tumors in NOD SCID mice, thereby being a normal immortal cell lines.

Growth curves
Transductant pools of MCF-10A cells were seeded at 2500 or 5000 cells per well in 96-well plates. Every 24 hours cells were fixed and stained with methylene blue as described previously [57], and the absorbance at 650 nm of the acid-extracted stain was measured on a plate reader (BioRad).

Cell cycle analysis
For cell cycle analysis MCF-10A cells transduced with the indicated viruses were trypsinized, washed twice with PBS and fixed in cold 70% ethanol at −20 °C for 30 minutes. Cells were then washed once with PBS and incubated for 45 minutes at room temperature with 250 μg/mL RNAse A and 50 μg/mL propidium iodide. Cell cycle was analyzed with FACScan flow cytometer.

Anoikis experiments
MCF-10A cells were transduced with the indicated retroviruses. Following selection, 1×10⁶ cells were resuspended in serum-free medium and incubated in 15ml polypropylene tubes with 360° rotation, average-speed of 25 rpm in a 37°C incubator chamber for 48hours. After 48 hours, cells were centrifuged at 1000g for 5 minutes and stained with trypan blue and live and dead cells counted.

Survival and apoptosis assays
MCF-10A or Panc-1 cells were transduced with the indicated retroviruses. Following selection, 1×10⁴ cells per well were seeded in 96-well plates. 24 hours later, the cells were serum starved for another 24 hours. At 24 hours (before treatment) one 96-plate was fixed and served as normalizing control ("Time 0"). After starvation the medium was replaced with starvation medium containing the indicated concentration of
anisomycin and the cells were incubated for an additional 24 hours. Cells were fixed and stained with methylene blue as described previously [57] and the absorbance at 650 nm of the acid-extracted stain was measured on a plate reader (BioRad) and was normalized to cell absorbance at "Time 0". For apoptosis, MCF-10A cells were seeded on 6-well plates (500×10^3 cells/well). 24 hours later cells were incubated with 0.5 or 1 µM anisomycin for 24 or 48 hours. Medium and PBS washes were collected together with cells trypsinized from each well into 15ml tubes and centrifuged at 1000 g for 5 minutes. Cells were washed with PBS and after another centrifugation were resuspended in 100 µl of PBS. 10 µl of the cell suspension was mixed with 10 µl of 4% trypan blue solution and live/dead cells were counted using a Bio-Rad TC-10 Automated Cell Counter. After counting, the remaining 90 µl of cell suspension was centrifuged, PBS was discarded and cells were resuspended in 90 µl of Laemmli buffer. Lysates were separated on SDS-PAGE and after Western blotting membranes were probed with antibodies against cleaved caspase 3 (Cell Signaling) to evaluate induction of apoptosis.

RT-PCR
Total RNA was extracted with Tri reagent (Sigma) and 2 µg of total RNA was reverse transcribed using the AffinityScript (Stratagene) reverse transcriptase. PCR was performed on 1/10 (2 µl) of the cDNA, in 50 µl reactions containing 0.2 mM dNTP mix, 10× PCR buffer with 15 mM MgCl₂ (ABI), 2.5 units of TaqGold (ABI) and 0.2 mM of each primer; 5% (v/v) DMSO was included in some reactions. PCR conditions were 95 °C for 5 minutes, then 33 cycles of 94°C for 30 seconds, 57°C for 30 seconds and 72°C for 45 seconds, followed by 10 minutes at 72°C. PCR products were separated on 1.5% or 2% agarose gels. Primers are listed in Table S1.

Q-RT-PCR
Total RNA was extracted with Tri reagent (Sigma) and 2 µg of total RNA was reverse transcribed using the AffinityScript (Stratagene) reverse transcriptase. We determined the mRNA levels of Mnk2a, Mnk2b, SRSF1, FOS, and COX-2 in MCF-10A breast cell lines by performing quantitative PCR with SYBER Green (SYBR® Premix Ex Taq™ # RR041A) using the CFX96 (Bio-Rad) machine. Unknown samples were
compared to a standard curve, which is established by serial dilutions of a known concentration of cDNA. The standard that was used is β-actin. Ct- Threshold cycle from β-actin and unknown samples were inserted to the standard curve formula and the final value was the ratio between the unknown sample divided by the β-actin standard gene. Primers are listed in Table S1. The PCR reaction is composed of the following steps: 1 cycle at 95°C for 10 seconds; 40 cycles of 95°C for 5 seconds and 48°C for 20 seconds.

**Colony survival assay**
MCF-10A cell populations transduced with retroviruses encoding for Mnk2 isoforms were transduced with pWZL-hygro-RAS\textsuperscript{V12}. Immediately after selection cells were diluted as indicated with fresh medium and seeded in 6-well plates for 14-20 days. Colonies were fixed and stained with methylene blue as described previously [57], for quantification, the number of colonies in each well was calculated.

**Tumor formation in mice**
Pools of Ras-MCF-10A cells expressing the indicated Mnk2 isoforms or shRNAs were injected into the rear flanks of NOD-SCID mice (2×10\textsuperscript{6} cells per site in 100 µl of serum free medium containing 0.25 v/v growth factor stripped matrigel (BD Bioscience) using a 26-gauge needle. Tumor growth was monitored as described previously [174].

**Immunoprecipitation**
Cells were lysed with CHAPS buffer [175], the lysates were cleared by centrifugation at 12,000g for 15 minutes, and the supernatant was passed through a 0.45-mm filter. For each cell line, 30 µl of 50% (v/v) protein G–sepharose beads was incubated with 2 µg of antibody against HA (Santa Cruz) or 2 µg of antibody against T7 (Novagen) for 1.5 hours at 4°C. The beads were then incubated while rotating with 0.7 mg of total protein from each lysate for 4 hours. The beads were washed three times with CHAPS buffer and boiled for 5 minutes in 50 µl of 2×SDS Laemmli buffer. After SDS-PAGE and transfer the membranes were probed with antibodies against Mnk2 and p38α.
**Immunofluorescence**

Cells plated on cover glass slides were rinsed twice with 1 X PBS+ containing Mg++/Ca++ and fixed with 4% PFA at room temperature for 30 minutes. Cells were permeabilized with 0.5% NP40 (Fluka) and then washed with 1X PBS+ containing 0.1% Tween 20 (PBS+T). Following permeabilization, cells were blocked with PBS+T containing 20% fetal bovine serum for 45 minutes. Fixed cells were incubated with the following primary antibodies in 1:1000-1:2000 dilutions: mouse anti T7-tag (Novagen), rabbit anti p38α (Santa Cruz), mouse anti HA-tag (Santa Cruz) over night at 4°C. Following incubation, cells were washed once with 1% ammonium chloride and then with PBS+T, and incubated with the following secondary antibodies in 1:1000 dilution: Alexa 594 goat anti-,(Invitrogen), Alexa donkey anti-rabbit 488 (Jackson) and incubated for 50 minutes at room temperature in the dark. Cover-glasses were washed with 1% ammonium chloride and then with PBS+T. Cover-glasses were placed onto slides with mounting solution (Thermo Scientific Cat# TA-030-FM) containing DAPI (1:1000, Sigma Cat#D9564)). Microscopy was performed using a NIKON ECLIPSE Ti / IntenseLight, C-HGFI using the NIS Elements digital system.

**Mnk2 in vitro kinase assay**

In vitro kinase assay was performed using recombinant Mnk2, eIF4E (Abcam) and kinase-dead p38α [176] as described [171] [170]. In brief, 300 ng of recombinant Mnk2a was incubated alone or with either 5 µg of recombinant kinase-dead p38α or 1 µg of recombinant eIF4E, in 20 µl reaction buffer containing: 20 µM cold ATP, 0.5 µCi ³²P ATP, 30 mM MgCl₂ 10 mM HEPES pH 7.5, 50 mM EGTA, 10 mM β-glycerophosphate, 5 mM NaVO4, 50 mM β-mercaptoethanol and 0.5 mM DTT. Reactions were shaken for 1 hour at 30°C. Reactions were stopped by the addition of 50 µl of cold DB (12.5 mM HEPES pH 7.5, 100 mM KCL, 0.5 mM DTT, 6.25% glycerol) followed by the addition of 30 µl 4X Laemmli buffer. 20 µl of the final volume was separated by SDS-PAGE, transferred to nitrocellulose by Western blotting and exposed to FUJI phosphorimager. After the radioactive exposure, the membrane was probed with the indicated antibodies to view the recombinant proteins in the reaction.
Modulation of MKNK2 splicing by Antisense-RNA oligos

Cy5-2'-O-methyl modifies RNA oligonucleotides were synthesized by and purchased from Sigma. 2.5 μM of each oligo were transfected by lipofectamine 2000 according to the manufacturer's instructions. For effects on alternative splicing, BEAS2B, MCF-10A-Ras or MCF-10A cells were harvested 48 hours after transfection and levels of Mnk2a and Mnk2b were analyzed by RT-PCR. For biological assays (colony survival, soft agar, apoptosis) cells were transfected as described and 24 hours after Oligos transfection were seeded for the indicated assay. Oligos sequences are indicated in Table-1.

**Table-1. Primers and sequences**

**Knockdown shRNA oligos**

shMnk2a-1:

CAGTGATTCATGTTTCGTAA

shMnk2a-2:

CAGGTGTTGAAGACGTCTACCA

**RT-PCR**

Mnk2:

Mnk2a/2b Forward: CCAAGTCCTGCAGCACCCCTG;
Mnk2a Reverse: GATGGGAGGGTCAGGCGTGGTC;
Mnk2b Reverse: GAGGAGGAAGTGACTGTCCCAC.

