Splicing factor hnRNP A2 activates the Ras-MAPK-ERK pathway by controlling A-Raf splicing in hepatocellular carcinoma development

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ABSTRACT

In recent years, it has become clear that splicing factors play a direct role in cancer development. We showed previously that splicing factors SRSF1, SRSF6, and hnRNP A2/B1 are up-regulated in several cancers and can act as oncogenes when up-regulated. Here we examined the role of splicing factors hnRNP A1/A1b and hnRNP A2/B1 in hepatocellular carcinoma (HCC). We show that the splicing factors hnRNP A1 and hnRNP A2 are up-regulated in HCC tumors derived from inflammation-induced liver cancer mouse model. Overexpression of hnRNP A1 or hnRNP A2, but not the splicing isoform hnRNP B1, induced tumor formation of immortalized liver progenitor cells, while knockdown of these proteins inhibited anchorage-independent growth and tumor growth of human liver cancer cell lines. In addition, we found that cells overexpressing hnRNP A2 showed constitutive activation of the Ras-MAPK-ERK pathway. In contrast, knockdown of hnRNP A2 inhibited the Ras-MAPK-ERK pathway and prevented ERK1/2 activation by EGF. Moreover, we found that hnRNP A2 regulates the splicing of *A-Raf*, reducing the production of a short dominant-negative isoform of A-Raf and elevating the full-length *A-Raf* transcript. Taken together, our data suggest that hnRNP A2 up-regulation in HCC induces an alternative splicing switch that down-regulates a dominant-negative isoform of A-Raf, leading to activation of the Raf-MEK-ERK pathway and cellular transformation.

Keywords: alternative splicing; hnRNP A2/B1; RNA processing; A-Raf; MAPK; liver cancer

INTRODUCTION

The process of alternative splicing is widely misregulated in cancer, and many tumors express new splicing isoforms that are either absent or expressed at low levels in the corresponding normal tissue (Roy et al. 2005; Xi et al. 2008; Venables et al. 2009; David and Manley 2010). 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 (Venables 2004; Srebrow and Kornblihtt 2006; Kim et al. 2008). Moreover, mutations in several components of the spliceosome were recently discovered in several cancers and are predicted to be driver mutations, providing further confirmation that splicing factors are indeed important players in cancer development (Papaemmanuil et al. 2011; Quesada et al. 2012). However, there is only limited information regarding the causal/functional role of alternative splicing regulators in cancer development and progression (Karni et al. 2007; Jia et al. 2010; Golan-Gerstl et al. 2011; Lefave et al. 2011; Anczukow et al. 2012; Cohen-Eliav et al. 2013).

hnRNP proteins are abundant RNA-binding proteins expressed in most human tissues (Hanamura et al. 1998; Cooper et al. 2009). The hnRNP A/B family is a subset of hnRNP proteins with closely related sequences and a conserved modular structure (Dreyfuss et al. 2002; He and Smith 2009). The structure of the hnRNP A/B proteins is composed of two major domains: two RNA-recognition motifs (RRMs) that bind specific RNA sequences, and a glycine-

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rich domain (G domain) (He and Smith 2009). *HNRNP A1* encodes for hnRNP A1 and its splicing variant hnRNP A1b, which contains an additional 52 amino acids in the C-terminal glycine-rich region (Buvoli et al. 1990; Blanchette and Chabot 1997; He and Smith 2009). *HNRNP A2/B1* encodes for hnRNP A2 and its splicing variant hnRNP B1, which contains an additional 12 amino acids near the N terminus (Burd et al. 1989; He and Smith 2009). An unsolved question is the biochemical and biological differences between hnRNP A/B protein family members and their splicing isoforms. To date, their splicing activities, both in vitro and in knockdown or transient transfection assays, showed similar effects on several substrates (Burd et al. 1989; Dreyfuss et al. 2002; Patry et al. 2003). Thus, it is not clear to what extent there is redundancy in their splicing targets and biological or oncogenic activities. Previous studies found overexpression of hnRNP A1 and hnRNP A2/B1 in lung and breast cancers (Fielding et al. 1999; Zhou et al. 2001b). Knockdown of hnRNP A1 and A2/B1 in breast cancer cells induced apoptosis that was specific for cancer cells (Patry et al. 2003). We reported recently the first direct evidence that hnRNP A2/B1 plays an important role as a driver oncogene in glioblastoma development (Golan-Gerstl et al. 2011). Recent studies found that hnRNP A1 and hnRNP A2/B1 modulate alternative splicing of the glycolytic PKM2 enzyme in cancer cells, suggesting a possible role for hnRNP A1 and hnRNP A2/B1 in the regulation of tumor metabolism (Clower et al. 2010; David et al. 2010).

Hepatocellular carcinoma (HCC) is the most common primary hepatic malignancy and the third most common cause of cancer-related death worldwide. Incidence remains highest and is steadily increasing across the developed world (Shiraha et al. 2013). The connection between chronic inflammation and liver carcinogenesis is well established (Pikarsky et al. 2004; Finkin and Pikarsky 2011; He and Karin 2011). A recent study showed that in HCC, hnRNP A1 overexpression enhances invasiveness (Zhou et al. 2013). Other splicing factors such as SRSF1 have also been shown to regulate alternative splicing of important HCC tumor suppressors and oncogenes (Munoz et al. 2012). Here we investigated the expression and roles of hnRNP A1, hnRNP A2, and their corresponding isoforms, hnRNP A1b and hnRNP B1, respectively, in HCC development. We found that hnRNP A1 and A2 are up-regulated in a mouse model of inflammation-induced HCC (Pikarsky et al. 2004). Moreover, transduction of immortal progenitor hepatocytes with hnRNP A1 or A2, but not its isoform B1, induced tumorigenesis, while hnRNP A1 or A2 knockdown in human HCC cells inhibited their transformation and tumorigenesis, indicating that hnRNP A1 and A2 are putative oncogenes in HCC development. Furthermore, we found that hnRNP A2 up-regulation caused constitutive activation of the RAS-Raf-MAPK-ERK pathway through regulation of A-Raf alternative splicing. Finally, activation of the RAS-Raf-MAPK-ERK pathway by hnRNP A2 renders HCC cells resistant to a MEK1 pharmacological inhibitor, suggesting that hnRNP A2 up-regulation might serve as a drug-resistance mechanism.

