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Avraham Maimon
Hebrew University of Jerusalem

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Mnk2 Alternative Splicing Modulates the p38-MAPK Pathway and Impacts Ras-Induced Transformation

Avraham Maimon,[1] Maxim Mogilevsky,[1] Asaf Shilo,[1] Regina Golan-Gerstl,[1] Akram Obiedat,[1] Vered Ben-Hur,[1] Ilana Lebenthal-Loinger,[1] Ilan Stein,[2] Reuven Reich,[3] Jonah Beenstock,[4] Eldar Zehorai,[5] Claus L. Andersen,[6] Kasper Thorsen,[6] Torben F. Ørntoft,[6] Roger J. Davis,[7] Ben Davidson,[8,9] David Mu,[10] and Rotem Karni[1,*]

[1]Department of Biochemistry and Molecular Biology, Institute for Medical Research Israel-Canada
[2]Department of Immunology and Cancer Research, Institute for Medical Research Israel-Canada
[3]Department of Pharmacology, Institute for Drug Research
[4]Department of Biological Chemistry
The Hebrew University-Hadassah Medical School, Jerusalem 91120, Israel
[5]Department of Biological Regulation, Weizmann Institute of Science, Rehovot 76100, Israel
[6]Department of Molecular Medicine, Aarhus University Hospital, Skejby, 8200 Aarhus N, Denmark
[7]Howard Hughes Medical Institute and Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA 01655, USA
[8]Department of Pathology, Oslo University Hospital, Norwegian Radium Hospital, Ullernchausseen 70, Oslo 0310, Norway
[9]University of Oslo, Faculty of Medicine, Institute of Clinical Medicine, Oslo 0424, Norway
[10]Department of Microbiology and Molecular Cell Biology, Leroy T. Canoles Jr. Cancer Research Center, Eastern Virginia Medical School, 651 Colley Avenue, Norfolk, VA 23501, USA
*Correspondence: rotemka@ekmd.huji.ac.il
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SUMMARY

The kinase Mnk2 is a substrate of the MAPK pathway and phosphorylates the translation initiation factor eIF4E. In humans, *MKNK2*, the gene encoding for Mnk2, is alternatively spliced yielding two splicing isoforms with differing last exons: Mnk2a, which contains a MAPK-binding domain, and Mnk2b, which lacks it. We found that the Mnk2a isoform is downregulated in breast, lung, and colon tumors and is tumor suppressive. 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. Alternatively, Mnk2b is pro-oncogenic and does not activate p38-MAPK, while still enhancing eIF4E phosphorylation. We further show that Mnk2a colocalization with p38α-MAPK in the nucleus is both required and sufficient for its tumor-suppressive activity. Thus, Mnk2a downregulation by alternative splicing is a tumor suppressor mechanism that is lost in some breast, lung, and colon tumors.

INTRODUCTION

The serine/threonine kinases Mnk1 and Mnk2 were discovered by their direct interaction with and activation by the mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinase (ERK) and p38-MAPK (Fukunaga and Hunter, 1997; Waskiewicz et al., 1997). Mnk1 and Mnk2 phosphorylate the translation initiation factor eIF4E on serine 209 (Fukunaga and Hunter, 1997; Waskiewicz et al., 1997). The eIF4E protein binds to the 5′ cap structure of mRNAs and is essential for cap-dependent translational initiation (Mamane et al., 2004). In mice lacking both kinases (MNK-double-knockout [DKO] mice), eIF4E is completely unphosphorylated on serine 209 (Shenberger et al., 2007; Ueda et al., 2004). Intriguingly, these mice develop and live normally, displaying no adverse phenotype (Shenberger et al., 2007; Ueda et al., 2004). Mnk1 and Mnk2 are 72% identical in their amino acid sequence (Fukunaga and Hunter, 1997; Waskiewicz et al., 1997). Biochemically, it has been shown that whereas Mnk1 activation is dependent on upstream MAPK signaling, Mnk2 possesses intrinsic basal activity when introduced into cells (Scheper et al., 2003). There is limited evidence connecting Mnk1/Mnk2 to human cancer, none directly. For example, Mnk1 was shown to be downregulated during differentiation of hematopoietic cells and upregulated in lymphomas (Worch et al., 2004). In a functional study, overexpression of a constitutively active Mnk1 mutant, or a phosphomimetic eIF4E mutant, promoted c-Myc-mediated lymphomagenesis in vivo (Wendel et al., 2007). Recently, it has been demonstrated that mice lacking both Mnk1 and Mnk2 are more resistant to tumor development when crossed with phosphatase and tensin homolog ($PTEN^{-/-}$) mice, and mouse embryonic fibroblast (MEF) cells from these mice are resistant to transformation by several oncogenes, suggesting that eIF4E phosphorylation or the presence of at least one Mnk is required for transformation (Furic et al., 2010; Ueda et al., 2010). The notion that Mnk1 and Mnk2 are positive drivers in human cancer stems from the important role their

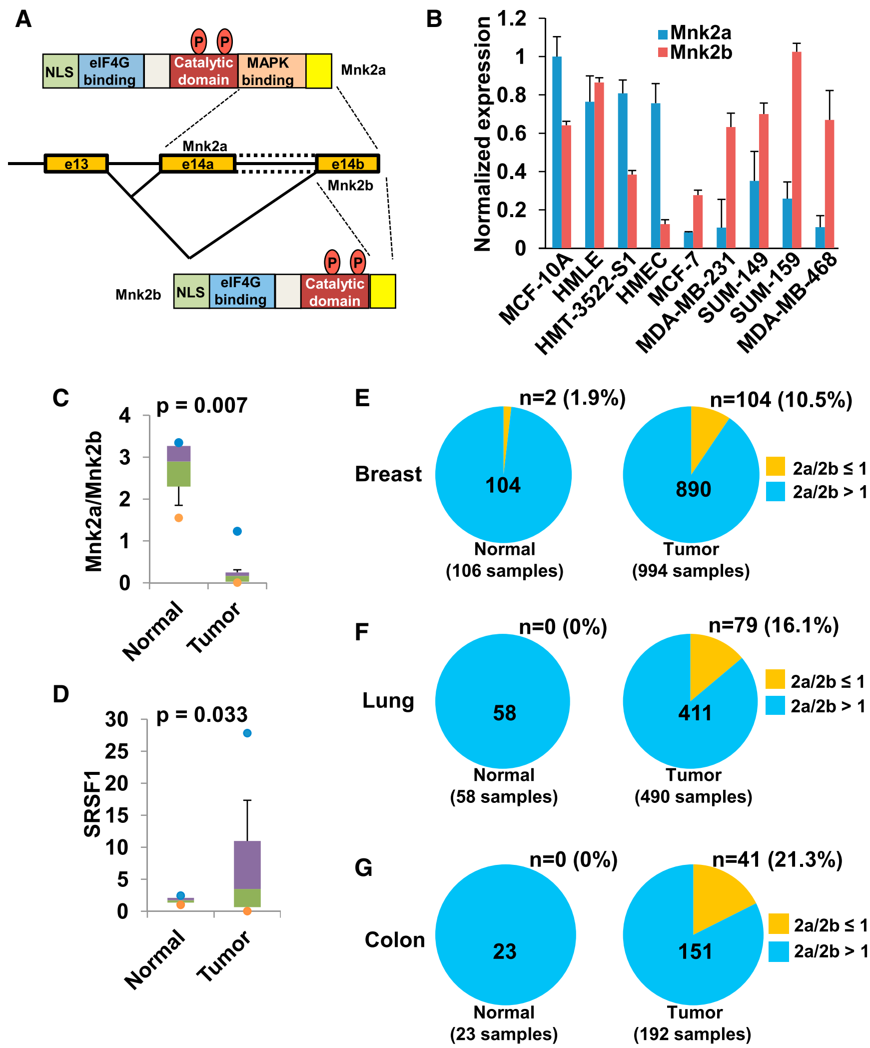

Figure 1. A Switch in *MKNK2* Alternative Splicing in Primary Tumors and Cancer Cell Lines

(A) 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 and T202) in the activation loop that need to be phosphorylated (P) by MAP kinases for kinase activation (Scheper et al., 2003). Mnk2a contains a binding site for MAP kinases located in the C terminus (Scheper et al., 2003). Mnk2b is generated by an alternative 3′ splice site in intron 14 that generates a shorter last exon (14b), lacking the MAPK-binding site (Scheper et al., 2003).

(B) RNA levels of Mnk2a and Mnk2b in the indicated primary or immortal breast and breast cancer cell lines.

(C) The ratios of Mnk2a/Mnk2b RNA in nontransformed breast cell lines (n = 4) and breast tumor samples (n = 13) are represented by a box plot. Top and bottom box edges represent the third and first quartile. Whiskers indicate 90th and 10th percentiles; yellow and blue dots represent minimum and maximum points, respectively. p value is based on t test two-tailed analysis.

(D) RNA levels of SRSF1 in breast tumor samples (n = 13) and nontransformed breast cell lines (n = 4) are represented by a box plot as depicted in (C). Median SRSF1 levels from nontransformed breast cell lines (4) and breast tumor samples (13) are shown. p value is based on t test two-tailed analysis.