GAPDH Forward: ATCAAGAAGGGTGGAGGCCATGT;
GAPDH Reverse: CTTACTCTTGGAGGCCATGT.

p38-MAPK target genes (Mouse):

mCOX2 Forward: TACAAGCAGTGGCAAGGCAAGGC
mCOX2 Reverse: CAGTATTGAGGAGACAGATGGG
mc-FOS Forward: GGCTTTCCAAAACCTTCGACC
mc-FOS Reverse: GGGGCTACAAAGCCAAAC
mGAPDH Forward: AATCAACGGCAGTTCAAGGC
mGAPDH Reverse: GAGGCAGGGATGATTTCTGG

p38-MAPK target genes (Human):
COX2 Forward: TCATTCACCAGGCAAATTGC
COX2 Reverse: TCTTTGATGATTTACAGGG
c-FOS Forward: GCTCGCCTGTCAACGCGCAG
c-FOS Reverse: TGAGGGGCTCTGGTCTGCGA

Q-RT-PCR
p38-MAPK target genes (Human):
COX2 Forward: CCGAGGTGTATGTATGAGTGT
COX2 Reverse: CTGTGTTTGGAGTGGTTTC
c-FOS Forward: GAACAGTTATCTCCAGAA
c-FOS Reverse: TTCTCATCTTCTAGTTGG

SRSF1:
SF2 Forward: GAGTTCGAGGACCCGAGACG
SF2 Reverse: GAGCTCCGCCACCTCCAC
Mnk2:
Mnk2a/2b Forward: TCCGTGAGCGCAACAGCAG
Mnk2a Reverse: GGTCTTTGGCAGACGCTG
Mnk2b Reverse: GAGGAAGTGACGTGTCACAC

Actin:
Actin Forward: CCCAGCACAATGAAGATCAA
Actin Reverse: TAGAAGCATTTGCGGTGGAC
GAPDH

GAPDH Forward: TCACCACCATGGAGAAGGC
GAPDH Reverse: GCTAAGCAGTTGGTGTTGCA

**Mnk2 Mutagenesis:**

**Mnk2aKD:**
L113A Forward: CAGGAGTACGCCGTGAGATCATGAGAAGGCAG
L113A Reverse: CTGCTTCTCATACTGACGACGGCGTACTACCTG

**Mnk2a L/S:**
L/S-Forward: TGCGACCTGTGGAGCATGGGCTCACTTGATATCCTACTCAGCG
L/S-Reverse: CGCTGAGTAGGATATAACTGATGACCGCACTGCTCCACAGGTCGCA

**Mnk2a KKR:**
KKR/AAA Forward:
GACGCCAAGAAGAGGGCAAGAAGGCGGCGCCGGCGGCCGACCCGACA
GCTTCTC

KKR/AAA Reverse:
GAGAAGCTGTGCCTGGCGCACTGCTTTCTGTCCCCCTCTTCTTGG
GCGTC

**Antisense RNA oligos:**

**MKNK2 2a block oligo:** CAGCTGTTCCTGGGAAACGG

**MKNK2 2b block oligo:** GGAAGTGACTGTCCCACCTT

**SCRAMBLED (Scr):** GCAATCCGCAATCCGCAATCC
Results

*Mnk2a mRNA is down-regulated in colon, breast and lung cancers.*

It has been shown recently that the splicing factor oncoprotein SRSF1 (SF2/ASF) is amplified in breast tumors, and is up-regulated in several other types of cancers such as lung and colon [57]. In addition, it has been shown that SRSF1 modulates the splicing of *MKNK2*. *MKNK2*, the gene encoding for Mnk2, is alternatively spliced to yield two isoforms Mnk2a and Mnk2b. Both isoforms are identical in their coding sequences except for their last exon which generates a different C-terminus [155, 177]. Mnk2a contains a MAPK binding domain in its C-terminus and Mnk2b does not (Fig. 1). To examine if changes in *MKNK2* splicing is a general phenomenon in cancer, we compared mRNA levels of Mnk2a and Mnk2b in immortal and primary breast cells to breast cancer cell lines and breast tumor samples. We detected higher or equal expression of Mnk2a compared to Mnk2b in immortal (MCF-10A, HMLE, HMT-3522-S1 [178]) and primary breast cells (HMEC). In contrast, Mnk2a expression was significantly decreased in tumor cell lines and tumor samples (Fig. 2). Reduced Mnk2a levels compared to Mnk2b could be observed also at the protein level in most of the breast cancer cell lines compared to immortal non-transformed cells (Fig. 3). In cancer cell lines, Mnk2a protein levels were decreased dramatically, while mRNA levels were decreased approximately 4 fold. This result suggests that Mnk2a protein might be further destabilized in cancer cells by a mechanism yet to be found. The average ratio of Mnk2a to Mnk2b mRNA was significantly lower in breast tumor samples (13 samples) as compared to immortal and primary breast cell lines (4 samples) (Fig. 4a). To expand our analysis we analyzed RNA-seq data from The Cancer Genome Atlas (TCGA) project (https://tcga-data.nci.nih.gov/tcga/). We compared Mnk2a/Mnk2b ratios from lung samples (58 samples) compared to lung adenocarcinoma (411 samples) and normal colon (23 samples) compared to colon carcinoma (151 samples). Examining the reads covering the Mnk2a or Mnk2b splice junctions, we found that in the normal lung group 0/58 (0%) of the patients had equal or lower than 1:1 ratios of Mnk2a/Mnk2b, while in the lung adenocarcinoma tumor group 79/490 (16%) of patients had similar ratios (Fig. 4b). In the normal colon group 0/23 (0%) of patients had equal or lower than 1:1 ratios of Mnk2a/Mnk2b,
Figure 1. Structure of human Mnk2 isoforms generated from MKNK2 alternative splicing.

The human splicing isoforms of MKNK2 contain a basic region important for eIF4G binding in their N-terminus as well as a putative NLS. The catalytic domain contains two conserved threonine residues (T197, T202) in the activation loop that need to be phosphorylated (P) by MAP kinases for kinase activation [151]. Mnk2a contains a binding site for MAP kinases located in the C-terminus [151]. Mnk2b is generated by an alternative 3’ splice site in intron 14 that generates a shorter final exon(14b), which lacks the MAPK binding site [151].
Figure 2. A switch in MKNK2 alternative splicing in primary tumors and cancer cell lines.

RNA from the indicated primary or immortal breast cell lines, breast cancer cell lines (a, b) or primary breast tumors (c, d) was extracted and the levels of Mnk2a and Mnk2b mRNA were detected by quantitative (a,c) or semi-quantitative (b,d) RT-PCR. PCR products were run on a 2% agarose gel (b,d). GAPDH (b,d) or beta-actin (a,c) mRNA was used as a control.
Figure 3. Alternative splicing switch correlates with reduced protein levels of Mnk2a while maintaining levels of Mnk2b.

Human breast immortal and tumor cell lines were subjected to Western blot analysis and the protein levels of Mnk2a and Mnk2b were measured. β-catenin was used as a loading control.
Figure 4. Mnk2a mRNA is lower and Mnk2b is higher in breast, lung and colon cancer samples.

(a) The median ratio of Mnk2a to Mnk2b mRNA in breast tumors and non-transformed breast cell lines. p value represent the average of Mnk2a/Mnk2b ratios from non-transformed breast cell lines (4) and breast tumor samples (13). (T-test two tail analysis). (b,c) Box plot representation of Mnk2a/Mnk2b ratios in lung (b) and colon (c) normal/tumor tissues based on RNA-seq analysis from The Cancer Genome Atlas (TCGA) project (https://tcga-data.nci.nih.gov/tcga/). Mnk2a/Mnk2b median ratios are represented by a box plot. Top and bottom box edges represent the third and first quartile. Whiskers indicate 90 and 10 percentile, asterisks represent minimum and maximum points. p values represent the median reads of Mnk2a/Mnk2b ratio between normal and tumor samples. [(b); T-test one tail analysis, (c); T-test two tail analysis].
while in the colon tumor group 41/191 (21%) of the patients had similar ratios (Fig. 4c). These results suggest that lower Mnk2a levels and/or higher Mnk2b levels are more prevalent in breast lung and colon tumors than in the corresponding normal tissues. Since the splicing factor SRSF1 controls the splicing of Mnk2, we examined if Mnk2 alternative splicing correlates with SRSF1 expression. We found that SRSF1 RNA and protein levels were higher in breast tumor samples than in immortal breast cell lines in correlation with Mnk2 alternative splicing (Fig. 5). SRSF1 is phosphorylated by SRPK1 and Clk1-4 on its RS domain, which might explain the shift in the corresponding band in some of the breast cancer cell lines [99]. Thus, upregulation of SRSF1 levels in cancer cells probably leads to reduced Mnk2a and elevated Mnk2b levels.

**The Ras oncogene regulates alternative splicing of the MKNK2 gene.**

It is well established that the Ras proto-oncogene is mutated in many human cancers. In pancreatic cancer about 70% of the tumors harbor K-Ras mutation [179, 180]. Ras activation is one of the hallmarks of cancer and is essential for cancer development in several cancer types [181-183]. When active, Ras is capable of inducing multiple downstream effector pathways such as: Raf-MEK-ERK, Akt-PI3K and the GDP-GTP exchange factor (GEFs) for the Ral small GTPases (RalGDS), which mediate proliferation and survival process [181, 184, 185]. Initially, we wanted to explore whether Ras signaling may have an effect on MKNK2 splicing. To this end we evaluated the ratio of Mnk2 isoforms in cancer cells harboring a mutation in Ras. Pancreatic cancer cells harboring a K-Ras mutation showed reduced Mnk2a levels compared to pancreatic cancer cells harboring wild-type Ras, suggesting that Ras activation affects Mnk2 alternative splicing (Fig. 6a,b). Moreover, pancreatic cancer cells harboring a K-Ras mutation also showed increased SRSF1 expression compared to cancer cells with WT-Ras (Fig. 6c). To further investigate if Ras activity modulates MKNK2 splicing, we transduced immortal breast cells MCF-10A cells or the Hepatocellular (HCC) cell line HuH7 with a retrovirus encoding an active Ras mutant (H-Ras V12). Levels of SRSF1 expression and Mnk2 isoform ratios were examined in these cells. We found that both cell lines expressing active Ras induced upregulation
Figure 5. Increased RNA and protein levels of SRSF1 are found in breast cancer tumors and cell lines.

(a) RNA from immortal breast cell lines and human breast tumors was extracted and quantitative RT-PCR was performed to detect SRSF1 mRNA levels. (b) Immortal breast cell lines and human breast cancer cell lines were lysed and proteins separated by SDS-PAGE. After Western blotting membranes were probed with the indicated antibody to detect the levels of SRSF1. Antibodies against GAPDH served as a control.
Figure 6. Alternative splicing of the MKNK2 gene and SRSF1 protein levels in pancreatic cancer cell lines harboring a Ras mutation.

RNA from the indicated pancreatic cancer cell lines was extracted and levels of Mnk2a and Mnk2b mRNA were detected by quantitative RT-PCR (a) and semi-quantitative RT-PCR (b). PCR products were run in a 2% agarose gel. GAPDH mRNA was used as a control. (c) Cells described in (a) were lysed and subjected to Western blot analysis. The levels of SRSF1 protein were detected by specific antibodies. GAPDH levels served as a loading control. (d) BXPC and Panc1 cells were lysed and subjected to Western blot analysis. The levels of SRSF1, Mnk2a AND Mnk2b were detected by specific antibodies. β-actin levels served as a loading control.
of SRSF1 mRNA (Fig. 7a,c), which also correlated with a decrease in Mnk2a and an increase in Mnk2b expression (Fig. 7b,d). In addition, the protein levels of SRSF1 also significantly increased in the cells expressing active Ras (Fig. 7e). SRSF1 elevation is probably mediated by activation of the MAPK-Sam68 pathway which was shown to modulate SRSF1 splicing by reducing the production of a transcript which is degraded by the nonsense-mediated decay (NMD) process and elevating the normal stable mRNA transcript [186]. When using specific primers for this splicing event we detected a reciprocal reduction in the NMD-prone isoform of SRSF1 and an increase in the stable SRSF1 transcript upon transduction with active Ras, suggesting that this mechanism contributes to SRSF1 upregulation by Ras (Fig. 7f). Thus, upregulation of SRSF1 expression in cancer cells by Ras activation or mutation, which reduces the unproductive NMD transcript of SRSF1, leads to reduced Mnk2a levels and elevated Mnk2b levels.