RESULTS

hnRNP A1/A1b and hnRNP A2/B1 proteins are up-regulated in inflammation-induced mouse HCCs

To examine if hnRNP A1/A1b or hnRNP A2/B1 plays a role in liver cancer development, we compared normal and tumor liver tissue samples from an inflammation-induced liver cancer $Mdr2^{-/-}$ mouse model. The mouse *Mdr2* gene encodes for the Abc4 protein. Knockout of this gene leads to chronic hepatic inflammatory disease (Pikarsky et al. 2004). When the mice are 6 to 9 mo of age, preneoplastic lesions develop in the liver, eventually progressing to metastatic liver cancer in the terminal phase. During progression into malignant lesions, $Mdr2^{-/-}$ tumor cells accumulate mutations and other genetic abrogations and are thus genetically variable. The $Mdr2^{-/-}$ mice therefore provide a tumor progression model of value for the study of hepatic carcinogenesis (Pikarsky et al. 2004). We found higher levels of hnRNP A1 mRNA and protein in most liver tumor samples and found higher levels of hnRNP A2 mRNA and protein in a large portion of liver tumors compared with normal mouse liver tissue from the $Mdr2^{-/-}$ mice (Fig. 1A–E). hnRNP B1 and hnRNP A1b were hardly detected in these samples. mRNA levels of hnRNP H, a close family member of the hnRNP A/B proteins, were elevated in some tumors (Supplemental Fig. S1A). Interestingly, most of the tumors that overexpress hnRNP H did not overexpress hnRNP A1 or A2/B1 (Supplemental Fig. S1B–D).

hnRNP A1/A1b and A2, but not hnRNP B1, transform progenitor liver cells

In order to examine the oncogenic activity of the different hnRNP A1/A2 isoforms, we expressed different hnRNP A/B isoforms in $TP53^{-/-}$ mouse embryonic progenitor hepatocytes overexpressing c-myc (PHM-1 cells) (Fig. 2A,B; Zender et al. 2006; Shimoni-Sebag et al. 2013). We then examined the growth rate of cells expressing the different isoforms. We found that while hnRNP A1 had a small proliferative effect, none of the other hnRNP A1/A2 isoforms changed significantly the proliferation rate of the cells (Fig. 2C). We injected nude/nude mice subcutaneously with pools of PHM-1 cells transduced with the different hnRNP A1/A2 isoforms. Mice injected with PHM-1 cells expressing either hnRNP A1 or hnRNP A2, and to a lesser degree hnRNP A1b, formed tumors, whereas mice injected with PHM-1 cells expressing hnRNP B1 did not form tumors (Fig. 2D,E).

hnRNP A2/B1 and hnRNP A1/A1b are required for HCC tumor maintenance

To better understand the involvement of the different isoforms in liver cancer and to examine if hnRNP A1/A1b or hnRNP A2/B1 is required for maintenance of the transformed

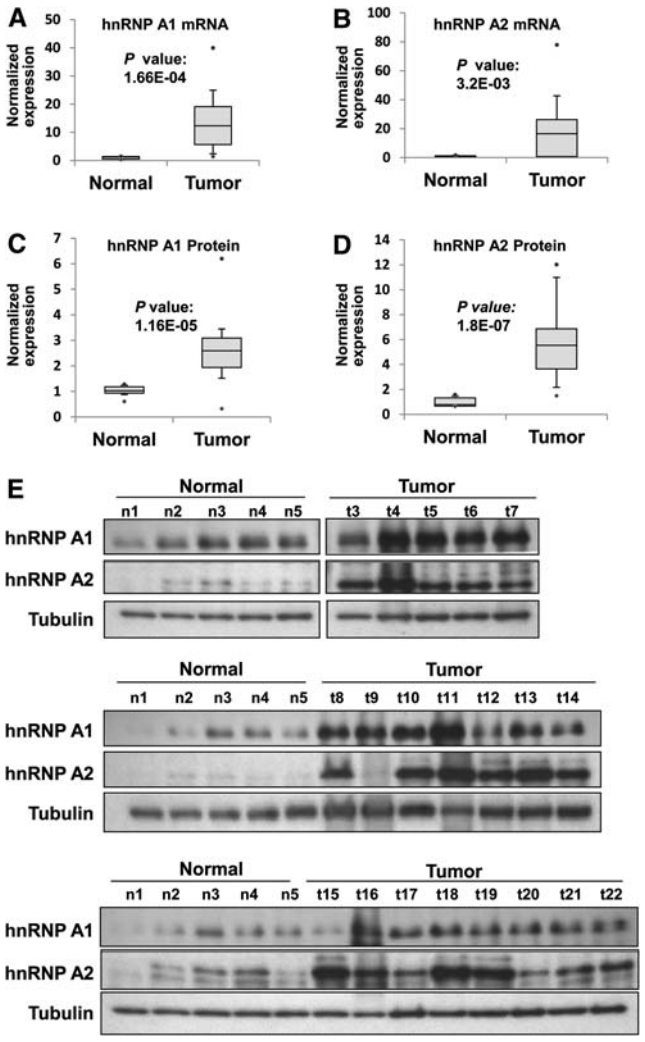

FIGURE 1. Elevated hnRNP A2 and hnRNP A1 expression in mouse liver tumors. (*A*,*B*) Box plot representation of qRT-PCR analysis of hnRNP A1 (*A*) and hnRNP A2 (*B*) RNA levels in $Mdr2^{-/-}$ mouse liver tumors ($n = 22$) and normal mouse liver tissue ($n = 5$). All samples were normalized to GAPDH mRNA levels, and the average expression in normal liver tissue was arbitrarily set at one. Medians are represented by solid black lines. *Top* and *bottom* box edges represent the third and first quartile. Whiskers indicate 90 and 10 percentile; asterisks, minimum and maximum points. (*C*,*D*) Box plot representation of protein levels for hnRNP A1 (*C*) and hnRNP A2 (*D*) in $Mdr2^{-/-}$ mouse liver tumors and normal liver tissue. All samples were normalized to tubulin protein levels and to the average expression in normal liver tissue, which was arbitrarily set at one. Medians are represented by solid black lines. *Top* and *bottom* box edges represent the third and first quartile. Whiskers indicate 90 and 10 percentile; asterisks, minimum and maximum points. (*E*) Western blot analysis of hnRNP A1 and hnRNP A2 protein levels in $Mdr2^{-/-}$ liver tumors (t) and normal (n) mouse liver tissue. Tubulin was used as loading control. Numbers indicate sample number.

phenotype, we knocked down either hnRNP A2/B1 or hnRNP A1/A1b expression in the human HCC cell line HuH7 (Fig. 3A). We found that cells knocked down for either hnRNP A1/A1b or hnRNP A2/B1 expression showed a reduced ability to form colonies in soft agar (Fig. 3B,C). Reduced anchorage-independent growth in soft agar can be the result of a combination of reduced invasion, sensitivity to apoptosis, and reduced proliferation. Detachment of most epithelial cells from the extracellular matrix induces programmed cell death called anoikis (Taddei et al. 2012). Unlike growth on a plastic cell culture dish, soft agar is a semi-liquid matrix and nontransformed cells are unable to grow, proliferate, and form colonies in the absence of a solid adhesive surface. Moreover, the semiliquid conditions may elicit anoikis of nontransformed cells as they cannot attach to a solid surface. To test whether hnRNP A1 and A2 play redundant or additive roles in transformation, we transduced HuH7 cells expressing hnRNP A1 shRNA with retroviruses encoding hnRNP A2 shRNA (Supplemental Fig. S2A). HuH7 cells with A1/A2 double knockdown formed fewer colonies in soft agar, suggesting that hnRNP A1 and A2 have additive and nonredundant roles in transformation (Supplemental Fig. S2B).