(E–G) Pie chart representation of percentage (%) of normal and tumor breast (E), lung (F), and colon (G) samples with Mnk2a/Mnk2b ratios ≤1 (yellow color) or >1 (blue color). Analysis is based on RNA-seq data from TCGA project (https://tcga-data.nci.nih.gov/tcga/).

known substrate, eIF4E, plays in cancer (Mamane et al., 2004), and not from direct functional evidence.

In humans, each of the *MKNK1* and *MKNK2* genes gives rise to at least two distinct proteins, with different C termini, as a consequence of 3′ prime alternative splicing (Parra-Palau et al., 2003; Parra et al., 2005; Scheper et al., 2003). The longer forms of human Mnk1 and Mnk2, referred to as Mnk1a and Mnk2a, respectively, possess a MAPK-binding motif that is absent from the shorter isoforms Mnk1b and Mnk2b. In the corresponding murine proteins, no short forms have yet been identified (Parra et al., 2005). A recent study showed that resistance of pancreatic cancer cells to gemcitabine is mediated by SRSF1 upregulation and a switch in Mnk2 alternative splicing, which enhances eIF4E phosphorylation, implicating this alternative splicing event with chemotherapy resistance (Adesso et al., 2013).

Here, we examined the expression of Mnk2-splicing isoforms in several types of cancer and manipulated the expression of each isoform in normal and transformed cells. We found that the Mnk2a isoform is downregulated in human cancers and is a tumor suppressor, which colocalizes, interacts with, phosphorylates, and activates p38-MAPK, leading to activation of its target genes and to p38α-mediated cell death. These results suggest that Mnk2a is a p38-MAPK kinase and acts as an upstream activator of p38-MAPK. Moreover, Mnk2a antagonizes Ras-induced transformation in vitro and in vivo. However, the Mnk2b isoform, or an inactive kinase-dead version of Mnk2a (Mnk2aKD), did not activate the p38-MAPK pathway and was pro-oncogenic. Taken together, our results suggest that Mnk2 alternative splicing serves as a switch in several cancers to downregulate a tumor suppressor isoform (Mnk2a) that activates the p38-MAPK stress pathway and to induce an isoform (Mnk2b) that does not activate this pathway and is pro-oncogenic.

RESULTS

A Switch in *MKNK2* Alternative Splicing in Breast, Lung, and Colon Cancers

MKNK2 is alternatively spliced to yield two isoforms: Mnk2a and Mnk2b (Figure 1A) (Parra-Palau et al., 2003; Parra et al., 2005;

Scheper et al., 2003). Recently, we have found that the splicing factor oncoprotein SRSF1 (SF2/ASF) modulates the splicing of *MKNK2*, to reduce Mnk2a and increase Mnk2b (Karni et al., 2007). To examine if changes in *MKNK2* splicing are a general phenomenon in cancer, we compared immortal and primary breast cells to breast cancer cell lines, as well as to breast tumor samples. We detected higher or equal expression of Mnk2a compared to Mnk2b in immortal (MCF-10A, HMLE, HMT-3522-S1) (Itoh et al., 2007) and primary breast cells (human mammary epithelial cells). In contrast, Mnk2a expression was significantly decreased, and in some cases, Mnk2b increased in tumor cell lines and tumor samples (Figures 1B, 1C, and S1A). In most of these tumors, SRSF1 was elevated (Figure 1D). To expand our analysis, we analyzed RNA-sequencing (RNA-seq) data from normal and cancer samples available from The Cancer Genome Atlas (TCGA) project (https://tcga-data.nci.nih.gov/tcga/). Comparison of reads covering Mnk2a or Mnk2b splice junctions in these samples shows that whereas only two out of 106 (1.9%) normal breast samples have a Mnk2a/Mnk2b ratio equal to or less than 1:1, 104 out of 994 (10.5%) breast tumor samples have such a ratio. This trend is also observed in lung and colon samples (Figures 1E–1G). In normal lung samples, Mnk2a level was higher and Mnk2b level was lower compared to lung tumors (Figures S2A–S2C). In the same manner, the Mnk2a/Mnk2b ratio in normal colon samples was higher compared to colon tumors (Figure S2E). These results suggest that a ratio of Mnk2a/Mnk2b less than 1:1, due to either reduced levels of Mnk2a and/or increased levels of Mnk2b, is much more prevalent in tumors than in normal tissue samples. We also examined the correlation of expression of several serine/arginine-rich (SR) and heterogeneous nuclear ribonucleoprotein (hnRNP) proteins with Mnk2a/Mnk2b ratios. We found that in breast tumors, expression of several SR and hnRNP A/B proteins (SRSF1, SRSF2, SRSF3, SRSF4, SRSF7, SRSF9, hnRNP A1, hnRNP A2/B1, and Tra2β) had a negative correlation (Pearson correlation smaller than −0.1) with Mnk2a/Mnk2b ratios. Expression of SRSF5 had a positive correlation with Mnk2a/Mnk2b ratios, whereas expression of SRSF6 had no correlation (Table S1).

We found a similar, but not identical, pattern in lung and colon tumors (Tables S2 and S3). Expression of the factors that showed negative correlation with Mnk2a/Mnk2b ratios was also elevated in breast tumors that showed a Mnk2a/Mnk2b ratio equal to or less than 1:1 (Figure S1I) and in lung and colon tumors when compared to corresponding normal tissues (Figures S2D and S2F).

To examine if oncogenic transformation affects *MKNK2* splicing, we compared MCF-10A cells to MCF-10A cells transformed by a mutant *Ras* oncogene ($H\text{-}Ras^{V12}$). We found that oncogenic Ras reduced Mnk2a and elevated Mnk2b as well as SRSF1 expression (Figures S1B and S1C). Similarly, pancreatic cancer cell lines harboring mutant *K-Ras* showed reduced Mnk2a and elevated Mnk2b levels compared to pancreatic cancer cell lines with wild-type (WT) *K-Ras* (Figure S1D). The pancreatic cancer cell line Panc-1, which harbors a *K-Ras* mutation, showed elevated levels of SRSF1 and Mnk2b and reduced Mnk2a at both the RNA and protein levels compared to BXPC-3 cells harboring WT *K-Ras* (Figures S1E–S1G). This altered splicing is also shown in another oncogenic-transformation model: immortal lung epithelial cells (BEAS-2B) transformed by SRSF1 (Shimoni-Sebag et al., 2013) (Figure S1H). These data suggest that oncogenic transformation, caused either by activated Ras or another oncogene, affects *MKNK2* alternative splicing.

Mnk2a Has Tumor-Suppressive Activity, whereas Mnk2b Is Pro-oncogenic In Vitro

To examine the role of Mnk2 alternative splicing in cellular transformation, we seeded nontransformed breast MCF-10A cells transduced with Mnk2-splicing isoforms into soft agar. Cells expressing Mnk2b or a Mnk2aKD were transformed and generated colonies in soft agar, whereas cells expressing Mnk2a did not (Figures 2A and 2C). Mnk2aKD probably acts in a dominant-negative manner by competing with Mnk2a for substrate binding, while incapable of phosphorylation. Similar results were obtained in another transformation model of NIH 3T3 cells (Figures S3A and S3B). Furthermore, when MCF-10A cells expressing Mnk2-splicing isoforms were transformed by oncogenic *Ras*, cells coexpressing Mnk2a showed reduced ability to form colonies in soft agar, indicating that Mnk2a can block Ras-induced transformation (Figures 2B and 2D). Similarly, Mnk2a inhibited colony formation in soft agar of the osteosarcoma cell line U2OS (Figures S3C and S3D). Knockdown of Mnk2a enhanced colony formation of MCF-10A and NCI-H460 cells in soft agar, suggesting that Mnk2a is tumor suppressive (Figures S3E–S3H). Neither Mnk2a nor Mnk2b expression changed significantly cell proliferation or cell-cycle distribution (Figures S3I–S3N). However, cells with Mnk2a knockdown had a slightly higher proliferation rate, indicating that Mnk2a reduction may enhance proliferation (Figure S3J). Taken together, 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 injected Ras-transformed MCF-10A cells transduced with Mnk2-splicing isoforms into nonobese diabetic-severe combined immunodeficiency (NOD-SCID) mice. We found that mice injected with Ras-MCF-10A cells expressing either empty vector or Mnk2b formed tumors (six out of six), whereas mice injected with Ras-MCF-10A cells expressing Mnk2a did not form any tumors (zero out of eight) (Figure 2E). Tumors from cells expressing Mnk2b showed an increased mitotic index (Figures 2G and 2H) compared with tumors from cells expressing Ras alone but did not show significant enhanced tumor growth (Figure 2E). Inversely, mice injected with Ras-MCF-10A cells expressing small hairpin RNA (shRNA) against Mnk2a showed enhanced tumor growth rate (Figure 2F) and increased mitotic index in the tumors (Figures 2I and 2J), indicating that Mnk2a depletion cooperates with and enhances Ras tumorigenicity. Collectively, our results suggest that Mnk2a has tumor suppressor activity, and it can antagonize Ras-mediated transformation in vitro and in vivo.