**Mnk2a has a tumor suppressive activity while Mnk2b is pro-oncogenic in vitro.**

The fact that Mnk2a is down-regulated in many cancers, led us to hypothesize that Mnk2 alternative splicing might contribute to cancer development and to the oncogenic activity of SRSF1. To examine the role of Mnk2 alternative splicing in cellular transformation we seeded non-transformed breast MCF-10A cells transduced with Mnk2 splicing isoforms into soft agar. Cells expressing Mnk2b or a kinase-dead Mnk2a were transformed and generated colonies in soft agar, while cells expressing Mnk2a did not (Fig. 8a-c). Kinase-dead Mnk2a probably acts in a dominant-negative manner by competing with Mnk2a for substrate binding, while incapable of phosphorylation. Furthermore, when MCF-10A cells expressing Mnk2 splicing isoforms were transformed by oncogenic Ras, cells co-expressing Mnk2a showed reduced ability to form colonies in soft agar indicating that Mnk2a can block Ras-induced transformation (Fig. 8d, e). Similar results were obtained using two other cell types; a cell transformation model NIH 3T3 cells (Fig. 9a, b) and the osteosarcoma cell line U2OS (Fig. 9c, d). Taken together these results suggest a pro-oncogenic activity of Mnk2b and a tumor suppressor activity of Mnk2a in vitro.
Figure 7. Active Ras regulates splicing of the MKNK2 gene by modulating SRSF1.

RNA from HuH7 (a,b) or MCF-10A (c,d) cell lines transduced either with empty vector or pWZL H-RASV12 retroviruses was extracted and the levels of SRSF1 and Mnk2 isoforms were detected by quantitative RT-PCR. (e) Transductant HuH7 and MCF-10A cells described above were lysed and subjected to Western blot analysis. The levels of SRSF1 were detected by specific antibodies. β-catenin levels served as a loading control. (f) RNA from cells described in (e) was subjected to RT-PCR with primers that detect the NMD-prone (NMD) or normal (FL-SRSF1) SRSF1 transcript. GAPDH was served as control.
Figure 8. Mnk2a has a tumor suppressive activity while Mnk2b is pro-oncogenic in vitro.

(a) MCF-10A cells were transduced with the indicated retroviruses encoding for Mnk2 isoforms and a kinase-dead version of Mnk2a (Mnk2aKD). After selection cells were lysed and Western blot membranes were probed with the indicated antibodies. (b) Cells described in (a) were seeded into soft agar (see Experimental procedures) in duplicate and colonies were grown for 20 days. Colonies in ten fields of each well were counted and the mean and standard deviation of number of colonies per well of 3 wells is shown (n=3). (c) Photographs of representative fields of colonies in soft agar described in (b). (d) Cells described in (a) were transduced with H-RAS<sup>V12</sup> and after selection with hygromycin cells were seeded into soft agar (see Experimental procedures) in duplicate and colonies were grown for 14 days. Colonies in ten fields of each well were counted and the mean and standard deviation of number of colonies per well of 3 wells is shown (n=3). (e) Photographs of representative fields of colonies in soft agar described in (d).
Figure 9. Both Mnk2 isoforms phosphorylate eIF4E but Mnk2a suppresses and Mnk2b increases tumorigenesis in vitro.

(a) NIH 3T3 cells were transduced with the indicated retroviruses encoding for Mnk2 isoforms and a kinase-dead version of Mnk2a (Mnk2aKD) and after selection were lysed and subjected to Western blot analysis. Membranes were probed with the indicated antibodies. (b) Cells described in (a) were seeded into soft agar in duplicate and colonies were grown for 14 days. Colonies in ten fields of each well were counted and the mean and standard deviation of number of colonies per well in a representative experiment is shown. (c) U2OS osteosarcoma cells were transduced with the indicated retroviruses encoding for Mnk2 isoforms and a kinase-dead version of Mnk2a (Mnk2aKD) and after selection were lysed and subjected to Western blot analysis. Membranes were probed with the indicated antibodies. (d) Cells described in (c) were seeded into soft agar in duplicate and colonies were grown for 14 days. Colonies in ten fields of each well were counted and the mean and standard deviation of number of colonies per well in a representative experiment is shown.
To further evaluate the tumor suppressor potential of Mnk2a, we decided to knockdown its expression using specific short hairpins (Fig. 10a, b), and test the transformation capacity of the cells by seeding them into soft agar. Abrogation of Mnk2a in immortalized MCF-10A breast cells or in NCI-H460 lung cancer cells enhanced colony formation, suggesting that Mnk2a possesses tumor suppressive activity (Figs. 10c and d). To examine the possibility that Mnk2a and Mnk2b exert their effects by changing cell proliferation, we examined the growth rate of cells expressing Mnk2 splicing isoforms. Neither Mnk2a nor Mnk2b expression changed significantly the proliferation rate of the cells or their cell cycle distribution (Fig. 11 and Fig. 12). However, cells with Mnk2a knockdown had a slightly higher proliferation rate, indicating that Mnk2a reduction may enhance proliferation (Fig. 11b). Collectively, these results suggest that the tumor-suppressive activity of Mnk2a is probably only partly mediated through its effects on cellular proliferation.

**Mnk2a has a tumor suppressor activity in vivo.**

In order to examine if Mnk2a possesses tumor-suppressor activity in vivo, we transformed MCF-10A cells transduced with Mnk2 splicing isoforms with active Ras and injected them into NOD-SCID mice. Analysis of Mnk2 isoforms and Ras expression was performed prior to injection (Fig. 13a). We found that mice injected with Ras-MCF-10A cells expressing either empty vector or Mnk2b formed tumors (6/6), whereas mice injected with Ras-MCF-10A cells expressing Mnk2a did not form any tumors (0/8) (Fig. 13b, c). Tumors from cells expressing Mnk2b showed an increased mitotic index (Fig. 14a, b) compared with tumors from cells expressing Ras alone but did not show significant enhanced tumor growth (Fig. 13c). Inversely, mice injected with Ras-MCF-10A cells expressing shRNA against Mnk2a showed enhanced tumor growth rate (Fig. 13d, e) and increased mitotic index in the tumors (Fig. 14c, d), indicating that Mnk2a depletion cooperates with and enhances Ras tumorigenicity. We also examined the effect of expression of Mnk2a and its kinase-dead form in the pancreatic cancer cell line Panc-1, which possesses mutant Ras as well as low levels of Mnk2a (Fig. 6a, b), and found that Mnk2a, but not its kinase-dead form, inhibited tumor growth in vivo (Fig. 15). Collectively, our results suggest
Figure 10. Knockdown of Mnk2a reduced activation of p38-MAPK and induced transformation in vitro.

(a) Pools of MCF-10A cells were transduced either with retroviruses encoding empty vector (MLP) or the indicated shRNAs against Mnk2a. After selection with puromycin, RNA was extracted and the levels of Mnk2a and Mnk2b mRNAs were measured by quantitative RT-PCR. (b) Cells described in (a) were lysed and proteins were subjected to Western blot analysis with the indicated antibodies. (c) Cells described in (a) were seeded into soft agar and 20 days later colonies were counted n=3. (d) NCI-H460 lung carcinoma cells were transduced with the indicated retroviruses encoding for Mnk2a specific shRNAs. Cells were seeded into soft agar in duplicate and colonies were grown for 14 days. Colonies in ten fields of each well were counted and the mean and standard deviation of number of colonies per well in a representative experiment is shown.
Figure 11. Proliferation analysis of MCF-10A cells transduced with viruses expressing either Mnk2 isoforms or Mnk2a specific shRNA.

MCF-10A cells transduced with retroviruses encoding for Mnk2 isoforms (a) or viruses expressing Mnk2a specific shRNAs (b) were seeded in sixplicates in 96 well plates and growth curves were measured, as described in Experimental procedures (n=6).
Figure 12. Cell cycle analysis of MCF-10A cells transduced with viruses expressing either Mnk2 isoforms or Mnk2a specific shRNA. (a-d) MCF-10A cells transduced with viruses expressing Mnk2 isoforms or the kinase dead version of Mnk2a (Mnk2aKD) were stained with PI and subjected to flow cytometry analysis for DNA content assessment. Percent of cells gated in each phase is indicated.
Figure 13. Mnk2a suppresses and Mnk2b promotes Ras-induced tumorigenesis.

(a) MCF-10A cells transformed with the indicated retroviruses encoding Mnk2 isoforms and transduced with H-RAS\textsuperscript{V12} were subjected to Western blot analysis. Membranes were probed with the indicated antibodies. (b, c) Pools of MCF-10A cells transformed with the indicated retroviruses encoding Mnk2 isoforms followed by H-RAS\textsuperscript{V12} transduction or (d, e) cells transduced with shRNA against Mnk2a (Sh2a-2) followed by transformation by H-RAS\textsuperscript{V12} were injected (2×10\textsuperscript{6} cells/injection, in matrigel) subcutaneously into NOD-SCID mice. Tumor growth curves were calculated as described in Experimental procedures. The number of tumors formed per injection is shown near the legend bars. (* p ≤ 0.01, T-test two tail analysis).
Figure 14. Knockdown of Mnk2a or overexpression of Mnk2b increased the mitotic index of tumors derived from Ras-MCF10A xenograft mice.

Formalin fixed, paraffin-embedded tissue sections from tumors derived from (a,b) MCF-10A cells transformed with retroviruses encoding either Mnk2b or empty vector followed by H-RAS<sup>V12</sup> transduction or (c,d) MCF-10A cells transduced with shRNA against Mnk2a (Sh2a-2) followed by transformation by H-RAS<sup>V12</sup> were stained with anti-phospho-H3 to detect mitotic cells. Graphs show the average and SD of p-H3 positive cells from 10 fields of 3 different tumors (a,c) (* p ≤ 0.01, T-test two tail analysis).
Figure 15. Mnk2a delays tumor growth of Panc-1 cancer cells in vivo.

(a) Pools of Panc-1 cells were transduced with the indicated retroviruses encoding either Mnk2a or its kinase-dead version (Mnk2aKD) and after selection were lysed and subjected to Western blot analysis. Membranes were probed with the indicated antibodies. (b) Cells described in (a) were injected (2×10⁶ cells/injection) subcutaneously into both rear flanks of nude mice. Tumor volume was calculated as described in Experimental procedures.
that Mnk2a has tumor-suppressor activity and can antagonize Ras-mediated transformation both in vitro and in vivo.