Moreover, knockdown of hnRNP A2 did not affect cell cycle distribution while there was a small increase in the number of cells in G1 in hnRNP A1 knockdown cells (Supplemental Fig. S3A–D). We did not observe a significant effect of most hnRNP A1/A2 isoforms on proliferation or cell cycle under normal growth conditions (10% serum) except for hnRNP A1, which had a small pro-proliferative activity (Fig. 2C; Supplemental Fig. S3A–D). Tumor cells in vivo are exposed to harsh conditions, including low concentrations of nutrients and growth factors. Thus, we examined if hnRNP A1/A1b and/or hnRNP A2/B1 might affect cell growth under low-serum conditions. To explore this possibility, we grew cells under low-serum conditions (0.1%) and measured cell number. Knockdown of either hnRNP A1/A1b or hnRNP A2/B1 significantly reduced the growth rate of HuH7 cells grown under low-serum conditions (Fig. 3D). Notably, we did not detect enhanced apoptosis under these low-serum conditions, suggesting that hnRNP A1 and A2 enable growth under low-nutrient conditions but do not affect cell death under these conditions (Supplemental Fig. S3E,F). We next examined if hnRNP A1/A1b or hnRNP A2/B1 expression is required to maintain transformation in vivo. We injected NOD-SCID mice with HuH7 cells expressing shRNAs against either hnRNP A1/A1b or hnRNP A2/B1. We found that mice injected with cells knocked down for either hnRNP A1/A1b or hnRNP A2/B1 formed fewer and smaller tumors compared with mice injected with empty vector (Fig. 3E). These results suggest that hnRNP A1 and hnRNP A2 are required for HCC tumor maintenance.

hnRNP A2 activates the Ras-MAPK-ERK pathway

In order to understand the mechanism by which these splicing factors drive transformation, we investigated the activation of several signal transduction pathways that are known to be involved in cancer development. The Ras-Raf-MEK-extracellular signal–regulated kinase (ERK) pathway is frequently activated in cancer, often due to activating mutations in Ras (Downward 2003; Karnoub and Weinberg 2008),

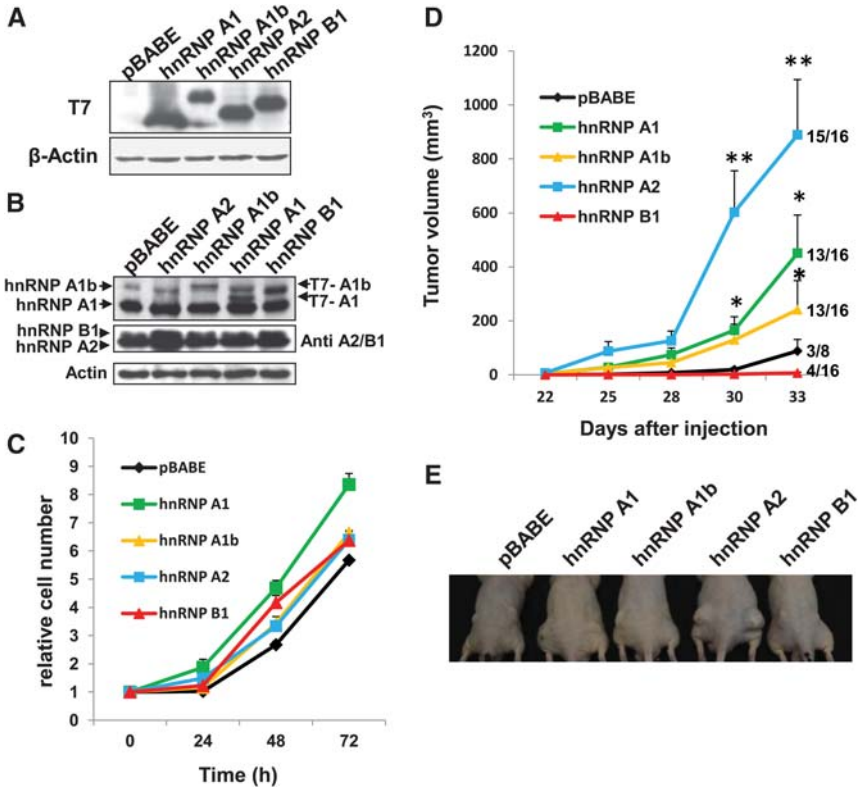

FIGURE 2. hnRNP A1/A1b and A2, but not B1, can transform PHM-1 cells in vivo. (*A*,*B*) PHM-1 cells transduced with retroviruses encoding empty vector (pBABE) or hnRNP A1, A1b, A2, and B1 were analyzed by Western blotting for hnRNP A1/A1b, hnRNP A2/B1, and T7-Tag protein to detect both endogenous and exogenous expression. β-Actin was used as a loading control. (*C*) Growth curves of cells described in *A* were measured by methylene blue staining. Error bars, SD ($n = 6$). (*D*) Cells described in *A* were injected (3×10^6 cells/site) subcutaneously near both rear flanks of nude/nude mice, and tumor volume was measured twice weekly (mean ± SEM; $n = 16$ or 8). (*) $P < 0.05$, (**) $P < 0.001$ (two-tailed *t*-test). (*E*) Representative mice described in *D* are shown.

B-Raf (Niault and Baccarini 2010; Pratilas and Solit 2010), MEK (Estep et al. 2007; Marks et al. 2008; Nikolaev et al. 2012), or amplification of several of these components (Little et al. 2011). We found that PHM-1 cells overexpressing hnRNP A2, but not its splicing isoform hnRNP B1, showed increased activation of the Ras-MAPK-ERK pathway, as seen by high levels of phospho-ERK1/2 and phospho-MEK1/2 even under low-serum conditions and after stimulation of the pathway by EGF (Fig. 4A; Supplemental Fig. S4A). In contrast, knockdown of hnRNP A2 in HuH7 cells inhibited the Ras-MAPK-ERK pathway and inhibited MEK1 and ERK1/2 activation by EGF (Fig. 4B; Supplemental Fig. S4B). Levels of phospho-ERK1/2 and phospho-MEK1 in hnRNP A1b overexpressing cells were elevated twofold compared with cells expressing the empty vector, while hnRNP A1 did not activate the MAPK-ERK pathway (Fig. 4A; Supplemental Fig. S4B). The differential ability of the isoforms to activate this signal transduction pathway can support a mechanism by which hnRNP A2 can initiate transformation in vivo while hnRNP B1 cannot.

hnRNP A2 regulates the splicing of *A-Raf* reducing the production of a dominant-negative A-Raf isoform