Mnk2a Sensitizes Cells to Stress-Induced Cell Death

Because overexpression or downregulation of Mnk2a did not affect cellular proliferation significantly (Figures S3I–S3N), we

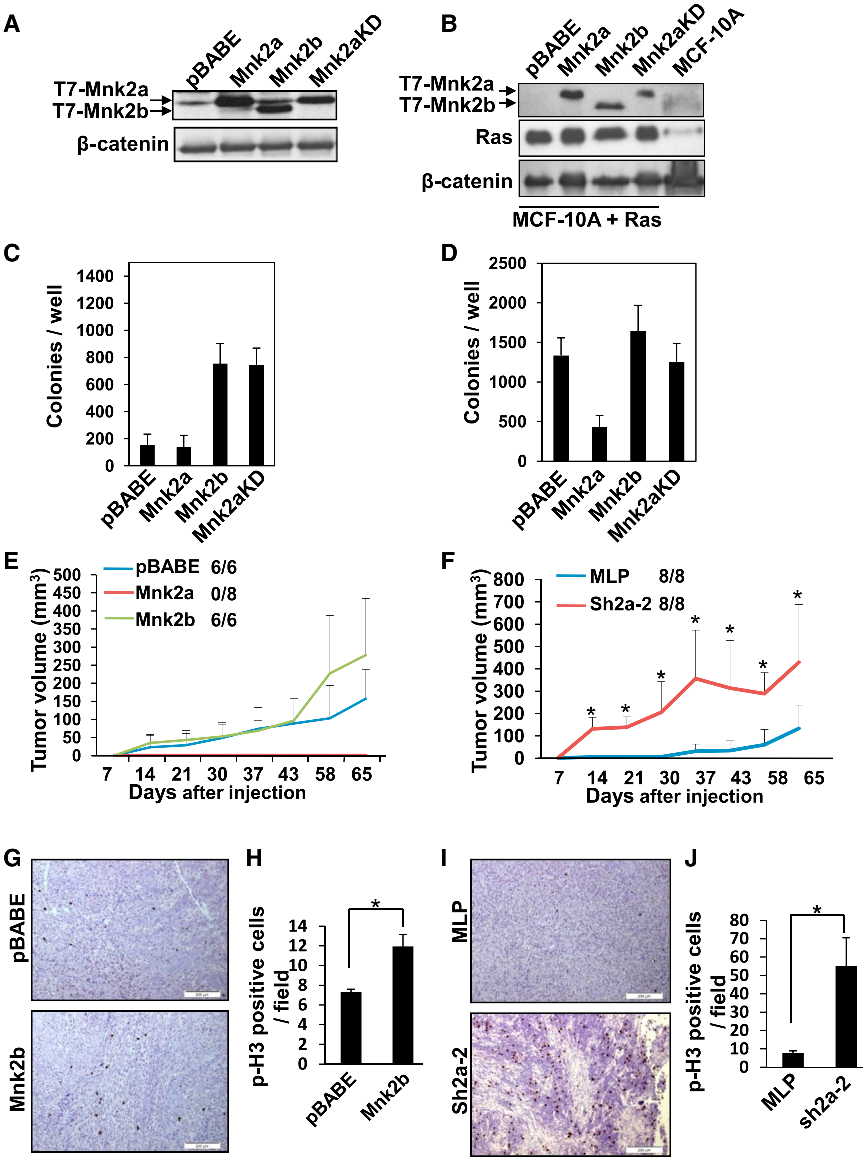

Figure 2. Mnk2a Inhibits Ras-Induced Transformation and Tumorigenesis

(A) Western blot of MCF-10A cells transduced with the indicated retroviruses encoding for Mnk2 isoforms and Mnk2aKD.

(B) Western blot of cells described in (A) transduced with a retrovirus encoding for *H-RAS*^{V12}.

(C) 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 SD of colonies per well of three wells are shown (n = 3).

(D) Cells described in (B) were seeded into soft agar and counted as in (C) (n = 3).

(E and F) Pools of MCF-10A cells transformed with the indicated retroviruses encoding Mnk2 isoforms followed by *H-RAS*^{V12} transduction (E) or MCF-10A cells transduced with shRNA against Mnk2a (Sh2a-2) followed by transformation by *H-RAS*^{V12} (F) were injected (2×10^6 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. *$p \leq 0.01$.

(G–J) Formalin-fixed, paraffin-embedded tissue sections from tumors derived from the indicated cell pools described in (E) and (F) were stained with anti-phospho-H3 to detect mitotic cells (G and I). Graphs show the average and SD of p-H3-positive cells from ten fields of three different tumors (H and J). *$p \leq 0.01$.

hypothesized that Mnk2a might enhance the sensitivity of cells to apoptosis. 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 Mnk2a, Mnk2b, or Mnk2aKD with different stress conditions. Although Mnk2a enhanced apoptotic cell death in response to anisomycin treatment, as measured by trypan blue exclusion and caspase-3 cleavage, Mnk2b and the Mnk2aKD protected against apoptosis (Figures 3A and 3D). Moreover, knockdown of Mnk2a protected MCF-10A cells from anisomycin-induced apoptosis (Figures 3B and 3E). The correlation of apoptosis and caspase-3 cleavage with p38-MAPK phosphorylation (Figures 3D and 3E) suggests that Mnk2a proapoptotic activity might involve activation of the p38-MAPK pathway. Mnk2a also reduced survival of Ras-transformed MCF-10A cells and MCF-10A cells forced to grow in suspension or stimulated with osmotic shock (Figures S4A and S4C). In contrast, knockdown of Mnk2a protected cells from osmotic shock, suggesting that Mnk2a mediated this stress response (Figure S4D). Mnk2a also inhibited the survival of MCF-10A cells transformed by oncogenic *Ras* when sparsely seeded for colony survival assay (Figures 3C and 3F), suggesting that Mnk2a sensitizes Ras-transformed cells to low-density stress conditions. One of the stress pathways induced by anisomycin and other cellular insults is the p38-MAPK pathway (Benhar et al., 2001; Cuadrado and Nebreda, 2010; Hazzalin et al., 1996). In order to examine if p38-MAPK activation is involved in Mnk2a-enhanced cell death, we blocked p38-MAPK kinase activity with the specific kinase inhibitor ATP competitor SB203580 (Badger et al., 1996; Benhar et al., 2001; Lee et al., 2000). p38-MAPK inhibition partially rescued cells expressing Mnk2a from anisomycin-induced cell death (Figure S4B). Neither transient expression nor stable expression of Mnk2a alone reduced survival of MCF-10A cells, suggesting that Mnk2a exerts its proapoptotic properties only under stress conditions (Figures S4F and S4G).