**Mnk2a sensitizes cells to stress-induced cell death.**

Although Mnk2a showed tumor suppressive activity in vitro and in vivo, its over-expression or down-regulation did not affect cellular proliferation significantly (Fig. 11 and Fig. 12). Thus, we hypothesized that Mnk2a might enhance the sensitivity of cells to apoptosis. Evasion from apoptosis and resistance to stress conditions are important properties of cancer cells. To examine the possible role of Mnk2 splicing isoforms in the response to cellular stress, we challenged immortalized breast cells (MCF-10A and Ras-transformed MCF-10A cells) transduced with retroviruses encoding either Mnk2a or Mnk2b or a kinase-dead mutant of Mnk2a with different stress conditions. While Mnk2a enhanced apoptotic cell death in response to anisomycin treatment, as measured by trypan blue exclusion and caspase 3 cleavage, Mnk2b and the kinase-dead Mnk2a protected the cells against apoptosis (Fig. 16a,b). Moreover, knockdown of Mnk2a protected MCF-10A cells from anisomycin-induced apoptosis (Fig. 16c,d). When evaluating activation of the p38-MAPK stress pathway we observed a positive correlation between the levels of caspase-3 cleavage with p38α phosphorylation (Fig. 16b, d). This result suggests that Mnk2a pro-apoptotic activity may exert an effect on the p38-MAPK pathway. Mnk2a also reduced survival of Ras-transformed MCF-10A cells when tested under two additional stress conditions; when sparsely seeded (Fig. 17a, b) or forced to grow in suspension (Fig. 17c). In addition, expression of Mnk2a in MCF-10A cells exposed to osmotic stress enhanced cell death (Fig. 18a). In contrast, knockdown of Mnk2a protected cells from osmotic shock, suggesting that Mnk2a mediated this stress response (Fig. 18b). One of the stress pathways induced by anisomycin and other cellular insults is the p38-MAPK pathway [187-189]. In order to examine if p38α activation is involved in Mnk2a-enhanced cell death, we blocked its activity with the specific inhibitor SB203580 [187]. p38α inhibition partially rescued cells expressing Mnk2a from anisomycin-induced cell death (Fig. 18c).
Figure 16. Mnk2a sensitizes MCF-10A cells to stress-induced apoptosis.

(a, c) MCF-10A cells transduced by the indicated retroviruses encoding for either Mnk2 isoforms or a kinase-dead version of Mnk2a (Mnk2aKD) (a) or MLP vectors encoding for shRNAs against the Mnk2a isoform (c) were seeded in 6-well plates. After 24 hours cells were starved in growth factor free media for an additional 24 hours. Following starvation cells were treated with 0.5 µM anisomycin for 24 hours (a) or 48 hours (c) and subjected to trypan-blue exclusion assay. (* p ≤ 0.01 , ** p ≤ 0.05, T-test two tail analysis). (b, d). Cells described in (a, c) were centrifuged and lysed with Laemmli buffer and proteins were analyzed by Western blot. Cleaved caspase-3 served as a marker for apoptosis. Levels of p-p38, p38, p-eIF4E and eIF4E were detected with the indicated antibodies. β-catenin served as a loading control.
Figure 17. Mnk2a decreased the survival of Ras transformed MCF-10A cells following stress.

(a) Pools of MCF-10A cells transduced with the indicated retroviruses encoding Mnk2 isoforms were transformed by H-RASV12. Cells were plated at low density (400 cells/well) 10 days after retroviral gene transduction and selection and assessed for colony formation with methylene blue staining 20 days later. Average and standard deviation of the number of colonies per well is shown. n=3. ( *, ** p ≤ 0.05, T-test two tail analysis). (b). Representative wells with colonies described in (a). (c) Cells described in (a) were maintained in growth factor free media in suspension without adherence for 48 hours. Cell death was measured using the trypan blue exclusion assay (n=2).
Figure 18. Mnk2a sensitizes cells to stress-induced apoptosis.

(a) MCF-10A cells transduced with the indicated retroviruses encoding Mnk2 isoforms were seeded on 6-well plates. 24 hours later, cells were starved in growth factor free media for 24 hours. Following starvation cells were treated with 0.5 M sorbitol (to induce osmotic shock) and 24 hours later were subjected to trypan-blue exclusion assay. (n=2). (b) MCF-10A cells transduced with the indicated retroviruses encoding for shRNAs against Mnk2a were seeded as in (a). Following starvation cells were treated with 0.25 M sorbitol and 24 hours later were fixed and stained with trypan blue (n=2). (*, p ≤ 0.01; ** p ≤ 0.05, T-test one tail analysis). (c) Human pancreatic cancer cells Panc-1 were seeded on 96-well plates (6000 cells/well). Following starvation cells were treated with 0.5 µM anisomycin in the presence or absence of 20 µM SB203580. 24 hours after, cells were subjected to trypan-blue exclusion assay. (n=3) (* p ≤ 0.05; ** p ≤ 0.01, T-test two tail analysis).
**Mnk2a but not Mnk2b enhances p38α-mediated cell death and suppression of Ras-induced transformation.**

Since Mnk2a, but not Mnk2b, contains a MAPK binding domain (Fig. 1) and can be activated by ERK and p38-MAPK [151, 155], we examined if Mnk2a can mediate stress responses emanating from activated p38-MAPK. MCF-10A cells, expressing either Mnk2 isoforms or knocked down for Mnk2a, were transduced with a constitutively-active p38α mutant [170-172] and grown in the absence or presence of the p38 inhibitor SB203580. Cells expressing Mnk2a showed increased cell death upon active p38α transduction, which was inhibited by SB203580 (Fig. 19a, b). Cells in which Mnk2a was knocked-down showed increased protection from p38-induced cell death (Fig. 19c, d). SB203580 efficiently inhibited p38 activity, as was measured by phosphorylation of its substrate MK-2 (Fig. 20). These results suggest that Mnk2a augments p38-MAPK stress activity. To examine if p38α activation by Mnk2a plays a role in its tumor-suppressive activity, we measured soft agar colony formation of MCF-10A cells co-transduced with Mnk2a isoforms and oncogenic Ras in the presence or absence of the p38 inhibitor SB203580. Inhibition of p38α by SB203580 rescued the ability of cells co-transduced with Mnk2a and Ras to form colonies in soft agar (Fig. 21), indicating that p38α activation by Mnk2a plays an important role in its ability to suppress Ras-induced transformation.

**Mnk2a interacts with, activates and induces nuclear translocation of p38-MAPK.**

The fact that Mnk2a might interact with p38α [151, 155, 177] and enhance p38α-mediated cell death (Fig. 16), suggests that it might regulate its activity. To determine if Mnk2a activates p38α, we examined the phosphorylation status of p38 on the known MKK 3/6 phosphorylation sites- Thr 180/Tyr 182 in cells expressing Mnk2 splicing isoforms [190-193]. As expected, phosphorylation of a known substrate of Mnk2, serine 209 of eIF4E, was induced by Mnk2a expression in MCF-10A cells (Fig. 22a) and two other cell lines (Fig. 9a,c). Even though previous reports have suggested that Mnk2b has a lower kinase activity than Mnk2a [151], we observed that it phosphorylates eIF4E to the same extent as Mnk2a (Fig. 22a and Fig. 9a,c). The kinase-dead version of Mnk2a did not enhance eIF4E phosphorylation (Fig. 9a, c and [65])
Figure 19. Mnk2a but not Mnk2b enhances p38α-mediated cell death.

(a) MCF-10A cells were transduced with the indicated Mnk2 isoforms and a kinase-dead version of Mnk2a (Mnk2aKD). Stable pools of cells were transduced with a constitutively active form of p38α or empty vector (pWZL), and selected with hygromycin in the presence or absence of 20 µM SB203580 for 48 hours. Following selection, the morphology of the cells was analyzed using a light microscope. (b) Methylene blue used for staining cells treated as in (a) was extracted and O.D. was measured as described below. (c, d) MCF-10A cells transduced with the indicated viruses expressing Mnk2a specific shRNAs were transduced with a constitutive active form of p38α or empty vector and analyzed as described in (a) and (b), respectively. Cell death in (b, d) was calculated by normalizing the absorbance values for cells co-transduced pWZL-active p38 mutant and Mnk2 isoforms to that of cells co-transduced with pWZL and Mnk2 isoforms. (n=4). (* p ≤ 0.01; ** p ≤ 0.05, T-test two tail analysis).
Figure 20. Expression of Mnk2a enhances the activation of a p38-downstream substrate following anisomycin induced stress.

MCF-10A cells transduced with retroviruses containing either the indicated Mnk2 isoforms or a kinase dead version of Mnk2a (Mnk2aKD) or with isoform specific shRNAs against Mnk2a were treated with 1 µM anisomycin, in the presence or absence of 10 µM SB203580. Cells were lysed and protein lysates were subjected to Western blot analysis with the indicated antibodies.
Figure 21. Mnk2a inhibits Ras transformation in a p38-dependent manner.

(a) MCF-10A cells were transduced with the indicated retroviruses followed by transduction with H-RASV12. After selection transductant cells were seeded into soft agar in the presence or absence of the indicated concentrations of SB203580 and colonies were counted 14 days later. (n=3). (*, **, *** p ≤ 0.01, T-test two tail analysis). (b) Photographs of representative fields of colonies in soft agar as described in (a).
Figure 22. Mnk2a interacts with p38-MAPK and leads to its activation and translocation into the nucleus.

(a) MCF-10A cells were transduced with the indicated retroviruses encoding for Mnk2 isoforms or Mnk2aKD. Total protein from stable pools of cells was subjected to Western blot analysis with the indicated antibodies. Numbers represent the ratio of p-p38/total p38 normalized to that of pBABE (arbitrarily set at 1) ± SD (n=2). (b) MCF-10A cells described in (a) were transduced by H-RASV12 and after selection cells were lysed and subjected to Western blot analysis with the indicated antibodies. Numbers representing the ratio of p-p38/total p38 were calculated as described in (a) (n=2). (c) MCF-10A cells were seeded in 6-well plates. 24 hours later cells were treated with either vehicle (DMSO) or the indicated concentrations of the Mnk1/2 kinase inhibitor, CGP 57380 for 4 hours, and subjected to Western blot analysis with the indicated antibodies. (d) HEK293 cells were cotransfected with the indicated Mnk2 isoforms together with HA-p38α-MAPK. HA-p38α or T7-Mnk2 isoforms were co-immunoprecipitated from lysates with either anti-HA or anti-T7 antibody. Input and precipitated proteins were subjected to Western blot analysis with the indicated antibodies. * represents a nonspecific band. (e) Distribution between cytoplasmic (C) and nuclear (N) fractions of p38α in MCF-10A cells transduced with retroviruses encoding the indicated Mnk2 isoforms. c-myc (nuclear) and caspase-2 (cytoplasmic) proteins served as controls for fractionation.
Fig. 22a). In contrast, only cells expressing Mnk2a showed increased p38α phosphorylation, indicating that the p38-MAPK pathway was activated (Fig. 22a,b), as it was shown previously that phosphorylation of p38-MAPK on these residues induces its activation [171, 191, 192]. Moreover, knockdown of Mnk2a in MCF-10A cells reduced p38α basal phosphorylation levels (Fig. 10b and Fig. 16d). In addition, we determined that the phosphorylation state of the p38α substrate MK2, was enhanced in cells expressing Mnk2a, but not Mnk2b or kinase-dead Mnk2a (Fig. 22a). To further examine if the kinase activity of Mnk2a is important for p38 phosphorylation, we treated MCF-10A cells with the Mnk1/2 kinase inhibitor CGP 57380 [169] [194]. Mnk1/2 kinase inhibition reduced p38α phosphorylation, similar to its effect on eIF4E S209 phosphorylation (Fig. 22c). Finally, we examined p38 phosphorylation in a panel of immortal breast cells and breast cancer cells and observed that in the latter (which tend to express low Mnk2a levels) (Fig. 2), phosphorylation of p38α is lower than in the non-transformed cells (Fig. 23a, b).