Elevated A-Raf expression levels have been observed in a number of malignancies, including astrocytomas (Hagemann et al. 2009), pancreatic ductal carcinoma (Kisanuki et al. 2005), angioimmunoblastic lymphadenopathies (Mark et al. 1986), head and neck squamous cell carcinomas, and colon carcinomas (Rauch et al. 2004, 2010). A recent study reported that *A-Raf* undergoes alternative splicing generating a short isoform which has a dominant-negative effect on A-Raf (Fig. 5A; Rauch et al. 2011). This short isoform, which contains the Ras binding domain and lacks the kinase domain, is predicted to bind to Ras but cannot transmit the signal without the kinase domain. Recently it has been shown that this splicing product is regulated by hnRNP H, a close family member of the hnRNP A/B proteins (Rauch et al. 2011). We examined if the differences we observed in the Ras-MAPK-ERK pathway in cells with hnRNP A2 overexpression or knockdown could be due to altered splicing of *A-Raf*. We found that in PHM-1 cells expressing hnRNP A2, the mRNA of the short isoform of A-Raf is less abundant while levels of full-length (FL) A-Raf were elevated compared with cells with empty vector (Fig. 5B–D). In contrast, in cells expressing hnRNP B1, the mRNA levels of the short isoform were higher than FL mRNA levels (Fig. 5B,C). Since FL-A-Raf levels were significantly higher in cells overexpressing hnRNP A2, we measured the total level of A-Raf transcripts. Total A-Raf transcript levels were significantly higher (Supplemental Fig. S5), suggesting that other mechanisms, such as enhanced stabilization or transcription of A-Raf, might be affected by hnRNP A2 overexpression. In accordance with higher levels of FL-A-Raf mRNA, we also found elevated FL-A-Raf protein in cells overexpressing hnRNP A2 (Fig. 5D). Moreover, knockdown of hnRNP A2 in HuH7 cells caused the reciprocal change and led to elevation of mRNA levels of the short isoform and down-regulation of FL-A-Raf levels (Fig. 5E). To examine if changes in *A-Raf* splicing can be detected at the protein level, we performed Western blotting on lysates from HuH7 cells transduced with retroviruses encoding shRNAs against hnRNP A1 or A2 using a specific antibody that can detect the A-Raf short isoform (Rauch et al. 2011). A-Raf short protein was elevated, while the FL-A-Raf protein was reduced in HuH7 cells expressing shRNAs

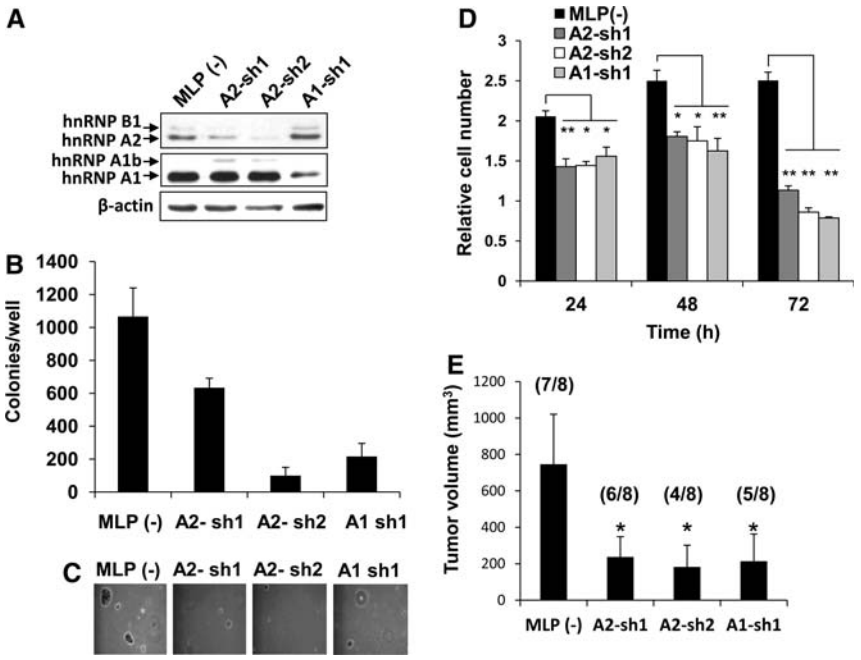

FIGURE 3. hnRNP A2/B1 and hnRNP A1/A1b are required for HCC transformation. (*A*) HuH7 cells were transduced with retroviruses encoding shRNAs against hnRNP A1/A1b, hnRNP A2/B1, or empty vector without the shRNA (MLP). Cells were analyzed by Western blotting for hnRNP A1/A1b and hnRNP A2/B1 protein expression. β-Actin was used as a loading control. (*B*) Cells described in *A* were seeded into soft agar in duplicate, and colonies were grown for 14 d. Colonies in 10 fields of each well were counted (mean ± SEM; $n = 2$). (*C*) Representative fields of colonies in soft agar described in *B*. (*D*) Cells described in *A* were starved (0.1% serum), and survival of cells was measured by methylene blue staining (mean ± SEM; $n = 6$). (*) $P < 0.05$, (**) $P < 0.001$ (two-tailed *t*-test). (*E*) Cells described in *A* were injected (2×10^6 cells/site) subcutaneously near both rear flanks of SCID mice, and a graph depicting tumor growth 29 d after injection is shown. The number of tumors formed per number of injections is shown in parentheses (mean ± SEM; $n = 8$). (*) $P < 0.05$ (one-tailed *t*-test).

against hnRNP A2 but not hnRNP A1 (Fig. 5F). Taken together these results suggest that A-Raf alternative splicing is regulated by hnRNP A2 and can be detected at both the mRNA and protein level. To further elucidate if hnRNP A2 levels correlate with *A-Raf* splicing in the mouse HCC tumor mouse model, we measured hnRNP A2 mRNA levels and A-Raf isoform levels in mouse liver normal and tumor tissues. We found that the ratio of FL-A-Raf mRNA to short-A-Raf mRNA was higher in most tumors compared to normal mouse livers (Supplemental Fig. S6A–C). Moreover, there was a positive correlation between hnRNP A2 expression and elevated ratios of FL- to short-A-Raf levels, while no such correlation existed between hnRNP H expression and ratios of A-Raf splicing isoforms (Supplemental Fig. S6D,E). These results suggest that hnRNP A2 up-regulation in tumors can induce an alternative splicing switch in *A-Raf*.

Inducible expression hnRNP A2 but not B1 can rescue anchorage independent growth and A-Raf splicing after hnRNP A2 knockdown

In order to further understand the roles of each hnRNP A1/A2 isoform in cellular transformation and *A-Raf* splicing, we used a HeLa tet-on inducible system to express each hnRNP A1/A2 isoform on the background of hnRNP A1 or A2 knockdown (Supplemental Fig. S7). This system enables expression of only one isoform in the absence of the other (Supplemental Fig. S7A,B). The shRNAs do not inhibit the expression of the inducible isoforms as they were designed against the 3′ UTR region of the isoform transcripts. Expression of hnRNP A1, but not A1b, could rescue colony formation in soft agar of cells with hnRNP A1/A1b knockdown (Supplemental Fig. S7C). Expression of hnRNP A2, but not B1, could rescue colony formation in soft agar of cells with hnRNP A2/B1 knockdown (Supplemental Fig. S7D). hnRNP A1 expression elevated FL-A-Raf, but did not change the ratio of A-Raf isoforms, and A1b elevated the short A-Raf isoform, suggesting that it might act antagonistically to A1 (Supplemental Fig. S7E,G). hnRNP A2 expression elevated FL-A-Raf and lowered short-A-Raf reciprocally, while hnRNP B1 did not rescue *A-Raf* splicing in cells with A2/B1 knockdown (Supplemental Fig. S7F,H). It should be noted that expression of hnRNP A1, A1b, and B1 was lower than hnRNP A2, and thus, it is possible that the ability of hnRNP A1 or B1 to rescue colony formation in soft agar and *A-Raf* splicing would be greater if equal expression was achieved. Since A1b expression was similar to that of A1, this isoform is probably unable to induce transformation or change *A-Raf* splicing as hnRNP A1.