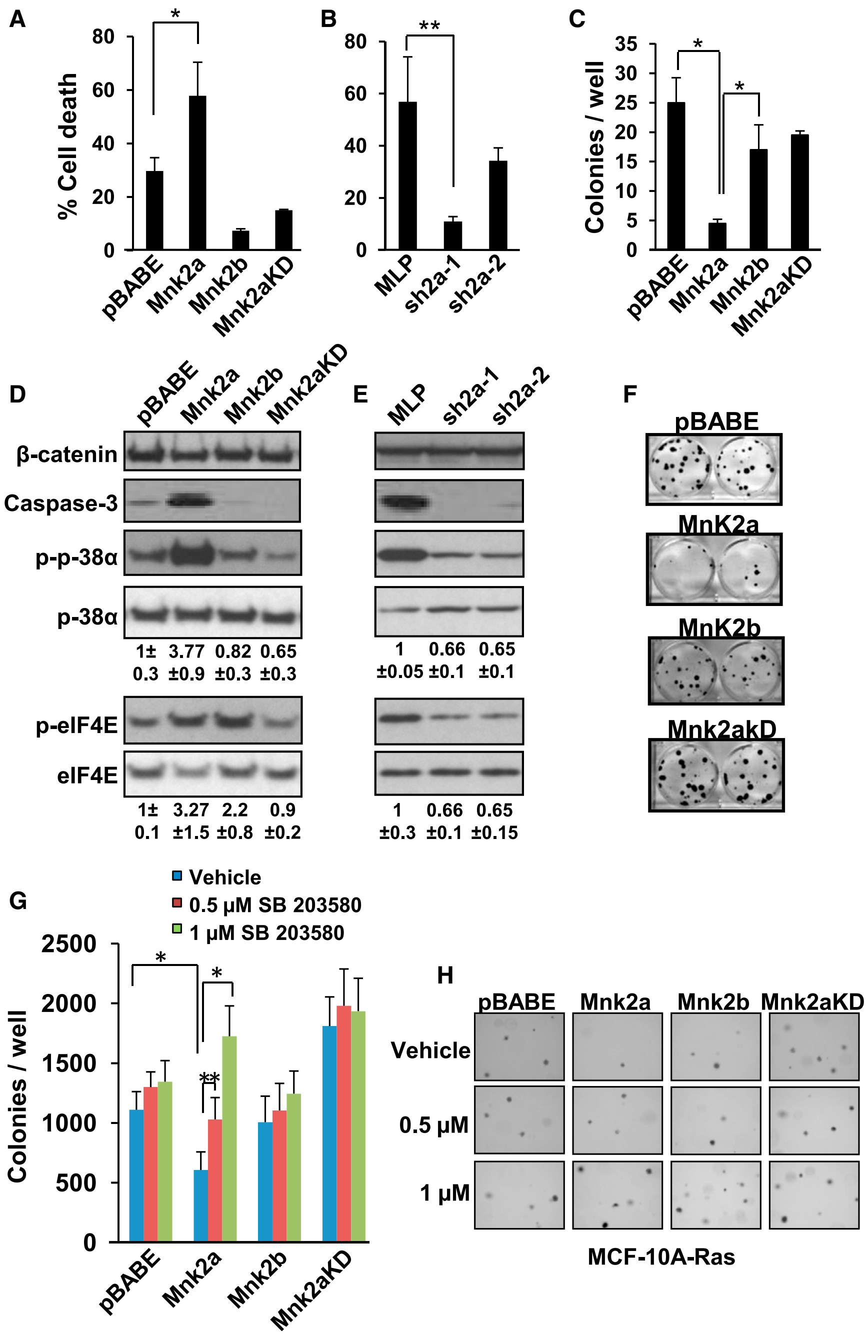

Figure 3. Mnk2a Sensitizes MCF-10A Cells to Stress-Induced Apoptosis

(A and B) Trypan blue exclusion assay of MCF-10A cells transduced by the indicated retroviruses. Following 24 hr in growth factor-free media, cells were treated with 0.5 μM anisomycin for 24 hr (A) or 48 hr (B). *p $\leq$ 0.05; **p $\leq$ 0.01.

(C) Colony formation assay of pools of MCF-10A cells transduced with the indicated retroviruses encoding Mnk2 isoforms and transformed by *H-RAS*^{V12}. Average and SD of the number of colonies per well are shown (n = 3). *p $\leq$ 0.05.

(D and E) Cells described in (A) and (B) 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 loading control. Numbers represent ratios of p-p38/total p38 and p-eIF4E/total eIF4E normalized to that of pBABE or MLP (arbitrarily set at 1) ± SD (n = 3).

(F) Representative wells with colonies described in (C).

(G) MCF-10A cells transduced with the indicated retroviruses followed by transduction with *H-RAS*^{V12} were seeded into soft agar in the presence or absence of the indicated concentrations of SB203580, and colonies were counted 14 days later. *p $\leq$ 0.01; **p $\leq$ 0.05 (n = 3).

(H) Photographs of representative fields of colonies in soft agar obtained as described in (G).

Mnk2a, but Not Mnk2b, Enhances p38α-Mediated Cell Death and Suppression of Ras-Induced Transformation

Because Mnk2a, but not Mnk2b, contains a MAPK-binding domain (Figure 1A) and can be activated by ERK and p38-MAPK (Parra et al., 2005; Scheper et al., 2003), 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 (Askari et al., 2007; Avitzour et al., 2007; Diskin et al., 2004) and grown in the absence or presence of the p38 kinase inhibitor SB203580. Cells expressing Mnk2a showed increased cell death upon active p38-MAPK transduction, which was inhibited by SB203580 (Figures S5A and S5B). Cells in which Mnk2a was knocked down showed increased protection from p38-induced cell death (Figures S5C and S5D). SB203580 efficiently inhibited p38 activity, as was measured by phosphorylation of its substrate MK2. However, it did not affect phosphorylation of p38-MAPK or eIF4E, indicating that SB203580 treatment did not inhibit upstream Mnk2 or MKK3/MKK6 activity (Figure S5E). These results suggest that Mnk2a augments p38-MAPK stress activity. Inhibition of p38-MAPK by SB203580 rescued the ability of cells cotransduced with Mnk2a and *Ras* to form colonies in soft agar, suggesting that p38 activation by Mnk2a plays an important role in its ability to suppress Ras-induced transformation (Figures 3G and 3H).

Mnk2a Interacts with, Activates, and Induces Nuclear Translocation of p38-MAPK

To determine if Mnk2a activates p38-MAPK, we examined the phosphorylation status of p38-MAPK in cells expressing Mnk2-splicing isoforms (Enslen et al., 1998). As expected,

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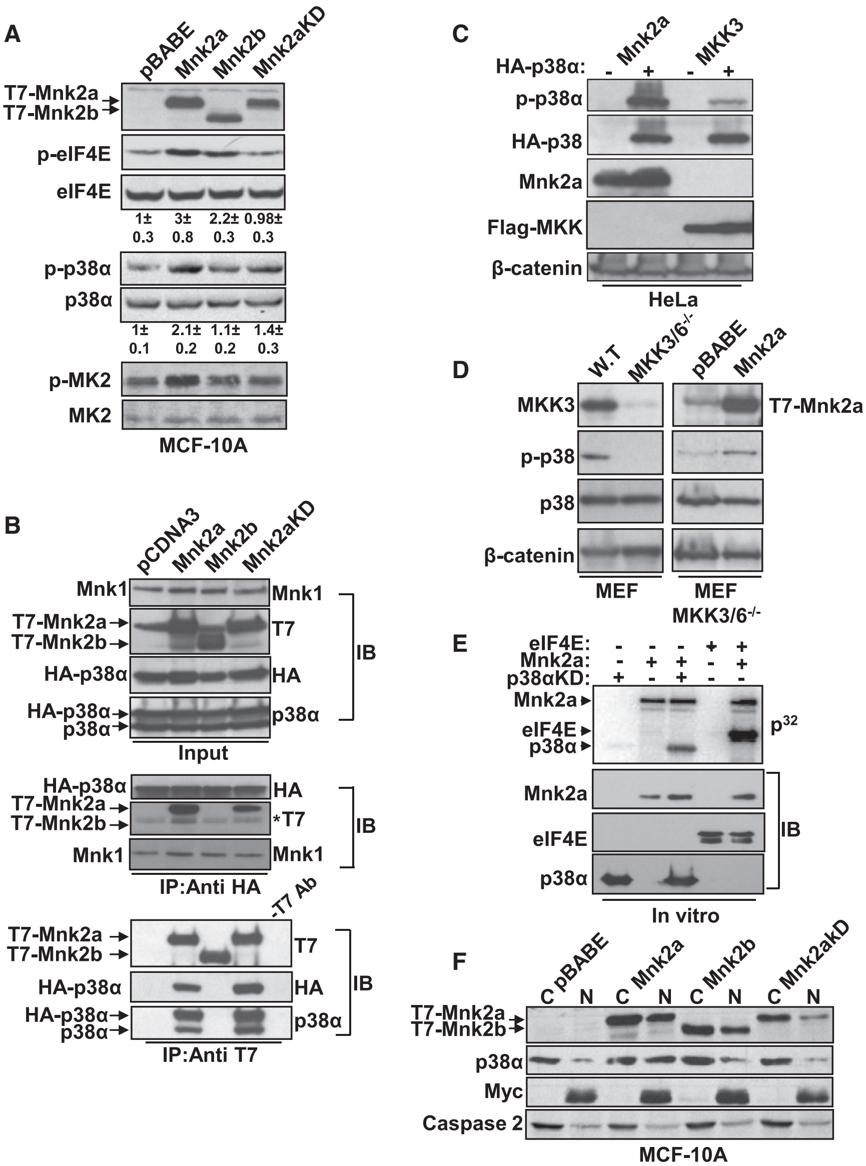

Figure 4. Mnk2a Interacts with p38-MAPK and Leads to Its Activation and Translocation into the Nucleus

(A) Western blot analysis of MCF-10A cells transduced with the indicated retroviruses encoding for Mnk2 isoforms or Mnk2aKD. Numbers represent the ratios of p-p38/total p38 and p-eIF4E/total eIF4E normalized to pBABE (arbitrarily set at 1) ± SD (n = 3).

(B) Coimmunoprecipitation of HA-p38α or T7-Mnk2 isoforms from HEK293 cells cotransfected with the indicated Mnk2 isoforms together with HA-p38α-MAPK. Precipitated and input proteins were subjected to western blot analysis. The asterisk (*) represents a nonspecific band. As a control, lysate from cells transfected with T7-Mnk2a was incubated with beads in the absence of the T7 antibody. IP, immunoprecipitation; IB, immunoblotting.

(C) Western blot analysis of HeLa cells cotransfected with either Mnk2a or MKK3 together with HA-p38α-MAPK. β-catenin was used as a loading control.

(D) Left: western blot analysis of MEF cells from WT mice (MKK3/MKK6 WT) and MKK3/MKK6$^{-/-}$ DKO mice. Right shows a western blot analysis of MEF cells from MKK3/MKK6$^{-/-}$ DKO mice (MEF MKK3/MKK6$^{-/-}$) transduced with the indicated retroviruses. β-catenin was used as a loading control.

(E) In vitro kinase assay using recombinant Mnk2a, recombinant kinase dead (KD) p38α, recombinant eIF4E alone, or in the indicated combinations. Reaction mixes were stopped, separated on SDS-PAGE, and transferred onto nitrocellulose membranes. Upper panel shows a radioactive exposure of the membrane detected by a Fuji phosphorimager cassette and visualized by MacBass phosphorimager. Bottom panel shows the same membrane as above that was probed with the indicated antibodies and detected by enhanced chemiluminescence.