These results suggest that Mnk2a is an upstream activator of p38-MAPK in normal and cancer cells. Collectively, our results suggest that the pro-apoptotic effect of Mnk2a or protective effects of its knockdown do not correlate with its effects on eIF4E phosphorylation, which has been assumed to promote survival or transformation, but is rather mediated by p38α.

We next examined whether Mnk2 isoforms can differentially interact with p38α MAPK in cells. Co-immunoprecipitation experiments, using transfected or endogenous p38α from HEK293 cells, demonstrated that Mnk2a and Mnk2aKD, unlike Mnk2b, efficiently bound p38α (Fig. 22d). Importantly, even though Mnk2aKD was bound to p38α (Fig. 22d), it did not cause activation of p38, as measured by p38α or MK2 phosphorylation (Fig. 16b; Fig. 20; and Fig. 22a,b).

Finally, to rule out the possibility that Mnk2 isoforms compete with Mnk1 for p38α binding, we examined Mnk1 binding to p38α and if it is affected by Mnk2 isoforms. We found that Mnk1 was bound to HA-p38α. However, its binding was not affected by any of the Mnk2 isoforms, suggesting either that they do not bind to the same residues in p38α or do not compete for its binding (Fig. 22d). Taken together, these results suggest that Mnk2a interacts with p38α and leads to its activation. Upon activation, p38α is translocated into the nucleus and phosphorylates transcription
factors that mediate some of its stress response [193, 195-197]. Both Mnk2 isoforms contain a putative NLS domain which might result in their nuclear localization (Fig. 1a). Previous reports suggested that there is a nuclear fraction of Mnk2b while Mnk2a is mostly cytoplasmic [151]. Using cytoplasmic and nuclear fractionation (Fig. 22e and Fig. 23d), as well as immunofluorescent staining (Fig. 24), we observed that both Mnk2a and Mnk2b can be detected in the nucleus. However, cells that express Mnk2a showed an increased nuclear fraction of total and phosphorylated p38α (Fig. 22e, Fig. 23d, Fig. 25, Fig. 26 and Fig. 27), indicating that Mnk2a leads to both p38α activation and its translocation into the nucleus. Importantly, in M KK3/6−/− MEF cells where p38α is mostly un-phosphorylated and mainly localized in the cytoplasm, ectopic expression of Mnk2a, enhances nuclear translocation of endogenous p38α (Fig. 26). Taken together, these results suggest that Mnk2a affects the activation and cellular distribution of p38α.

**Mnk2a co-localizes with p38-MAPK and affects its cellular localization.**

In order to examine if Mnk2a affects p38-MAPK cellular localization, we generated two Mnk2a mutants. In the first mutant (KKR) we mutated the putative nuclear localization signal of Mnk2a (69-KKRGKKKKR-77) to (KKRGKAAA), in which the last KKR was replaced with three alanines (AAA). This mutant is expected to be mostly cytoplasmic, as was shown for the homologous mutation in Mnk1 [177]. In the second mutant (L/S) we mutated the putative nuclear export signal (NES) of Mnk2. Although in Mnk1 the NES motif is localized to a different region [177], we identified a similar motif (LxxxLxxL) in Mnk2 in the C-terminal region (starting at amino acid 281 of Mnk2a) and mutated the last two lysines to serines. Mutating the nuclear export signal is expected to render this Mnk2a mutant mostly nuclear. Indeed, when transfected into HeLa cells or transduced into MCF-10A cells these Mnk2a mutants showed the expected localization: the nuclear localization of Mnk2 L/S was enhanced, while that of Mnk2 KKR was decreased, when compared to that of Mnk2a (Fig. 24, Fig. 25, Fig. 26 and Fig. 27). When co-transfected with HA-tagged or GFP-tagged p38-MAPK, Mnk2a colocalized with p38-MAPK. Expression of Mnk2a L/S rendered p38-MAPK mostly nuclear and co-localized with it in the nucleus. Mnk2a KKR co-localized with p38-MAPK in both the cytoplasm and nucleus, but was less nuclear than Mnk2a (Fig. 25 and Fig. 27). In addition, the L/S and KKR mutants had
Figure 23. Phosphorylation of p38-MAPK and eIF4E is regulated by Mnk2 kinase activity and modulates p38α localization.

(a) Immortal (MCF10A, HMLE) or cancer (MCF-7, MDA-MB-231, SUM-149, SUM-159, MDA-MB-468) breast cell lines were seeded in 10cm plates and 24 hours later were lysed and subjected to Western blot analysis with the indicated antibodies. (b, c) The level of phospho-p38 (b) and eIF4E (c) were quantitated and normalized to the levels of total p38 and total eIF4E, respectively. Results are presented as an average ± SD (n=2). (d) Distribution between cytoplasmic (C) and nuclear (N) fractions of p38α, p-p38α and T7-Mnk2 isoforms in MCF-10A cells transduced with Mnk2 isoforms or a kinase-dead version of Mnk2a (Mnk2aKD). C-myc (nuclear) and caspase-2 (cytoplasmic) served as fractionation controls.
Figure 24. Cellular localization of Mnk2 isoforms and Mnk2aKKR and Mnk2aL/S mutants.

HeLa cells were seeded on coverslips in 12-well plates and transfected with pCDNA3 vectors encoding for T7-Mnk2 isoforms, Mnk2aKD, Mnk2aL/S and Mnk2aKKR. 24 hours after transfection cells were fixed with 4% paraformaldehyde and subjected to an immunofluorescence assay as described in Experimental procedures.
Figure 25. Mnk2a co-localizes with GFP-p38α and affects its cellular localization.

(a) HeLa cells were seeded on coverslips in 12-well plates and co-transfected with either empty pcDNA3 vector or pcDNA3-based expression vectors for T7-tagged Mnk2a, Mnk2aL/S or Mnk2aKKR, together with pCDNA3-GFP-p38α (WT). 48 hours after transfection cells were fixed with 4% paraformaldehyde, stained with the indicated antibodies and subjected to an immunofluorescence assay, as described in Experimental Procedures (T7-tag was stained red and GFP-p38α is green). (b) Quantitation of cytoplasmic/nuclear distribution of GFP-p38α in cells similar to those described in (a). n=40 for each mutant. (*, ** p ≤ 0.01, T-test two tail analysis). (c) Correlation between T7-Mnk2a localization (Cy3) and GFP-p38α localization (FITC) in HeLa cells co-transfected with T7-Mnk2a and GFP-p38α as described in (a). Pearson correlation = 0.358. (n=40).
Figure 26. Mnk2a co-localizes with endogenous p38α and induces nuclear translocation.

MKK3/MKK6−/− MEF cells transduced with the indicated retroviruses were seeded, fixed and subjected to an immunofluorescence assay, as described in Experimental Procedures to detect endogenous p38α. T7 tag was stained red, and p38α was stained green. Right view shows quantification of cytoplasmic/nuclear distribution of p38α in cells similar to those described. (n = 57 for pBABE; n = 47 for Mnk2a). * p ≤ 0.001.
Figure 2. Mnk2a mutants co-localize and affect cellular localization of HA-p38.

(a) HeLa cells were seeded on a coverslip in 12-well plates and co-transfected with pCDNA3 (empty vector) or with pCDNA3 encoding for T7-Mnk2a, Mnk2aL/S and Mnk2aKKR and HA-tagged p38α. 48 hours after transfection cells were fixed with 4% paraformaldehyde and subjected to an immunofluorescence assay, as described in Experimental procedures (Mnk2 was stained green and HA-tag was stained red).

(b) Quantitation of cytoplasmic/nuclear distribution of HA-p38α in cells similar to those described in (a). (T7-tag was stained green and HA-p38α was stained red). n=35 cells for each mutant. (*, ** p ≤ 0.01, T-test two tail analysis).
similar effects on the localization of endogenous p38α in MCF-10A cells transduced with retroviruses expressing these mutants (Fig. 26). Mnk2a, KKR and the L/S mutants can interact with HA-tagged and endogenous p38α as demonstrated by co-immunoprecipitation (Fig. 28) and can pull-down phospho-p38α (Fig. 28). Overall, these results suggest that Mnk2a and p38-MAPK are colocalized in both the cytoplasm and nucleus, and that Mnk2a can affect the cellular localization of p38α.

**Mnk2a can phosphorylate p38-MAPK directly in vitro and in vivo.**

To examine if Mnk2a can directly phosphorylate p38α, we performed an in vitro kinase assay with recombinant Mnk2a and recombinant p38α. We used a kinase-dead version of p38α to avoid background of autophosphorylation. As a positive control we used recombinant eIF4E, the known substrate of Mnk2. We determined that Mnk2a can directly phosphorylate p38α (Fig. 29a). Next, we examined if Mnk2a can phosphorylate p38α in the absence of its primary activators MKK3 and MKK6. To address this question, we infected MKK3/6−/− MEF cells with retroviruses expressing either Mnk2a or empty vector and analyzed the phosphorylation status of p38α. Remarkably, Mnk2a transductant cells were capable of inducing phosphorylation of p38α similar to wild type MKK3/6 MEFs (Fig. 29b). These results suggest that the mechanism of p38α activation by Mnk2a is probably by direct phosphorylation which is independent of MKK3/6.

**Mnk2a localization and kinase activity affects induction of p38-MAPK target genes and apoptosis.**

To examine if p38α activation and nuclear translocation induced by Mnk2a leads to induction of p38α target genes, we examined the expression of FOS and COX-2, both targets of the p38α- stress response [198]. Expression of both genes was induced in MCF-10A cells expressing Mnk2a and reduced in cells expressing Mnk2b or kinase-dead Mnk2a (Fig. 30a). Moreover, knockdown of Mnk2a greatly decreased FOS and COX-2 expression (Fig. 30b). Interestingly, while Mnk2a L/S could activate p38-MAPK target genes similarly to Mnk2a, the KKR mutant did not activate the expression of FOS and COX2 (Fig. 30c).
Figure 28. Mnk2a mutants interact with p38 and can induce its phosphorylation.

HeLa cells were seeded in 10cm plates and 24 hours later were co-transfected with pCDNA3 vectors encoding for T7-Mnk2a, Mnk2aL/S or Mnk2aKKR and HA-tagged-p38α. Cells were lysed 48 hours later and the Mnk2a mutants and p38α were co-immunoprecipitated as described in Experimental procedures. Input (top panel) and immunoprecipitated (bottom panel) proteins were subjected to Western blot analysis with the indicated antibodies. Numbers representing the ratio of HA-p-p38/total HA-p38 normalized to that of pCDNA3 (arbitrarily set at 1) ± SD (n=3).
Figure 29. Mnk2a directly phosphorylates p38α-MAPK in vitro and in cells.

(a) Recombinant Mnk2a, p38α kinase dead (KD) or eIF4E were incubated alone or in the indicated combinations, and subjected to an in vitro kinase assay as described in Experimental procedures. After the reaction was stopped, reaction mixes were separated on SDS-PAGE and transferred onto nitrocellulose membranes. Upper panel: Radioactive membrane was exposed to Fuji phosphorimager cassette and visualized by MacBass phosphorimager. Bottom panel: Membranes were probed with the indicated antibodies and detected by ECL. (b) Left; MEF MKK3/6 W.T cells were seeded in 10cm plates and after 24h total protein was extracted and subjected to Western blot analysis with the indicated antibodies. Right; MEF MKK3/6−/− knockout cells were transduced with the indicated retroviruses. Total protein from stable pools was extracted and subjected to Western blot analysis with the indicated antibodies. β-catenin was used as a loading control.
Figure 30. Mnk2a localization and kinase activity are required for induction of p38α target genes.