HCC cells with hnRNP A1/A2 overexpression are resistant to apoptosis induced by MEK inhibition

To clarify the importance of the Ras-MAPK-ERK pathway for the oncogenic potential of HCC cells overexpressing hnRNP A1/A2 proteins, we examined the relationship between hnRNP A1/A2 expression level, activation of the Raf-MEK-ERK pathway, and sensitivity to a MEK1 inhibitor. Human and mouse immortal (Hc3716-hTERT, PHM-1) cells and HCC cells (BNL-1ME, Hep3B, and FLC-4) showed similar levels of hnRNP A1/A1b and hnRNP A2/B1expression, while HuH7 and HepG2 HCC cells showed elevated levels of hnRNP A1/A1b and hnRNP A2/B1 proteins (Fig. 6A). HuH7 and HepG2 cells also showed elevated levels of FL-A-Raf protein and strong activation of the Raf-MEK-ERK pathway as measured by MEK1/2 and ERK1/2 phosphorylation (Fig. 6A). We treated the seven human and mouse cell lines with the MEK inhibitor U0126 and measured cell death

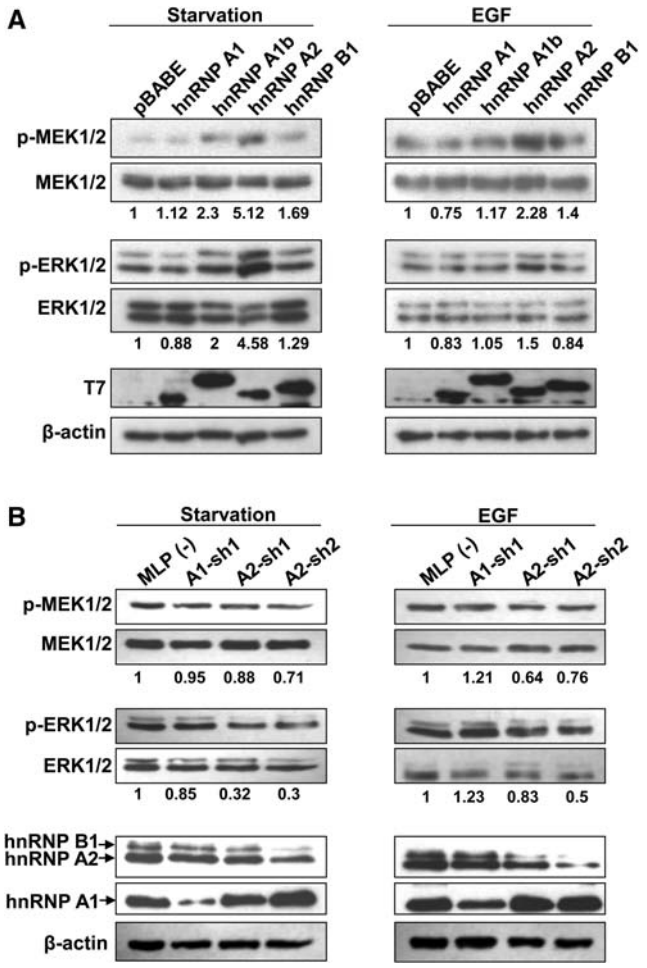

FIGURE 4. hnRNP A2 activates the Ras-MAPK-ERK pathway. (*A*,*B*) PHM-1 (*A*) and HuH7 (*B*) cells transduced with the indicated retroviruses were seeded in six-well plates (1×10^5 cells/well for PHM-1 cells, 2×10^5 cells/well for HuH7 cells). Cells were serum-starved for 24 h (0.1% FCS) and stimulated with EGF (10 nM) for 30 min. Cells were lysed and immunoblotted for expression of phosphorylated and total MEK1/2, ERK1/2 and T7-Tag (*A*) or hnRNP A2/B1 and hnRNP A1/A1b (*B*). β-Actin was used as a loading control. Fold increase of ERK1/2 or MEK1/2 was normalized (phosphorylated/total protein levels) to that of untreated empty vector, which was arbitrarily set at one. Shown here is one representative experiment out of three repeats (see also Supplemental Fig. S4).

induced by MEK inhibition. HuH7 and HepG2 cells, which express higher levels of hnRNP A1 and A2, were resistant to apoptosis induced by the MEK inhibitor U0126. PHM-1, BNL-1ME, Hc3716-hTERT, Hep3B, and FLC-4 cells, which did not have lower levels of hnRNP A1/A2, were more sensitive to MEK inhibitor–induced apoptosis (Fig. 6B,C). Staining for cleaved caspase-3 confirmed that cell death was due to apoptosis (Fig. 6C). These results suggest that HCC tumors that overexpress hnRNP A1 and A2 might be more resistant to Raf and MEK inhibitors, and thus, hnRNP A1 and A2 levels might serve as a biomarker for resistance to such drugs. To examine if hnRNP A2 mediates the resistance of HuH7 to MEK inhibition, we treated HuH7 cells with or without hnRNP A2 knockdown with the MEK1 inhibitor U0126. We found that cells with hnRNP A2 knockdown had a modest increase in apoptosis and a significant decrease in total cell number upon treatment with the MEK1 inhibitor U0126 (Fig. 6D,E). These results suggest that hnRNP A2 up-regulation in HCC cancer cells contributes to resistance to MEK1 inhibition.

DISCUSSION

A growing body of evidence suggests that alternative splicing factors play a major role in cancer development and progression (Karni et al. 2007; Golan-Gerstl et al. 2011; Quesada et al. 2012; Cohen-Eliav et al. 2013). Moreover, genome-wide deep sequencing studies conducted recently on several types of cancer or premalignant diseases discovered mutations in splicing factors and suggest that these mutations are driver mutations (Papaemmanuil et al. 2011; Imielinski et al. 2012; Quesada et al. 2012). Furthermore, previous studies from our laboratory and others demonstrated functional oncogenic roles for several splicing factors, including hnRNP A2/B1 (Karni et al. 2007; Golan-Gerstl et al. 2011; Lefave et al. 2011; Cohen-Eliav et al. 2013). Overexpression of hnRNP A1/A2 proteins has been reported in several cancers (Zhou et al. 2001a, 2013; Ushigome et al. 2005; Li et al. 2009; Ma et al. 2009; Boukakis et al. 2010; Wang et al. 2011). In HCC, the splicing factor SRSF1 has been shown to regulate splicing of tumor suppressors such as KLF-6 (Munoz et al. 2012). Splicing factor hnRNP A1 was shown to enhance invasiveness and to correlate with metastasis and poor survival in HCC patients (Zhou et al. 2013).