(F) Cellular distribution of p38α in MCF-10A cells, transduced with retroviruses encoding the indicated Mnk2 isoforms. N, nuclear fraction; C, cytoplasmic fraction. *c-myc* (nuclear) and caspase-2 (cytoplasmic) served as fractionation controls.

phosphorylation of a known substrate of Mnk2, serine 209 of eIF4E, was induced by Mnk2a expression (Figures 4A, S3A, and S3C). Although previous reports have suggested that Mnk2b has a lower kinase activity than Mnk2a (Scheper et al., 2003), we observed that it phosphorylates eIF4E to a similar extent as Mnk2a (Figures 4A, S3A, SC3, and S5E). The Mnk2aKD did not enhance eIF4E phosphorylation (Figures 4A, S3A, and S3C). In contrast, only cells expressing Mnk2a showed increased p38-MAPK phosphorylation, suggesting that p38-MAPK is activated by Mnk2a (Figures 4A, 4C, 4D, S5E, and S6A) (Avitzour et al., 2007; Enslen et al., 1998). Moreover, knockdown of Mnk2a in MCF-10A cells reduced p38-MAPK basal phosphorylation level (Figures 3E and S3E). In addition, we determined that the phosphorylation state of the p38-MAPK substrate MK2 was enhanced only in cells expressing Mnk2a, but not Mnk2b or Mnk2aKD (Figure 4A). Knockdown of Mnk2a inhibited MK2 phosphorylation, and inhibition of p38-MAPK by the kinase inhibitor SB203580 abolished MK2 phosphorylation in cells expressing Mnk2a (Figure S5E). To further examine if the kinase activity of Mnk2a is important for p38 phosphorylation, we treated MCF-10A cells with the Mnk1/Mnk2 kinase inhibitor CGP 57380 (Knauf et al., 2001) (Chrestensen et al., 2007). Mnk1/Mnk2 kinase inhibition reduced p38-MAPK phosphorylation on the known MEK3/MEK6 phosphorylation sites T180 and Y182, similar to its effect on eIF4E S209 phosphorylation (Figure S6B). Finally, we examined p38 phosphorylation in immortal breast and breast cancer cell lines and observed that in the latter (which tend to express lower Mnk2a levels) (Figure 1B), both p38-MAPK and eIF4E phosphorylation is lower than in the nontransformed cells (Figures S6C–S6E).

We next examined whether Mnk2 isoforms can differentially interact with p38α-MAPK in cells. Coimmunoprecipitation of transfected or endogenous p38α from HEK293 cells demonstrated that Mnk2a and Mnk2aKD, unlike Mnk2b, efficiently bound p38α (Figure 4B). Importantly, even though Mnk2aKD was bound to p38α, it did not cause activation of p38α, as measured by p38α or MK2 phosphorylation (Figures 3D, S5E, and 4A). Finally, to rule out the possibility that Mnk2 isoforms compete with Mnk1 for p38α binding, we examined Mnk1 binding to p38α. Mnk1 was bound to HA-p38α; however, its binding was not affected by any of the Mnk2 isoforms (Figure 4B). Taken together, these results suggest that Mnk2a interacts with p38α and leads to its activation.

Several p38-MAPK isoforms other than p38α exist and are involved in stress signaling and apoptosis (Cuadrado and Nebreda, 2010; Turjanski et al., 2007). Thus, we examined if Mnk2a enhances the phosphorylation of other p38-MAPK family members when cotransfected into HeLa cells. We found that Mnk2a enhanced the phosphorylation of p38α and p38β, only weakly elevated phosphorylation of p38γ, and had no significant effect on phosphorylation of p38δ (Figure S6F). We next examined the interaction of Mnk2a with the other p38-MAPK members by coimmunoprecipitation. In correlation with the ability of Mnk2a to phosphorylate the different p38-MAPK isoforms, we found that Mnk2a interacts with p38α and p38β, but we could not detect interaction with p38γ or p38δ (Figures S6G and S6H). These results suggest that Mnk2a kinase activity is specific to p38α and p38β.

Cotransfection of HeLa cells with p38α and WT Mnk2a showed higher p38α phosphorylation levels than cells cotransfected with WT MKK3, indicating that (without upstream activation) Mnk2a's ability to phosphorylate p38α is as strong or stronger than MKK3 (Figure 4C). To examine if Mnk2a can directly phosphorylate p38-MAPK in the absence of the known p38-MAPK kinases MKK3 and MKK6 (Enslen et al., 1998; Rincón and Davis, 2009), we transduced MEF cells from MKK3/MKK6 DKO mice (Brancho et al., 2003) with retroviruses encoding for Mnk2a or an empty vector. Mnk2a induced p38-MAPK phosphorylation in the absence of MKK3 and MKK6, suggesting that it can directly phosphorylate p38-MAPK (Figure 4D). To further examine if Mnk2a directly phosphorylates p38-MAPK, we performed an in vitro kinase assay with recombinant WT Mnk2a and kinase-dead p38α proteins and found that Mnk2a can directly phosphorylate p38α-MAPK (Figure 4E). Taken together, these results suggest that Mnk2a is a direct p38α-MAPK activator kinase.

Mnk2a Colocalizes with p38-MAPK and Affects Its Cellular Localization

Upon activation, p38-MAPK is translocated to the nucleus and phosphorylates transcription factors that mediate some of its stress response (Aplin et al., 2002; Gong et al., 2010; Pfundt et al., 2001; Plotnikov et al., 2011). Using cytoplasmic and nuclear fractionation, as well as immunofluorescent staining, we observed that both Mnk2a and Mnk2b can be detected in the nucleus (Figures 4F, S6I, and S7). However, cells that express Mnk2a showed an increased nuclear fraction of total and phosphorylated p38-MAPK (Figures 4F, 5B, S6I, S7A, and S7B), indicating that Mnk2a leads to both p38-MAPK activation and its translocation into the nucleus.

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 KKRGKKAAA, 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(Parra-Palau et al., 2003). 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 (Parra-Palau et al., 2003), we identified a similar motif (LxxxLxxL) in the C-terminal region of Mnk2 (starting at amino acid 281 of Mnk2a) and mutated the last two lysines to serines. Transfection of these mutants into HeLa cells or transduction into MCF-10A cells showed the expected localization: the nuclear localization of Mnk2a L/S was enhanced, whereas that of Mnk2a KKR was decreased, when compared to that of Mnk2a (Figures 5A and S7). When cotransfected with HA-tagged or GFP-tagged p38-MAPK, Mnk2a colocalized with p38-MAPK. Mnk2a L/S rendered p38-MAPK mostly nuclear and colocalized with it in the nucleus. Mnk2a KKR colocalized with p38-MAPK in both the cytoplasm and nucleus but was less nuclear than Mnk2a (Figures 5A, S7A–S7D, and S7F). Both Mnk2a KKR and the L/S mutants can interact with HA-tagged and endogenous p38α, as was demonstrated by coimmunoprecipitation, and can pull down phospho-p38α (Figure S7E). In addition, the effects of the L/S and KKR mutants on the localization of endogenous p38α were similar to their effects on HA-p38 in MCF-10A cells transduced with retroviruses expressing these mutants (Figure S7A). 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-MAPK.

Mnk2a Localization Affects Induction of p38-MAPK Target Genes and Apoptosis

We next examined the expression of *FOS* and cyclo-oxygenase 2 (*COX-2*), both targets of p38-MAPK stress response (Ferreiro et al., 2010). Expression of both genes was induced in MCF-10A cells expressing Mnk2a and reduced in cells expressing Mnk2b or Mnk2aKD (Figure 6A). Moreover, knockdown of Mnk2a inhibited *FOS* and *COX-2* expression (Figure 6B). Interestingly, whereas Mnk2a L/S could activate p38-MAPK target genes similarly to Mnk2a, the KKR mutant did not increase the expression of *FOS* and *COX-2* (Figure 6C). To examine if Mnk1/Mnk2 kinase activity modulates the expression of p38α target genes, we treated MCF-10A cells with the Mnk1/Mnk2 kinase inhibitor CGP 57380. Inhibition of Mnk1/Mnk2 kinase activity, which reduced p38-MAPK phosphorylation (Figure S6B), also reduced the expression p38-MAPK target genes (Figure 6D). MEF cells from mice with a DKO of both activators of p38-MAPK, MKK3 and MKK6 (MKK3/MKK6 DKO), show very low basal phosphorylation of p38-MAPK compared to WT cells (Brancho et al., 2003) (Figure 4D). Introduction of Mnk2a into MKK3/MKK6 DKO MEFs elevated p38-MAPK phosphorylation (Figure 4D) and also induced the expression of *FOS* and *COX-2* (Figure 6E). Mnk2b inhibited the expression of *FOS* and *COX-2* below the basal level (Figure 6A). Mnk2aKD inhibited