(a, b) RNA from MCF-10A cells transduced with the indicated Mnk2 isoforms and a kinase-dead version of Mnk2a (Mnk2aKD) or with MLP vectors encoding for shRNAs against Mnk2a isoform was extracted and the levels of FOS and COX-2 were measured by quantitative RT-PCR. (c) RNA from MCF-10A cells transduced with the indicated Mnk2 isoforms and the indicated Mnk2a mutants was extracted and the levels of FOS and COX-2 were measured as described above. (d) MCF-10A cells were seeded in 10 cm plates. 24 hours later cells were serum starved for an additional 24 hours, then treated with Mnk1/2 inhibitor CGP 57380 at the indicated concentrations for 8-10 hours. RNA from cells was extracted and the levels of p38α-targets genes, FOS and COX-2, were measured by quantitative RT-PCR as described above. (e) MEF MKK3/6−/− knockout cells were transduced with the indicated retroviruses. After selection with puromycin RNA from stable pool of cells was extracted and the levels of FOS and COX-2 were measured by RT-PCR. (n=3). (*, ** p ≤ 0.05, T-test two tail analysis).
To examine if Mnk1/2 kinase activity modulates the expression of p38α target genes, we treated MCF-10A cells with the Mnk1/2 kinase inhibitor CGP 57380. Inhibition of Mnk1/2 kinase activity reduced both p38-MAPK phosphorylation (Fig. 22c) and also reduced the expression of p38-MAPK target genes (Fig. 30d). Importantly, MKK3/6−/− MEF cells expressing Mnk2a induced FOS and COX-2 expression (Fig. 30e). These results suggest that p38-MAPK activation by Mnk2a may play a critical role in the induction of p38α targets genes. Levels of MKNK2 transcripts is sufficient to achieve similar results as overexpression or knockdown of each isoform. Mnk2b inhibited the expression of FOS and COX-2 below the basal level (Fig. 30a). To examine if Mnk2a localization can affect its pro-apoptotic activity we treated cells expressing Mnk2a, Mnk2b and Mnk2a KKR and L/S mutants with anisomycin and measured cell death. We also seeded these cells sparsely for colony survival assay. We found that while Mnk2a and the Mnk2a L/S mutant sensitized cells to apoptosis, the Mnk2a KKR mutant inhibited apoptosis and did not significantly decrease colony survival, in correlation with its inability to induce p38α target genes (Fig. 31 and Fig. 30c). Taken together, these results indicate that Mnk2a, not only activates p38α, but can also regulate p38α localization and in this manner control its target genes which induce apoptosis.

**Modulation of MKNK2 splicing by splice-site competitive antisense RNA oligos affects sensitivity to apoptosis and cellular transformation.**

To examine the effects of modulating the endogenous MKNK2 alternative splicing, I designed Cy5 labeled 2’-O-met-RNA antisense oligonucleotides to mask either of the MKNK2 splice sites, shifting the splicing balance between Mnk2a and Mnk2b. We tested these oligos in two experimental systems in which Mnk2a/Mnk2b ratios were altered because of cellular transformation: MCF-10A cells transformed by oncogenic Ras and immortal lung bronchial epithelial cells (BEAS-2B) transformed by SRSF1 overexpression (Fig. 32) [199]. Elevation of Mnk2b and concomitant reduction of Mnk2a, by the oligo that competes with Mnk2a intron-exon junction, protected the cells from anisomycin-induced apoptosis and increased colony survival (Fig. 33a-f). However, this oligo did not increase anchorage-independent growth of the cells in soft agar (Fig. 33g,h), probably due to the fact that cells expressing oncogenic Ras or
Figure 31. Co-localization of Mnk2a and p38 is required for the induction of p38α-mediated apoptosis.

(a) MCF-10A cells transduced with the indicated Mnk2 isoforms and the indicated Mnk2a mutants were serum starved for 24 hours. After starvation cells were treated with 0.5 µM anisomycin for another 24 hours and subjected to trypan-blue exclusion assay (graph). Cells described in (a) were centrifuged and lysed with Laemmli buffer and proteins were subjected to Western blot analysis (bottom panel). Cleaved caspase-3 served as a marker for apoptosis and β-catenin as a loading control. (b) Transductant cells described in (a) were plated at low density (400 cells/well) 10 days after retroviral gene transduction and selection and assessed for colony formation with methylene blue staining 20 days later. Average and standard deviation of the number of colonies per well is shown. n=2.
Figure 32. Modulation of MKNK2 splicing by splice-site competitive antisense RNA oligos

(a) Schematic diagram of splice site interfering RNA oligonucleotides. 2'-O-methyl RNA oligos are labeled with Cy5 at the 5' and interfere with U1/U2 binding to the 5' or 3' splice site respectively. (b) Representative photographs of Ras transformed MCF-10A and BEAS2B cells expressing SRSF1 transfected with labeled 2'-O-methyl RNA oligos. Cells were visualized under fluorescent microscope 24h after oligo transfection.
Figure 33. Modulation of MKNK2 splicing by splice-site competitive antisense RNA oligos affects sensitivity to apoptosis and cellular transformation.

(a) Ras transformed MCF-10A cells were transfected with the indicated oligos. After 48h, RNA was extracted and the levels of Mnk2a and Mnk2b were measured by RT-PCR. Numbers represent ratio of Mnk2a/Mnk2b normalized to that of Scrambled oligo (Scr) (arbitrarily set at 1) ± SD (n=2). (b-e) MCF-10A (b,c) and Ras transformed MCF-10A (d,e) cells were transfected as described in (a). 24 hours later cells were treated with 1 µM anisomycin for 48 hours and subjected to trypan-blue exclusion assay and quantified by the BioRad cell counter (c,e). Cells described in (c,e) were lysed with Laemmli buffer and analyzed by Western blot. Cleaved caspase-3 served as a marker for apoptosis. β-catenin served as a loading control. (f) BEAS-2B transformed with SRSF1 were transfected with the indicated oligos. After 48 hours cells were plated at low density (40 or 400 cells/well, bars marked with blue and red colors respectively). 10 days after oligo transfection plates were assessed for colony formation with methylene blue staining. Average and standard deviation of the number of colonies per well is shown. n=3. (g). Cells described in (a) were seeded into soft agar (see Experimental procedures) in duplicates and colonies were allowed to grow for 14 days. Colonies in ten fields of each well were counted and the mean and standard deviation of colonies per well of 3 wells is shown (n=3). (h). Photographs of representative fields of colonies in soft agar obtained as described in (g).
SRSF1 are already highly invasive and tumorigenic (see Fig. 8 for Ras, and [199] for SRSF1). Elevation of Mnk2a and reduction of Mnk2b by the oligo that blocks production of Mnk2b sensitized the cells to anisomycin-induced apoptosis, reduced colony survival and inhibited soft agar colony formation in both the Ras and SRSF1 transformed cells (Fig. 33). This data suggest that manipulation of the endogenous ratios of Mnk2a and Mnk2b affects the oncogenic potential of cells transformed by Ras and SRSF1.

We propose that in cancer cells, constitutive active Ras modulates alternative splicing of the MKNK2 gene to decrease Mnk2a levels and increase those of Mnk2b (Scheme 3). By inhibiting Mnk2a, cancer cells with activated Ras escape from activation of the p38-MAPK stress pathway, which can lead to activation of stress response and apoptosis Mnk2b (Scheme 3).

Discussion

The process of alternative splicing is widely misregulated in cancer and many tumors express new splicing isoforms which are absent in the corresponding normal tissue [195, 200, 201]. Many oncogenes and tumor suppressor genes are differentially spliced in cancer cells and it has been shown that many of these cancer-specific isoforms contribute to the transformed phenotype of cancer cells [42, 44, 46]. The work presented here, adds to our knowledge of genes that are differentially spliced in cancer cells and play a role in the cancer process. Here we have shown that \textit{MKNK2} alternative splicing is modulated in cancer cells to down-regulate the expression of the tumor suppressive isoform Mnk2a and enhance the expression of the pro-oncogenic isoform Mnk2b. Both splicing isoforms phosphorylate the translation initiation factor eIF4E. However, only Mnk2a binds to and activates p38\textsubscript{\alpha} leading to enhanced activation of the p38-MAPK stress pathway, induction of its target genes and enhanced cell death.

The serine/threonine kinases Mnk1 and Mnk2 are the only known kinases that phosphorylate eIF4E on serine 209 [116, 117, 150, 202]. This phosphorylation, although not essential for proper normal development [150, 202], may contribute to cellular transformation as demonstrated in several mouse models [145, 147]. Only a handful of other substrates of Mnk1/2 are known and their contribution to transformation or cancer development is not clear [203-206]. The \textit{MKNK1}/\textit{MKNK2} genes in mouse generate one known protein each [151, 155, 177]. However in humans, both genes have at least two splicing isoforms resulting in different C-termini [151, 203] (Fig. 1). Biochemical assays showed that Mnk1 activity is highly dependent on activation by the Ras- ERK or p38 pathways while Mnk2 possesses higher basal activity without the need for upstream activation [151, 155, 203]. The biochemical properties of the two splicing isoforms of Mnk2 have been studied mostly in vitro and it has been shown that Mnk2a has stronger catalytic activity towards eIF4E than Mnk2b [151, 203]. However, the biological roles of the different isoforms and their contribution to transformation and cancer were unknown.