In this study, we examined the oncogenic potential of four isoforms of the hnRNP A1/A2 proteins (hnRNP A1, A1b, A2, and B1). The differences or redundancies between these isoforms in terms of splicing activity, oncogenic activities, or biological functions are currently unknown. We found that hnRNP A1 and hnRNP A2 are up-regulated in an inflammation-induced HCC mouse model at both the RNA and protein levels (Fig. 1). hnRNP A1 and A2 up-regulation was observed in other types of cancer due to gene amplification (Golan-Gerstl et al. 2011) or transcriptional activation by c-Myc (David et al. 2010). c-Myc is frequently up-regulated in HCC and might be the cause of hnRNP A1 and A2 up-regulation (Tiniakos et al. 1989; Chan et al. 2004). Moreover, transduction of immortal liver progenitors (PHM-1 cells) (Zender et al. 2006) with hnRNP A1 and A2 caused cellular transformation, and these cells were tumorigenic in mice (Fig. 2). Interestingly, hnRNP A2 was more oncogenic than hnRNP A1, but its splicing variant, hnRNP B1 was inactive as an oncogene and could not induce transformation although it is only 36 bases longer (Fig. 2; He and Smith 2009). To examine if hnRNP A1 and A2 are required for tumor maintenance in addition to their oncogenic role in cancer initiation, we knocked down hnRNP A1 and A2 in the human HCC cell line HuH7 (Nakabayashi et al. 1982). We found that knockdown of both genes inhibited colony formation in soft

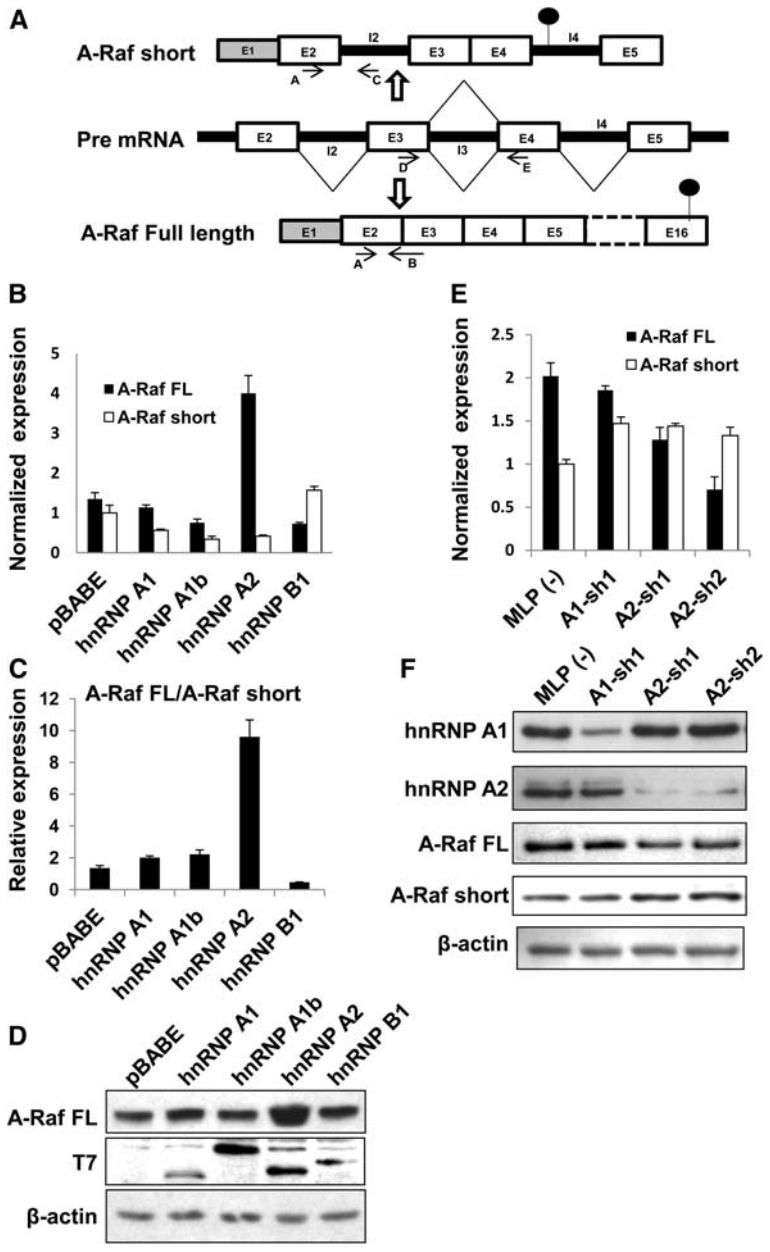

FIGURE 5. hnRNP A2/B1 regulates *A-Raf* alternative splicing. (*A*) A diagram showing intronic and exonic regions of *A-Raf* regulated by alternative splicing generating the dominant-negative A-Raf short isoform or the FL transcript. Black lines represent introns, empty boxes represent coding exons, gray boxes represent 5′ UTR, and black circles represent stop codons. Arrows represent primer positions. Primer pair A–B was used to detect A-Raf FL isoform. Primer pair A–C was used to detect A-Raf short isoform. Primer pair D–E was used to detect total A-Raf transcripts (see also Supplemental Fig. S5). (*B*,*C*) qRT-PCR analysis of A-Raf mRNA isoform expression (*B*) or the expression ratio of A-Raf FL/A-Raf short (*C*) in PHM-1 cells transduced with the indicated retroviruses. All samples were normalized to GAPDH mRNA levels and to the expression of the A-Raf short isoform in control (empty vector, pBABE) cells (*B*) or to the A-Raf FL/A-Raf short ratio in control (pBABE) cells (*C*), which was arbitrarily set at one (mean ± SEM; $n = 3$). (*D*) Immunoblot analysis of A-Raf FL and T7-TAG in cells described in *B* and *C*; β-actin was used as loading control. (*E*) qRT-PCR analysis of A-Raf isoforms in HuH7 cells transduced with retroviruses encoding for specific shRNAs against either hnRNP A1 or hnRNP A2. All samples were normalized to β-actin mRNA levels and to the expression of the A-Raf short isoform in control (empty vector without shRNA, MLP) cells, which was arbitrarily set at one (mean ± SEM; $n = 3$). (*F*) Total protein from cells described in *E* was extracted, and the expression levels of hnRNP A1/A1b, hnRNP A2/B1, A-Raf FL, and A-Raf short were assessed by Western blotting. β-Actin was used as a loading control.

agar (Fig. 3B,C) and tumorigenesis (Fig. 3E). The tumorigenic effects of hnRNP A1 and A2 overexpression (Fig. 2) suggest that they act as proto-oncogenes in HCC development, and the knockdown experiments (Fig. 3) suggest that they are required for tumor maintenance.