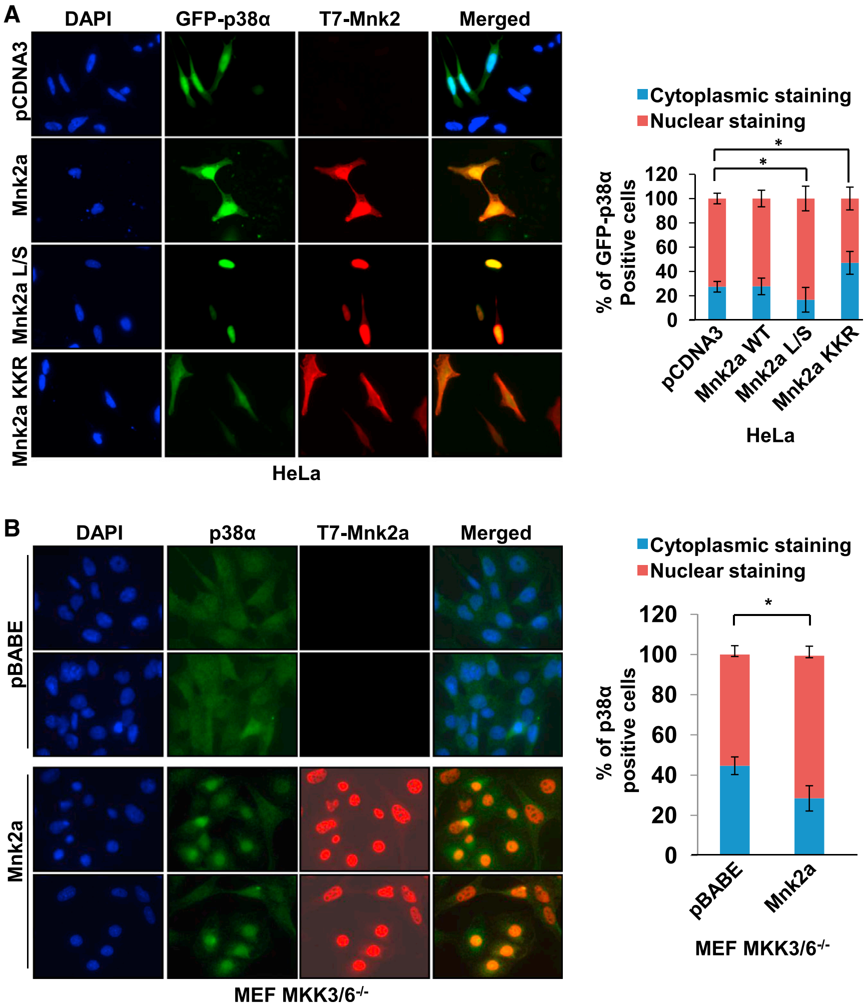

Figure 5. Mnk2a Colocalizes with p38-MAPK and Affects Its Cellular Localization

(A) Immunofluorescence of HeLa cells 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) as described in Experimental Procedures. T7 tag is red, and GFP-p38α is green. Right view shows quantification of cytoplasmic/nuclear distribution of GFP-p38α in cells similar to those described (n = 40 for each mutant). $^{*}p \leq 0.01$.

(B) Immunofluorescence of MKK3/MKK6$^{-/-}$ MEF cells transduced with the indicated retroviruses. 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). $^{*}p = 9.6 \times 10^{-17}$.

the expression of *COX-2* and had no effect on the expression of *FOS* (Figure 6A). This is possibly due to other regulatory elements in the *FOS* promoter making it less responsive to inhibition of MNK2a, as seen from the kinase inhibitor experiment (Figure 6D). To examine if Mnk2a localization can affect its proapoptotic activity, we treated cells expressing Mnk2a isoforms and the KKR and L/S mutants with anisomycin and measured cell death. We found that whereas Mnk2a and the L/S mutant sensitized cells to apoptosis, the KKR mutant inhibited apoptosis and did not significantly decrease colony survival, in correlation with its inability to induce p38α target genes (Figures 6F and S4E). Taken together, these results indicate that Mnk2a not only activates p38-MAPK, but it also regulates the expression of p38-MAPK target genes, altering the transcriptional program of the cells and leading to induction of apoptosis.

Modulation of *MKNK2* Splicing by Splice Site-Competitive Antisense RNA Oligos Affects Sensitivity to Apoptosis and Cellular Transformation

To examine if modulation of endogenous *MKNK2* alternative splicing affects the oncogenic properties of cells, we used two experimental systems in which Mnk2a/Mnk2b ratios were altered due to cellular transformation: MCF-10A cells transformed by oncogenic *Ras* (Figures S1B and S1C), and immortal lung bronchial epithelial cells, BEAS-2B, transformed by SRSF1 overexpression (Shimoni-Sebag et al., 2013) (Figure S1H). We designed Cy5-labeled 2′-O-met-RNA antisense oligos to mask the *MKNK2* splice sites, shifting the splicing balance between Mnk2a and Mnk2b (Figures 7A and 7B). Elevation of Mnk2a and reduction of Mnk2b by the oligo that blocks production of Mnk2b (2b Block) sensitized cells to anisomycin-induced apoptosis, reduced colony survival, and inhibited soft agar colony formation in both Ras-transformed MCF-10A cells (Figure 7) and SRSF1-transformed BEAS-2B cells (Figure S8). The 2b block oligo also enhanced anisomycin-induced apoptosis of nontransformed MCF-10A cells (Figure 7G). Elevation of Mnk2b and concomitant reduction of Mnk2a, by the oligo that competes with Mnk2a intron-exon junction (2a block), protected Ras-transformed MCF-10A cells and SRSF1-transformed BEAS-2B cells from anisomycin-induced apoptosis, and increased colony survival (Figures 7E, 7F, S8E, and S8F). Introduction of the 2a block oligo also protected non-transformed MCF-10A cells from anisomycin-induced apoptosis (Figure 7G). The 2a block oligo did not increase anchorage-independent growth in soft agar (Figures 7C, 7D, S8C, and S8D), probably because cells expressing oncogenic Ras or SRSF1 are already highly invasive and tumorigenic (see Figure 2D for Ras-transformed and Shimoni-Sebag et al., 2013 for SRSF1-transformed cells). As in the case of Mnk2a or Mnk2b overexpression (Figures S4F and S4G), in normal conditions without stress, modulation of *MKNK2* splicing by the antisense oligos did not induce apoptosis on its own (Figures S8G and S8H). These data suggest that manipulation of the endogenous ratios of

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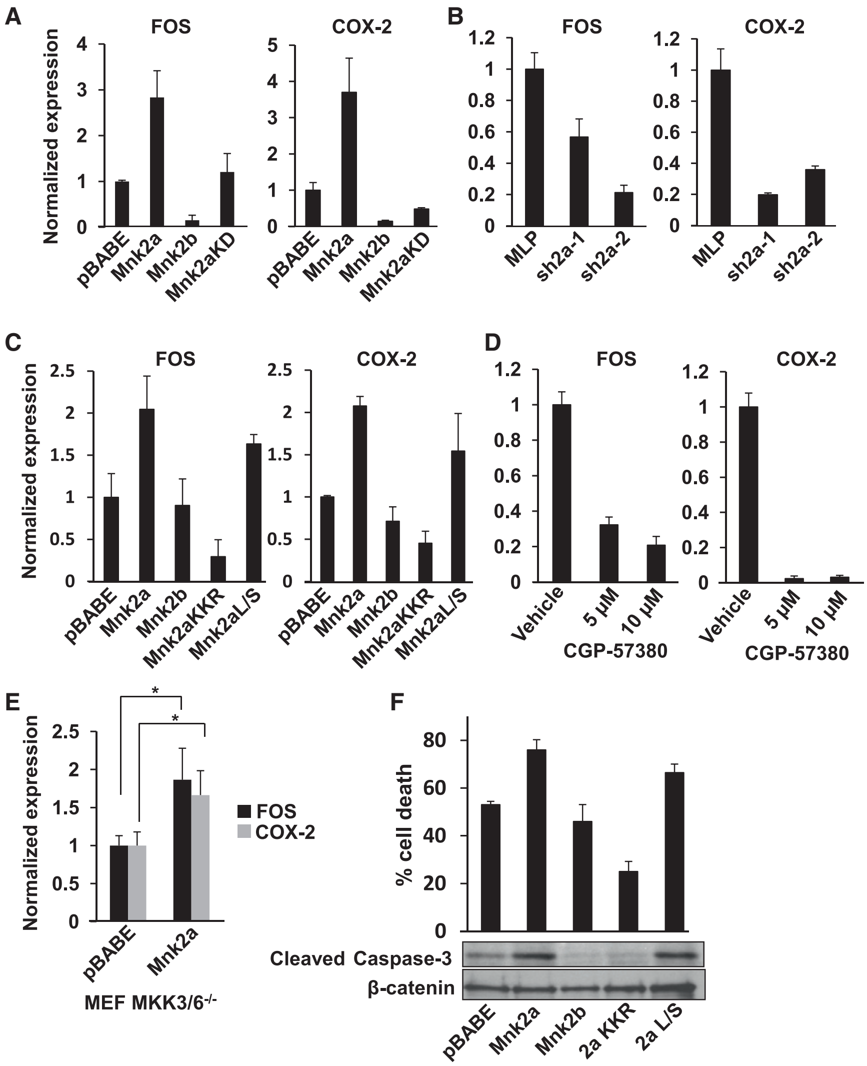

Figure 6. Mnk2a Localization and Kinase Activity Are Required for Induction of p38α Target Genes and Apoptosis

(A and B) Quantitative RT-PCR (qRT-PCR) of RNA from MCF-10A cells transduced either with the indicated Mnk2 isoforms or Mnk2aKD or with MLP vectors encoding for shRNAs against Mnk2a isoform.