[86]
Oncogenic signals regulate alternative splicing of MKNK2

The splicing factor SRSF1 was previously identified as a potent proto-oncogene and MKNK2 was reported as one of its splicing targets [57]. Enhanced expression of SRSF1 reduced the levels of Mnk2a and increased the levels of Mnk2b while knockdown of SRSF1 caused a reciprocal change in MKNK2 splicing [57]. We sought to examine the role of Mnk2 alternative splicing in cancer and the biological and biochemical properties of the Mnk2a and 2b splicing isoforms. We found that Mnk2a is down-regulated while in some cases Mnk2b is up-regulated in several cancers such as colon, breast and lung cancers (Fig. 2, Fig. 3 and Fig. 4). Mnk2 alternative splicing switch correlated with SRSF1 levels both in cancer cell lines and tumors and upon Ras transformation (Figs. 5, and Fig. 7a-d). In pancreatic cancer cell lines, K-Ras mutation status correlates with MKNK2 splicing (Fig. 6). Introduction of active Ras (H-RasV12) into cells induced a splicing switch reducing Mnk2a levels and elevating Mnk2b levels similar to the shift found in cancer cells (Fig. 7b,d). Furthermore, we found that Ras increased the levels of the stable SRSF1 transcript and protein by reducing the level of the nonsense-mediated decay-prone transcript of SRSF1 (Fig. 7e,f). Our results showing that mutant/oncogenic Ras reduces the level of the tumor suppressive Mnk2a isoform, suggests that this may be one of the important oncogenic activities of Ras. However, SRSF1 upregulation, independent of the Ras-MAPK pathway, can be caused by additional mechanisms such as gene amplification [57] and transcriptional activation [207]. In addition to the above mechanisms it was shown by Adesso et al. [156] that resistance of pancreatic ductal adenocarcinoma cells to Gemcitabine stems from increased expression of SRSF1, which induces a shift in Mnk2 splicing to reduce Mnk2a and increase Mnk2b. Inhibition of Mnk1/2 pharmacologically or by Mnk2 siRNA reduced eIF4E phosphorylation and increased the sensitivity of the cells to Gemcitabine, suggesting a role for Mnk2 alternative splicing in chemotherapy resistance [156]. Furthermore, upregulation of SRSF1 was recently discovered during the process of EMT [186]. Valacca et al. showed that the splicing factor Sam68 binds to the 3' UTR of the SRSF1 transcript promoting recognition of this region as an intron and producing a transcript which is subjected to degradation by the nonsense-mediated decay pathway. Activation of the Ras-MEK-ERK pathway by EMT inhibited Sam68 binding and led
to SRSF1 up-regulation by increasing its stable mRNA transcript [186]. In summary, the results presented here show that activated or mutated Ras induces down-regulation of Mnk2a by elevating the production of a stable SRSF1 transcript leading to elevated expression of SRSF1 protein which affects Mnk2 alternative splicing. We postulate that splicing of additional targets of SRSF1 is modulated upon Ras activation/mutation (Fig. 7), which requires further investigation in the future.

**The role of Mnk2 isoforms in cellular transformation**

After establishing that Mnk2a is downregulated and Mnk2b is partially upregulated in several human tumors we wanted to study the potential role of Mnk2 isoforms in cellular transformation. As Mnk1 and Mnk2 are the only known kinases of eIF4E we sought to investigate the pathways that control eIF4E activity. Two signaling pathways are known to regulate eIF4E activity: PI3K-Akt and the Ras-MAPK pathways. Akt oncogenic activity depends on the serine/threonine kinase mammalian target of rapamycin (mTOR), which phosphorylates eIF4E binding proteins (4E-BPs) causing their dissociation from eIF4E and promoting the assembly of the eIF4F translation complex [121, 122, 208, 209]. As a consequence of this, enhanced eIF4E activation contributes to cancer by selective translation of capped-mRNAs encoding for proteins which participate in cell proliferation and survival. The oncogenic potential of eIF4E is completely dependent on ser209 phosphorylation, and thus a potential oncogenic activity for Mnk kinases [129, 144, 145, 147]. Importantly, a Mnk inhibitor was more effective than rapamycin in blocking proliferation of Pten-expressing cells, whereas a combination of the two inhibitors suppressed cell cycle progression in prostate cancer lines [137]. The fact that SRSF1 regulates MKNK2 splicing and enhances eIF4E activation may serve as a bypass mechanism independent of upstream signals such as the Ras-MAPK or PTEN-Akt pathways. Therefore, the relevance of Mnk2a/Mnk2b and Mnk1 as exclusive kinases of eIF4E in the context of tumorigenesis is extremely important. Given that eIF4E phosphorylation functions in a variety of biological process such as cell-proliferation, survival and cell cycle progression, we decided to focus our attention on the biological and biochemical differences, if any, between the two isoforms. To elucidate the role of Mnk2 splicing isoforms in cellular transformation we examined the
oncogenic activity of the Mnk2 splicing isoforms both in vitro and in vivo. We found that Mnk2b and the kinase-dead form of Mnk2a could transform MCF-10A cells which could then form colonies in soft agar. Mnk2a knockdown also increased colony formation in soft agar (Fig. 10c,d). Furthermore, Mnk2a could antagonize Ras-mediated transformation leading to inhibition of colony formation in soft agar of cells co-expressing Mnk2a and an active mutant of H-Ras (Fig. 8d,e).

In light of the above results, we addressed whether Mnk2a anti- and Mnk2b pro-oncogenic activity could be ascribed to their impact on the cell cycle and/or proliferation. To examine if the ability to form colonies is dependent on increased proliferation we measured the growth rate and cell cycle distribution of cells with overexpression or knockdown of Mnk2a and found that Mnk2 isoforms did not significantly affect the proliferation of the cells (Fig. 11a). However, cells with a Mnk2a knockdown had a modest increase in their proliferation suggesting that Mnk2a loss might contribute to enhanced proliferation (Fig. 11b). Expression of Mnk2 isoforms did not alter the cell cycle distribution of cells (Fig. 12). These results suggest that Mnk2a tumor-suppressive activity is only partly mediated by a cell-cycle inhibitory activity.

To examine the tumor suppressive activity of Mnk2a in vivo we injected MCF-10A cells co-expressing an active Ras mutant and Mnk2 splicing isoforms or shRNAs against Mnk2a and Mnk2b into NOD-SCID mice. We found that Mnk2a expression caused a complete inhibition of tumor formation in mice while its knockdown enhanced the tumorigenic activity of Ras (Fig. 13). These results suggest that Mnk2a is a tumor suppressor that inhibits Ras oncogenic activity in vivo. Moreover, re-introduction of Mnk2a into Panc-1 cells, where Mnk2a levels are low, sensitized these cells to stress (Fig. 18c) and inhibited their ability to form tumors in mice (Fig. 15), suggesting that Mnk2a down-regulation in pancreatic cancer cells with mutant Ras has physiological implications and can modulate the tumorigenic potential of these cells.

**Mnk2a modulates the p38-MAPK stress pathway**

Since Mnk2 isoforms did not significantly affect cellular proliferation, we hypothesized that Mnk2a anti- and Mnk2b pro-oncogenic activity is mediated by their
effects on cell death and stress-resistance or sensitivity to stress. We thus examined the response of cells, with either up-regulation or knock down of Mnk2 isoforms, to cellular stress and transformation assays. We found that Mnk2a, but not Mnk2b or a Mnk2a kinase-dead version, enhanced cellular response to stress and augmented the apoptotic activity of an active mutant form of p38α. Moreover, knockdown of Mnk2a enhanced the survival of cells transduced with the active mutant form of p38α (Figs., 16, 17, 18 and 19). These results indicate that Mnk2 alternative splicing can modulate the p38-MAPK stress response. Furthermore, inhibition of p38α by the pharmacological inhibitor SB203580 rescued Ras transformed MCF-10A cells from suppression of anchorage independent growth due to Mnk2a expression (Fig. 21).

Collectively, this data suggests that the tumor suppressive activity of Mnk2a is mediated by activation of the p38-MAPK pathway. We then examined if Mnk2 isoforms can activate the p38-MAPK pathway. We found that only Mnk2a could enhance p38-MAPK phosphorylation, translocation into the nucleus and phosphorylation of its substrate MK2 (Fig. 22). Moreover, cells with Mnk2a knockdown showed reduced p38-MAPK phosphorylation (Fig. 10). Interestingly, both Mnk2a and Mnk2b, but not the kinase-dead mutant of Mnk2a, phosphorylated eIF4E to a similar extent suggesting that eIF4E phosphorylation cannot account for their different biological activities (Figs. 9, 16b and 22a,b). Two recent studies report that MKNK1/2-double knockout mice (Mnk1/2-DKO) are resistant to lymphomagenesis developed by crossing to PTEN−/− mice and that Mnk1/2-DKO MEFs are resistant to Ras-induced transformation in vitro [146, 147]. In the MNK1/2-DKO mouse model both Mnk1 and Mnk2 genes are missing and there is no eIF4E phosphorylation. In cancer, when alternative splicing results in up-regulation of Mnk2b or when it is manipulated artificially, as we have done, eIF4E is still phosphorylated due to the presence of Mnk2b and Mnk1 (Fig. 23a-c); however there is no activation of the p38-MAPK pathway which is mediated by Mnk2a. Thus our results suggest that Mnk2b uncouples eIF4E phosphorylation from activation of the p38-MAPK stress pathway and thus sustains only the pro-oncogenic arm of the pathway.
We further found that Mnk2 isoforms can differentially interact with p38α MAPK in cells. Using co-immunoprecipitation assays we detected Mnk2a binding to p38α while Mnk2b did not bind p38α efficiently suggesting that this interaction might be important for p38α activation by Mnk2a (Fig. 22d). Importantly, even though Mnk2aKD bound to p38α (Fig. 22d) it did not cause activation or translocation of p38α as measured by p38α or MK2 phosphorylation and by cellular fractionation (Figs. 22a, 22e). We also found that the kinase activity of Mnk2a is required for activation of p38α and its target genes as both the kinase-dead form of Mnk2a or application of the Mnk1/2 kinase inhibitor CGP 57380 [169], [194] inhibited these activities (Figs. 22c, 30a,d).

In all of our experimental systems we demonstrated that while both p38α and eIF4E phosphorylation is enhanced by Mnk2a over-expression and reduced by its knockdown, only p38α phosphorylation correlates with the degree of apoptosis (Figs. 9, 16 and 22). Results from these gain/loss of function experimental systems suggest that p38α, but not eIF4E, phosphorylation/activation determines the fate of these cells. This is supported by the finding that breast cancer cell lines examined in this study had lower levels of p38α (and eIF4E) compared to two immortal breast cell lines (Fig. 23), suggesting that there is clinical relevance to these findings. One of the important discoveries of this study is that Mnk2a is a new p38-MAPK kinase (Fig. 29). The only known kinases of p38-MAPK are MKK3 and MKK6 (and in some rare cases MKK4) [192, 193]. Thus, Mnk2a is the first non-MKK kinase that phosphorylates p38-MAPK. Further investigation is required to determine if Mnk1 can also phosphorylate p38-MAPK. The fact that Mnk2a can directly phosphorylate p38-MAPK provides a mechanistic explanation for how Mnk2a activates the p38-MAPK stress pathway and manifests its tumor suppressor activity.