Interestingly, hnRNP A2 knockdown or overexpression did not significantly affect cell proliferation under normal growth conditions, while expression of hnRNP A1 had a small positive effect on proliferation (Fig. 2C; Supplemental Fig. S3). To examine if hnRNP A1/A2 knockdown sensitizes HCC cells to stress conditions, we starved HuH7 cells transduced with shRNAs against hnRNP A1 and A2 and measured cell proliferation. Indeed, hnRNP A1 and A2 knockdown decreased HuH7 cells proliferation under serum starvation conditions (Fig. 3D), suggesting that their oncogenic effect might rely on characteristics of transformation such as growth factor–independent proliferation, motility, invasion, and others (Hanahan and Weinberg 2011). Activation of the Ras-Raf-MAPK-ERK signaling pathway is one of the hallmarks of cancer cells, including many HCC tumors. Ras-Raf-MAPK-ERK activation can occur due to Ras or Raf mutations, inactivation of Ras GTPases, or activation of upstream growth factor receptors (Challen et al. 1992; Hopfner et al. 2004; Calvisi et al. 2006, 2011; Colombino et al. 2012). We examined if hnRNP A1, A2, or their isoforms affect the activity of this pathway. We found that in correlation with its oncogenic potential, hnRNP A2 was the strongest activator of the Ras-MAPK-ERK pathway as measured by MEK1 and ERK phosphorylation, while its isoform hnRNP B1 did not activate this pathway at all (Fig. 4A). Knockdown of hnRNP A2 prevented MEK1 and ERK1/2 activation by EGF, suggesting that hnRNP A2 is required for activation of this pathway in HCC cells (Fig. 4B). Notably, hnRNP A1b seems to be a weak activator of the Ras-MAPK-ERK pathway, while hnRNP A1 did not affect the MEK-ERK pathway (Fig. 4). Recent studies showed that upon c-Myc up-regulation, A-Raf is alternatively spliced to reduce a dominant-negative short isoform

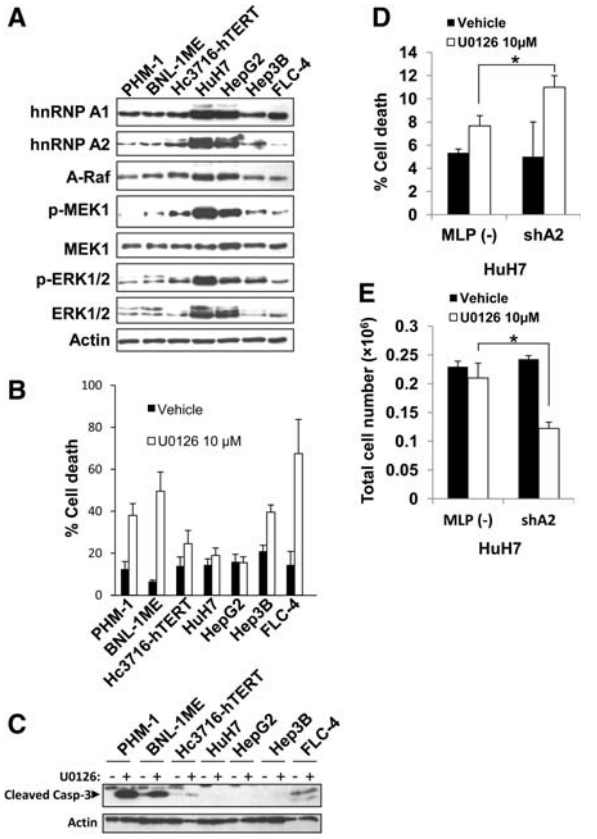

FIGURE 6. Sensitivity of HCC cells to MEK inhibitor–induced apoptosis correlates with hnRNP A1 and A2 levels. (*A*) Cells were lysed, and protein levels of hnRNP A2/B1, hnRNP A1/A1b, A-Raf FL and total and phosphorylated MEK1/2 and ERK1/2 were examined using Western blot. β-Actin was used as loading control. (*B*) HCC cell lines were treated either with vehicle or MEK inhibitor U0126 (10 μM) for 24 h and subjected to trypan blue exclusion assay. (*C*) Cells described in *A* and *B* were analyzed by Western blot for cleaved caspase-3, a marker for apoptosis. β-Actin served as loading control. (*D*) HuH7 cells transduced with the indicated retroviruses were seeded (120×10^4 cells/well in a six-well plate in triplicates) and treated with either vehicle or MEK inhibitor U0126 (10 μM) for 48 h and subjected to trypan blue exclusion assay. (*) Increased apoptosis after U0126 treatment in hnRNP A2 knockdown compared with MLP. $P = 0.053$. (*E*) Cells described in *D* were counted (total cell number) by a BioRad cell counter. (*) Reduced cell number after U0126 treatment in hnRNP A2 knockdown compared with MLP. $P = 0.033$. Experiments described in *D* and *E* were repeated at least twice.

and up-regulate the transcript encoding active A-Raf (Rauch et al. 2010, 2011). We examined if hnRNP A1, A2, or their splicing isoforms affect *A-Raf* alternative splicing, and we found that hnRNP A1, A1b, and A2 down-regulated the dominant-negative short A-Raf isoform, while hnRNP B1 did not (Fig. 5). hnRNP A2 overexpression induced elevated levels of FL-A-Raf beyond a reciprocal change in alternative splicing, suggesting that it might also affect mRNA stability or transcription of A-Raf transcripts (Fig. 5B; Supplemental Fig. S5). These results suggest that hnRNP A2 activates the Ras-Raf-MEK-ERK pathway by its effect on *A-Raf* splicing downstream from Ras (Fig. 5; Supplemental Fig. S7).

To further pinpoint the roles of each hnRNP A1/A2 isoform in cellular transformation and *A-Raf* splicing, we used a HeLa tet-on inducible system to express each hnRNP A1/A2 isoform on the background of hnRNP A1 or A2 knockdown. This system enables expression of one isoform while the other is absent (Supplemental Fig. S7). Results from the inducible system show that hnRNP A2, but not B1, expression can rescue colony formation in soft agar and A-Raf splicing (Supplemental Fig. S7). hnRNP A1 could partially rescue colony formation but not *A-Raf* splicing. Expression of hnRNP A1 and A1b was lower than hnRNP A2, and thus, it is possible that the ability of hnRNP A1 to rescue colony formation and *A-Raf* splicing is greater if equal expression was achieved.

To examine if activation of the Raf-MEK-ERK pathway plays a role in the oncogenic activity of cells overexpressing hnRNP A1 and A2, we treated seven human and mouse HCC cell lines with the MEK inhibitor U0126 and measured cell death induced by MEK inhibition. Surprisingly, HuH7 and HepG2 cells that express high levels of hnRNP A1 and A2 showed elevated FL-A-Raf levels, enhanced phosphorylation of MEK1/2 and ERK 1/2, and were resistant to apoptosis induced by the MEK inhibitor U0126 (Fig. 6). To examine if hnRNP A2 mediates the resistance of HuH7 cells to MEK1 inhibition, we treated HuH7 cells with or without hnRNP A2 knockdown with the MEK1 inhibitor U0126. Since hnRNP A2 knockdown reduced cell proliferation upon treatment with the MEK1 inhibitor U0126 (Fig. 6), the results suggest that hnRNP A2 up-regulation in HCC cells contributes to resistance to MEK1 inhibition. Thus, hnRNP A2 up-regulation might serve as a biomarker for resistance to Raf-MEK inhibitors and as a new target for resensitization of HCC tumors resistant to MEK1 inhibitors.