(C) qRT-PCR of RNA from MCF-10A cells transduced with either the indicated Mnk2 isoforms or the indicated Mnk2a mutants.

(D) qRT-PCR of RNA from serum-starved MCF-10A cells treated with Mnk1/Mnk2 inhibitor CGP 57380 at the indicated concentrations for 8–10 hr.

(E). qRT-PCR of RNA from MKK3/MKK6$^{-/-}$ MEF cells transduced with the indicated retroviruses. $^{*}p \leq 0.01$.

(F) Trypan blue exclusion assay of MCF-10A cells described in (C) serum starved for 24 hr and then treated with 0.5 μM anisomycin for 24 hr (graph). Bottom panel shows a western blot analysis of the cells described. Cleaved caspase-3 served as a marker for apoptosis and β-catenin as a loading control.

Mnk2a and Mnk2b affects the oncogenic potential of cells transformed by Ras and SRSF1.

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 (Venables et al., 2009; Xi et al., 2008). Many oncogenes and tumor suppressors 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 (Srebrow and Kornblihtt, 2006; Venables, 2004). Here, we have shown that *MKNK2* alternative splicing is modulated in cancer cells to downregulate 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-MAPK, leading to enhanced activation of the p38 stress pathway, induction of its target genes, and enhanced cell death.

Previously, we identified the splicing factor SRSF1 (SF2/ASF) as a potent proto-oncogene and reported *MKNK2* as one of its splicing targets (Karni et al., 2007) (Anczuków et al., 2012). Enhanced expression of SRSF1 reduced the levels of Mnk2a and increased the levels of Mnk2b, whereas knockdown of SRSF1 caused a reciprocal change in *MKNK2* splicing (Karni et al., 2007) (Anczuków et al., 2012). Here, we found that Mnk2a is downregulated, whereas in some cases, Mnk2b is upregulated, and the level of SRSF1 is upregulated in breast cancer cell lines and tumors (Figures 1A–1D). Moreover, oncogenic transformation by Ras elevated the levels of SRSF1 and induced a shift in *MKNK2* splicing (Figures S1B and S1C). Pancreatic cancer cell lines harboring mutant *K-Ras* (such as Panc-1) had elevated SRSF1 levels and altered *MKNK2* splicing compared to a pancreatic cancer cell line with WT *K-Ras* (Figures S1D–S1G). In another model system of oncogenic transformation, by SRSF1 (Shimoni-Sebag et al., 2013), *MKNK2* alternative splicing was also shifted (Figure S1H), suggesting that changes in MKNK2 splicing occur upon transformation with oncogenes such as Ras and SRSF1.

Recently, it has been shown that Mnk2, but not Mnk1, inhibits protein translation through its negative effect on eIF4G Ser1108 phosphorylation and by inhibiting mammalian target of rapamycin activity (Hu et al., 2012). An earlier study detected similar effects of Mnk2 on translation (Knauf et al., 2001). These results suggest that Mnk2 might possess a tumor-suppressive activity by inhibiting translation, an additional mechanism to the one

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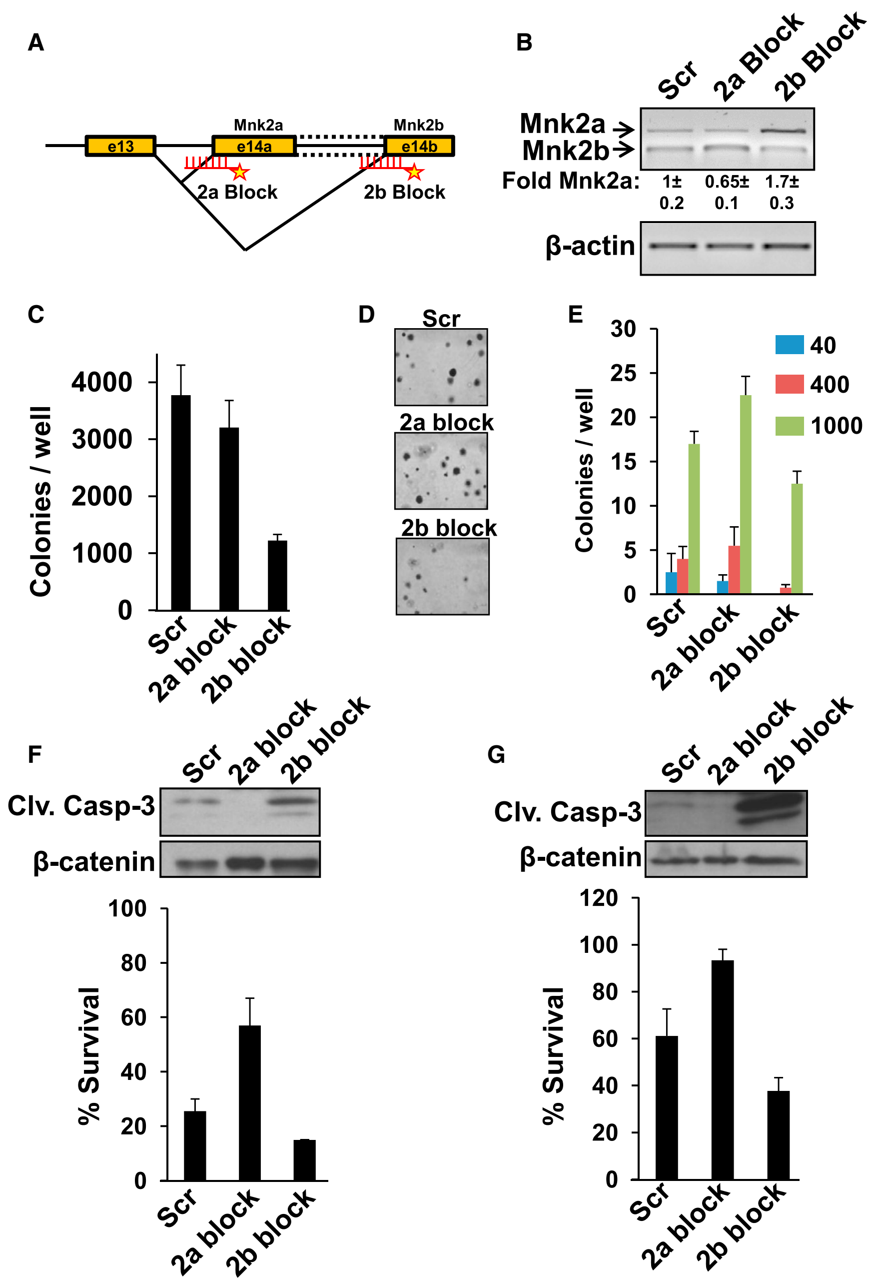

Figure 7. Modulation of *MKNK2* Splicing by Splice Site-Competitive Antisense RNA Oligos Affects Sensitivity to Apoptosis and Cellular Transformation

(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 3′ splice site of either exon 14a (2a Block) or exon 14b (2b Block).

(B) RT-PCR of RNA from Ras-transformed MCF-10A cells transfected with the indicated Cy5-labeled antisense 2′-O-methyl RNA oligos. Numbers ± SD represent ratio of Mnk2a/Mnk2b normalized to that of cells transfected with the scrambled (Scr) oligo (arbitrarily set at 1) (n = 3).

(C) Soft agar growth assay of cells transfected as in (B). Colonies in ten fields of each well were counted, and the mean and SD of colonies per well of three wells are shown (n = 3).

(D) Photographs of representative fields of colonies in soft agar obtained as described in (C).

(E) Colony formation assay of cells transfected as in (B). Average and SD of the number of colonies per well are shown (n = 3).

(F) Cell survival assay and western blot analysis of MCF-10A-Ras cells transfected as in (B) incubated in the presence of anisomycin for 36 hr. Cleaved caspase-3 (Clv. Casp-3) is a marker for apoptosis, and β-catenin was used as a loading control.

(G) Cell survival assay and western blot analysis of MCF-10A cells transfected and treated as in (F).

proposed in our study. Analyzing RNA-seq data from hundreds of tumors from patients with breast, colon, and lung cancer deposited in the TCGA database, we found that the *MKNK2* alternative splicing switch occurs in a significant portion of breast, lung, and colon tumors (Figures 1E–1G and S1) and is either weakly correlated or inversely correlated with expression of several splicing factors from the SR and hnRNP A/B families (Tables S1–S3). These results suggest that a combinatorial network of splicing factors (rather than a single splicing factor) controls *MKNK2* alternative splicing in tumors. Upregulation of oncogenic-splicing factors such as SRSF1 can be caused by several mechanisms such as gene amplification (Karni et al., 2007) or transcriptional activation (Das et al., 2012) and may lead to a change in *MKNK2* alternative splicing as has been shown previously (Karni et al., 2007).