In order to examine if Mnk2a affects p38α cellular localization and if this effect mediates p38α activation, we generated two Mnk2a mutants. A NLS mutant of Mnk2a (KKR) is expected to be mostly cytoplasmic as was shown for the homologous mutation in Mnk1[177]. A second mutant (L/S), in which the putative nuclear export signal (NES) of Mnk2a is mutated, is expected to render this mutant mostly nuclear. Indeed, when transfected into HeLa cells or transduced into MCF-10A cells, these Mnk2a mutants showed the expected localization; Mnk2a L/S is
nuclear while Mnk2a KKR is more cytoplasmic when compared to Mnk2a (Fig. 24). When co-transfected with HA-tagged or GFP-tagged p38α, Mnk2a colocalized with p38α both in the cytoplasm and nucleus (Figs. 25 and 27). Mnk2a L/S rendered p38α mostly nuclear and colocalized with it in the nucleus. Mnk2a KKR colocalized with p38α in both the cytoplasm and nucleus but was less nuclear than Mnk2a (Figs. 25, 26 and 27). Both Mnk2a mutants could interact with p38α, including the p38α phosphorylated form as was measured by co-immunoprecipitation (Fig. 28). These results imply that, in addition to Mnk2a’s ability to interact and enhance activation of p38α, it can also affect the cellular distribution of p38α in the cell. Although Mnk2a modulated the localization of p38α, it is not clear if p38α translocation into the nucleus is the result of protein-protein interactions with Mnk2a or the enhanced phosphorylation of p38α which is mediated by Mnk2a kinase activity. To address these questions it would be necessary to block Mnk2 activity with the kinase inhibitor (CGP 57380) and examine p38α localization. Alternatively, manipulation of Mnk2a L/S mutant by substitution of the crucial lysine residue (K113) located in the ATP binding site to alanine would result in a kinase inactive isoform (Mnk2a L/S, KD). In this way, expression and co-localization analysis with GFP-p38α could reveal if p38α translocation is dependent on the kinase activity of Mnk2a. In general, localization of signaling proteins involve the classical nuclear localization signal which is mediated through importin α (Impα) and importin β (Imp-β) [210]. Nonetheless, other signaling proteins might be using a self Imp-like activity [211] or alternatively rely on shuttling proteins other than Imp α/β [212]. Recently the molecular pathway controlling ERK translocation was deciphered [213, 214]. As opposed to ERK, the mechanisms that mediate p38-MAPK translocation are ambiguous. On one hand, it was established that in resting cells, anchoring proteins retain p38-MAPK in the cytoplasm and following stimuli release it to translocate into the nucleus [23, 215]. But on the other hand it was shown that p38-MAPK localized in the nucleus of resting cells and following stimulation it is exported to the cytoplasm [216]. When taking into consideration the kinase activity and subcellular localization of Mnk2a, it is possible that the importins are targets of phosphorylation mediated by Mnk2a. This hypothetical direct mechanism might regulate the shuttling of p38-MAPK (e.g. facilitate binding to nuclear pore complex). Alternatively, p38α phosphorylation by Mnk2a might enhance
the interaction with importin similarly to ERK translocation. In this model, casein kinase 2 (CK2) phosphorylates ERK within the nuclear translocation signal (NTS) to facilitate recognition for Imp7 [213]. Further investigation is required in order to better understand these molecular events.

**Nuclear activity of p38-MAPK**

Upon activation, p38α is thought to translocate to the nucleus and phosphorylate several transcription factors which are involved in the induction of the stress response [193, 195-197]. To examine if p38α activation and nuclear translocation changes the transcriptional targets of p38-MAPK we examined the expression of FOS and COX-2, both known targets of the p38-MAPK stress response [198]. Expression of both genes were induced in MCF-10A cells expressing Mnk2a and reduced in cells expressing Mnk2b or kinase-dead Mnk2a (Fig. 30a). Moreover, knockdown of Mnk2a decreased FOS and COX-2 expression (Fig. 30b) indicating that Mnk2a alters the transcriptional program of the cells by regulating the expression of p38-MAPK target genes (Fig. 30). Importantly, we found that the kinase activity of Mnk2a is required for induction of p38α-target genes as both the kinase-dead form of Mnk2a or application of the Mnk1/2 kinase inhibitor CGP 57380 inhibited this activity (Figs. 22c, 30d).

Only Mnk2a and the nuclear Mnk2a L/S mutant could activate both p38α target genes (Fig. 30c) and induce apoptosis (Fig. 31a) while the cytoplasmic mutant Mnk2a KKR mutant could not activate either and even reduced apoptosis below the level of cells transfected with empty vector (Fig. 31a). These results suggest that Mnk2a’s ability to translocate p38α into the nucleus is both required and sufficient to mediate its tumor suppressor activity as an inducer of p38α-MAPK target genes and apoptosis. Mnk2b inhibited the expression of FOS and COX-2 below the basal level. Since Mnk2b does not bind p38α, we hypothesize that it does not act in a dominant-negative fashion. Rather, other downstream effects of this isoform may affect the promoters of FOS and COX-2 to inhibit their transcription. Further investigation is required to examine this question.
**p38-MAPK involvement in cancer**

The role of p38α in cancer is ambiguous. On one hand, it possesses an anticancer activity mostly by negative regulation of proliferation and induction of apoptosis. While on the other hand, it can modulate pro-inflammatory responses which could potentially contribute to cancer progression. p38α can activate transcription factors and regulate the production of cytokines such as; IL-1, IL-6 and TNF-α [217], which are involved in pro-survival and angiogenic activities. Apart from inflammation-dependent processes, p38α directly affects tumor invasion and angiogenesis through the induction of matrix-metalloproteinase (MMPs) 1,2 and 3 which regulate matrix remodeling and degradation by metastatic cancer cells, as well as vascular endothelial growth factor A (VEGF-A), a potent inducer of tumor survival and angiogenesis [218, 219]. Additionally, p38α can activate hypoxia inducible factor 1 (HIF-1), which has a key role in hypoxia-driven expression of angiogenic factors [220]. Interestingly, activation of p38α also triggers premature senescence following oncogene activation in primary cells as an antitumorigenic defense mechanism [221]. Indeed, p38α and γ are essential components in Ras-induced senescence which involves the p53 and/or p16INK4A pathways [221]. The finding that Mnk2a was capable of suppressing the growth of Ras V12 MCF-10A cells when sparsely seeded or grown in suspension (Fig. 17a,b), suggests that it can augment p38-mediated tumor suppressive processes. It would be intriguing to explore whether deletion of the Mnk2a gene in primary cells can delay oncogene-induced senescence.

Several in vivo experiments suggest a tumor suppressor activity to p38-MAPK. It was shown that MEF cells knocked out for p38α or MKK3 and MKK6 lead to increased tumor formation in an oncogene-induced model in nude mice [222, 223]. In parallel, the potential role of p38α as a tumor suppressor has been examined using genetically modified mice in which protein phosphatase 1D (PPM1D; also known as WIP1) is inactivated. PPM1D is transcriptionally regulated by p53 and can target p38 MAPKs, among other substrates. Genetic inactivation of PPM1D reduces mammary gland tumorigenesis in mice that express the Erbb2 or H-ras oncogenes, which are under the control of the mouse mammary tumor virus (MMTV) promoter. More direct evidence for the negative regulation of tumorigenesis by p38 MAPKs in mice has been provided by studies using conditional p38α alleles. p38α deficient mice are sensitized
to *Kras* induced lung tumorigenesis, which has been attributed to the immature and hyper-proliferative lung epithelium that results from p38α inactivation [224]. A recent study showed that p38-MAPK upstream kinase MKK3 is deleted/lost in breast cancer tumors and acts as a bona fide tumor suppressor [225]. Finally, the role for the tumor suppressor activity of p38α was recently demonstrated in hepatocellular carcinoma and colon cancer development. In both cases, tissue-specific knockout of p38α in each of these tissues led to cancer development in vivo [226] [227].

From our experimental model system it appears that p38α exerts its apoptotic activity only when translocated to the nucleus rather than when it is present mostly in the cytoplasm, even though it is still phosphorylated (Fig. 23d, Fig. 29, 30 and 31). This raises the question of whether p38α possesses a dual role which depends on its cellular distribution. Proteomic analysis can be used to compare cytoplasmic and nuclear fractions from cells that over-express either the nuclear (L/S) or cytoplasmic (KKR) Mnk2a mutant. This analysis would enable the identification of novel Mnk2a substrates which are involved in its tumor suppressor activity. From a clinical aspect, therapeutic molecules which enhance Mnk2a activity, can theoretically be used against p38-sensitive tumors.

**MKNK2 alternative splicing and cancer**

An alternative splicing switch to eliminate a tumor suppressor is fast and cost-effective for the cell and can serve as an additional level of regulation. Several examples of such regulation of tumor suppressors already exist; *BIN1, MDM2, Caspase-9, BCL-2* family members and others [45, 154, 228-231]. Many tumor suppressor genes are deleted from the genomes of cancer cells. Such an event would be unfavorable in the case of Mnk2 since absence of both isoforms would result in reduced eIF4E phosphorylation and cells would be less oncogenic as in the case of Mnk1/2-DKO cells [146, 147]. However, elimination of only Mnk2a and expression of Mnk2b instead, would sustain eIF4E phosphorylation without activation of the p38 stress pathway. Although we did not analyze MKNK2 DNA copy number variation in tumors, we predict that the main mechanism to eliminate Mnk2a expression in tumors is through modulation of alternative splicing rather than deletion of the *MKNK2* gene.
Cells can also control splicing using stress signals rather than oncogenic signals. Several years ago it was reported that the MKK3/6-p38 pathway can regulate splicing factor hnRNP A1 activity by affecting its cellular distribution [104]. Given that Mnk2a activates p38α, it might theoretically enhance the export of hnRNP A1 from the nucleus to the cytoplasm, reducing the availability and binding of hnRNP A1 to its splicing targets and thus changing the alternative splicing program. Importantly, the finding that Mnk2a is reduced in tumors which harbor a Ras mutation, implies that its Mnk2a levels may be used as a diagnostic biomarker for the activity of this pathway. Furthermore, manipulation of the splicing choice, in favor of Mnk2a over Mnk2b production, in tumors has potential as a future therapeutic tool in the clinic.

Although the tumor suppressor activity of Mnk2a does not involve inhibition of eIF4E, it was recently shown that Mnk2, but not Mnk1, inhibits protein translation through its negative effect on eIF4G Ser1108 phosphorylation and by inhibiting mTOR activity [168]. A previous study which detected negative effects of Mnk2 on translation also supports this finding [169]. These results suggest that Mnk2, and maybe Mnk2a specifically, might possess a tumor suppressive activity by inhibiting translation, an additional mechanism to the one proposed in our study.

In conclusion, we have identified a signaling cascade that emanates from oncogenic (mutant) or activated (by upstream signals) Ras, which increases the production of the splicing factor SRSF1 which in turn alters the splicing of MKNK2 to reduce Mnk2a and increase Mnk2b expression. Mnk2a interacts with, phosphorylates and induces translocation of p38α into the nucleus inducing transcription of its target genes which results in increased apoptosis (Scheme 3). Both Mnk2a and Mnk2b phosphorylate eIF4E on serine 209, which contributes to cellular transformation, but Mnk2b which cannot bind p38-MAPK uncouples this phosphorylation from induction of the p38-MAPK stress response. Our results identify Mnk2 alternative splicing as a mechanism to eliminate a tumor suppressor (Mnk2a) which is a modulator of the p38-MAPK stress pathway and to generate the non tumor suppressive isoform (Mnk2b).
Scheme 3: Alternative Splicing of MKNK2 is manipulated by cancer cells through the Ras - SRSF1 route.

Physiological levels of SRSF1 in normal cells maintain an equal or larger ratio of Mnk2a/2b which ensures activation of the tumor suppressor arm of the p38-MAPK pathway. Mnk2a interacts with and phosphorylates p38-MAPK, increasing its nuclear localization and activation of its target genes which leads to increased apoptosis. In transformed cancer cells, oncogenic or activated Ras increases the production of the splicing factor SRSF1 which in turn modulates the splicing of MKNK2 to reduce Mnk2a and increase the Mnk2b isoform. Both Mnk2a and Mnk2b phosphorylate eIF4E on serine 209, which contributes to cellular transformation, but Mnk2b which cannot bind p38-MAPK, uncouples this phosphorylation from induction of the p38-MAPK stress response.
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