To elucidate the role of Mnk2-splicing isoforms in cancer development, we examined the oncogenic activity of Mnk2-splicing isoforms in vitro and in vivo. Results of these experiments suggest that Mnk2a possesses a tumor-suppressive activity in vitro and in vivo, and this activity of Mnk2a is mediated by activation of the p38-MAPK pathway.

Interestingly, both Mnk2a and Mnk2b, but not the Mnk2aKD, phosphorylated eIF4E to a similar extent, suggesting that eIF4E phosphorylation cannot account for their different biological activity (Figures 4A, S3A, and S3C). In cancer cells, when alternative splicing results in reduced expression of Mnk2a and increased expression of Mnk2b or when it is manipulated artificially, as we have done here, there is still phosphorylation of eIF4E (Figures 3D and 4A), but 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 (Figures 3, 4, S5, and S6). However, we cannot rule out the possibility that Mnk2b can phosphorylate other substrates (yet to be found) that contribute to its oncogenic activity and, in this manner, acts as a pro-oncogenic factor. We further found that Mnk2 isoforms can differentially interact with p38α-MAPK in cells. In coimmunoprecipitation assays, Mnk2a binds p38α, whereas Mnk2b does not bind p38α efficiently, suggesting that this interaction might be important for p38α activation by Mnk2a (Figure 4B). The ability of Mnk2a to phosphorylate p38-MAPK was similar in strength or even stronger than MKK3 (Figure 4C), and Mnk2a can induce p38-MAPK phosphorylation in the absence of MKK3 and MKK6 (Figure 4D). These results combined with the results of an in vitro kinase assay (Figure 4E) suggest that Mnk2a is a direct p38α-MAPK activator kinase. To the best of our knowledge, Mnk2a is the first direct kinase, other than MKK3, MKK6, and MKK4, that can phosphorylate and activate p38-MAPK. Some scaffold proteins can enhance p38-MAPK autophosphorylation and induce its activation (De Nicola et al., 2013). However, this cannot explain our finding because we used a kinase-dead recombinant p38α and recombinant Mnk2a in the in vitro kinase assay (Figure 4E). We also found that Mnk2a can bind and phosphorylate both p38α and p38β, but not p38γ or p38δ, suggesting that Mnk2a has specificity toward these two substrates (Figures 4A, 4B, and S6F–S6H).

We further confirmed that the kinase activity of Mnk2a is required for activation of p38α and its target genes because both the Mnk2aKD or application of the Mnk1/Mnk2 kinase inhibitor CGP 57380 (Knauf et al., 2001; Chrestensen et al., 2007) inhibited these activities (Figures S6B and 6). Taken together, these results indicate that Mnk2 kinase activity is required for the activation of p38-MAPK and its downstream targets.

In all of our experimental systems, we demonstrated that whereas both p38 phosphorylation and eIF4E phosphorylation are enhanced by Mnk2a overexpression and reduced by its knockdown, only p38 phosphorylation correlates with the degree of apoptosis (Figure 3). Results from these gain- and loss-of-function experimental systems suggest that p38-MAPK, but not eIF4E, phosphorylation/activation determines the fate of these cells. Finally, in the breast cancer cell lines examined in this paper, there are lower levels of p38 (and of eIF4E) compared with two immortal breast cell lines (Figure S6), suggesting clinical relevance of these findings. The role of the tumor suppressor activity of p38α was recently demonstrated in hepatocellular carcinoma and colon cancer development. In both cases, tissue-specific knockout of p38α led to cancer development in vivo (Sakurai et al., 2013) (Wakeman et al., 2012). Moreover, a recent study showed that the gene encoding MKK3, a known p38-MAPK upstream kinase, is lost in some breast tumors and that MKK3 acts as a tumor suppressor in breast cancer (MacNeil et al., 2014). Because Mnk2a acts as another p38-MAPK kinase, it is reasonable to assume that it acts as a tumor suppressor in a similar manner.

The generation of Mnk2a mutants mutated in either the NLS or NES allowed us to examine if Mnk2a affects p38-MAPK cellular localization and if this effect mediates p38α activation. When cotransfected with HA-tagged or GFP-tagged p38-MAPK, Mnk2a colocalized with p38-MAPK both in the cytoplasm and nucleus (Figures 5A and S7C). However, transduction of MKK3$^{-/-}$/MKK6$^{-/-}$ MEFs where p38-MAPK is unphosphorylated (Figure 4D) with Mnk2a induced nuclear accumulation of p38-MAPK (Figure 5B). These results suggest that physiological expression levels of Mnk2a can induce translocation of the endogenous p38-MAPK into the nucleus. Both Mnk2a mutants can interact with p38α, including with its phosphorylated form, as was measured by coimmunoprecipitation (Figure S7E). However, only Mnk2a and the nuclear L/S mutant could activate both p38-MAPK target genes (Figure 6C) and induce apoptosis (Figure 6F), whereas the cytoplasmic mutant KKR could not activate either and even reduced apoptosis below the empty vector levels (Figures 6C and 6F). 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. Because Mnk2b does not bind p38α, we hypothesize that it does not act in a dominant-negative manner to Mnk2a. 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.

We manipulated *MKNK2* alternative splicing by antisense RNA oligonucleotides that mask either the Mnk2a or the Mnk2b splice sites (Figure 7A). Results from these experiments suggest that manipulation of the endogenous ratios of Mnk2a and Mnk2b affects the oncogenic potential of cells transformed by Ras and SRSF1. Moreover, these results suggest that *MKNK2* alternative splicing is a critical event in both Ras- and SRSF1-induced transformation, similar to other oncogenic alternative splicing events regulated by SRSF1 (Ben-Hur et al., 2013).

An alternative splicing switch to eliminate a tumor suppressor is fast and cost effective and serves as an additional level of regulation. Several examples of such regulation of important tumor suppressors already exist: *BIN1*, *MDM2*, *Caspase-9*, *BCL-2* family members, and others (Bae et al., 2000; Ge et al., 1999; Hossini et al., 2006; Kim et al., 2008; Srinivasula et al., 1999; Steinman et al., 2004). Many tumor suppressor genes are deleted from the genomes of cancer cells. Such an event would be unfavorable in the case of Mnk2 because absence of both isoforms would result in reduced eIF4E phosphorylation, and cells would be less oncogenic as in the case of Mnk1/Mnk2-DKO cells (Furic et al., 2010; Ueda et al., 2010). However, eliminating only Mnk2a and expressing 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 expect that the main mechanism to eliminate Mnk2a in tumors will be through modulation of alternative splicing rather than deletion of the *MKNK2* gene.

In conclusion, we have identified a mechanism in which Mnk2a interacts with, phosphorylates, and induces translocation of p38-MAPK into the nucleus, and thus induces transcription of its target genes, which results in increased apoptosis. 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 for elimination of a tumor suppressor (Mnk2a), which is a modulator of the p38-MAPK stress pathway, and for generating the pro-oncogenic isoform (Mnk2b).

EXPERIMENTAL PROCEDURES

Animal Care

All animal experiments were performed in accordance with the guidelines of the Hebrew University committee for the use of animals for research. Veterinary care was provided to all animals by the Hebrew University animal care facility staff in accord with AAALAC standard procedures and as approved by the Hebrew University Ethics committee.

In Vitro Kinase Assay

In vitro kinase assay was performed using recombinant Mnk2, eIF4E (Abcam), and kinase-dead p38α (Diskin et al., 2007) as described by Askari et al. (2007) and Avitzour et al. (2007). 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 dithiothreitol (DTT). Reactions were shaken for 1 hr at 30°C. Reactions were stopped by the addition of 50 μl of cold dialysis buffer (12.5 mM HEPES [pH 7.5], 100 mM KCL, 0.5 mM DTT, and 6.25% glycerol) followed by the addition of 30 μl 4× Laemmli buffer. A total of 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 visualize the recombinant proteins in the reaction.

Modulation of *MKNK2* Splicing by Antisense-RNA Oligos

Cy5-labeled 2′-O-methyl-modified RNA oligonucleotides were synthesized by Sigma-Aldrich. A total of 2.5 μM of each oligo was transfected using Lipofectamine 2000 according to the manufacturer's instructions. For determination of RNA levels, BEAS-2B, MCF-10A-Ras, or MCF-10A cells were harvested 48 hr after transfection, and levels of Mnk2a and Mnk2b were analyzed by RT-PCR. For biological assays (colony survival, soft agar, and apoptosis), cells were transfected as described and 24 hr after transfection were seeded for the indicated assay. Oligo sequences, cell lines, plasmids, shRNA, primer sequences, and additional experimental procedures used are described in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, eight figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.03.041.

AUTHOR CONTRIBUTIONS

A.M. and R.K. designed the experiments. A.M., A.S., A.O., M.M., V.B.-H., I.S., E.Z., R.G.-G., and D.M. performed experiments. J.B., C.L.A., K.T., T.F.Ø., R.R., R.J.D., I.L.-L., and B.D. contributed reagents and technical help. A.M. and R.K. analyzed the data and wrote the manuscript.

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