Taken together, our data suggest that hnRNP A1 and more profoundly hnRNP A2 up-regulation in HCC tumors induces an alternative splicing switch that down-regulates a dominant-negative isoform of A-Raf and up-regulation of the FL-A-Raf, leading to activation of the Raf-MEK-ERK pathway (Figs. 5, 6). Another implication for these results is that tumors overexpressing hnRNP A1 and A2 might be more resistant to Raf, MEK1, or ERK inhibitors.

MATERIALS AND METHODS

Mice

Mice with a homozygous disruption of the *Mdr2* gene, *Mdr2*$^{-/-}$ (Jackson Laboratory, stock no. 002539), with a FVB/N genetic background were used. Liver tumors were derived from 14-mo-old *Mdr2*$^{-/-}$ mice. Age-matched FVB/N mice were used as a source for normal liver tissue. Animals were killed by a lethal dose of anesthesia. Mice were held in specific pathogen-free conditions. All animal experiments were performed in accordance with the guidelines of the Hebrew University committee for the use of animals for research.

Cells

Liver progenitor cells from embryonic day 18 fetal livers from *TP53*$^{-/-}$ mice were isolated and immortalized with MSCV-based retroviruses expressing Myc–IRES-GFP as previously described (Zender et al. 2006) to generate *TP53*$^{-/-}$ hepatocytes myc (PHM-1) cells. PHM-1, HuH7, HepG2, Hep3B FLC-4, BNL-1ME, Hc3716-hTERT (Waki et al. 2010), and HeLa Tet-on (Clontech) cells were grown in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 0.1 mg/mL penicillin, and 0.1 mg/mL streptomycin. All cell lines have been tested and authenticated by the biosynthesis DNA Identity Testing Center on July 29, 2013.

Stable cell lines

To generate stable cell lines, PHM-1 and HeLa Tet-on cells were transduced with-pBABE-puro or STP-puro retroviral vectors, respectively, expressing T7-tagged hnRNP A1, hnRNP A1b, hnRNP A2, or hnRNP B1 cDNA. Medium was replaced 24 h after infection, and 24 h later, infected cells were selected with puromycin (2 μg/mL) for 72 h. Stable pools of HeLa Tet-on cell lines were transduced with pSin-Hygro-miR30-shRNA vectors (Narita et al. 2006). Medium was replaced 24 h after infection, and 24 h later, infected cells were selected for with hygromycin (200 μg/mL) for 72 h. shRNA sequences are as follows: *HNRNP A2/B1*-sh2, CCA TGGGCTTCACTGTATA; *HNRNP A1*-sh1, GACTGTATTTGTGA CTAAT. In the case of infection with MLP-puro-shRNA vectors, HuH7 cell transductants were selected with puromycin (2 μg/mL) for 96 h. shRNA sequences are as follows: *HNRNP A2/B1*-sh1, CTGTTTGTTGGCGGAATTA; sh2, CCATGGGCTTCACTGTA TA; and *HNRNP A1*-sh1, GACTGTATTTGTGACTAAT.

EGF stimulation

We seeded 8×10^5 PHM-1 or 1.2×10^6 HuH7 cells in 10-cm plates. Twenty-four hours later, cells were washed with PBS, and medium was replaced with medium containing 0.1% serum for 24 h. Cells were stimulated with EGF 50 ng/mL for 30 min, after which, cells were lysed in Laemmli buffer for Western blot analysis.

Immunoblotting

Cells were lysed in Laemmli buffer and analyzed for total protein concentration as described (Karni et al. 2007). Fifty micrograms of total protein from each cell lysate was separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were blocked with 5% milk and probed with specific antibodies. Bands were visualized using enhanced chemiluminescence detection. Primary antibodies are as follows: hnRNP A1 (mAb A1/55, 1:1000) (Allemand et al. 2005), hnRNP A2/B1 (1:1000, Santa Cruz), β-tubulin (1:1000, Sigma), β-catenin (1:2000, Sigma), β-actin (1:1000, Santa Cruz), T7 tag (1:5000, Novagen), phospho-MEK S217/221 (1:1000, Cell Signaling), total MEK (1:1000, Cell Signaling), phospho-ERK T202/Y204 (1:1000 Sigma), total ERK1/2 (1:1000, Cell Signaling), A-Raf (1:1000, Cell Signaling), and A-Raf short (1:500) (Rauch et al. 2011). Secondary antibodies are as follows: HRP-conjugated goat anti-mouse, goat anti-rabbit, donkey anti-goat IgG (H+L; 1:10,000 Jackson Laboratories).

Anchorage-independent growth

Colony formation in soft agar was assayed as described previously (Karni et al. 2007). In the case of HeLa Tet-on cell lines, 5 μg/mL doxycycline (Dox) was added to the top media. Plates were incubated at 37°C with 5% CO_2. After 10–21 d, colonies were counted from 10 different fields in each of two wells for each transductant pool, and the average number of colonies per well was calculated. The colonies were stained as described (Karni et al. 2007) and photographed under a light microscope at 100×magnification.

Growth curves

Transductant pools of PHM-1 cells were seeded at 1800 cells per well in 96-well plates. Every 24 h, cells were fixed and stained with methylene blue as described previously (Karni et al. 2007), and the absorbance at 650 nm of the acid-extracted stain was measured by a plate reader (BioRad).

Survival and apoptosis assays

HuH7 cells were transduced with the indicated retroviruses. Following selection, 4500 cells per well were seeded in 96-well plates. Twenty-four hours later, the medium was replaced to medium containing 0.1% serum. At 24 h (before treatment), one 96-plate was fixed and served as a normalizing control ("Time 0"). Every 24 h, cells were fixed and stained with methylene blue as described previously (Karni et al. 2007) and the absorbance at 650 nm of the acid-extracted stain was measured by a plate reader (BioRad) and normalized to cell absorbance at Time 0. HCC cells were seeded in six-well plates (2×10^5 cells/well). Twenty-four hours later, cells were incubated with 10 μM U0126 for 24 h. Medium and PBS washes were collected together with cells trypsinized from each well into 15-mL tubes and centrifuged at 1000*g* for 5 min. Cells were washed with PBS and after centrifugation were resuspended in 100 μL of PBS. Ten microliters of the cell suspension was mixed with 10 μL of 4% trypan blue solution, and live/dead cells were counted in Bio-Rad TC-10 automated cell counter. After counting, the remaining 90 μL of cell suspension was centrifuged, 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 to evaluate induction of apoptosis.

Quantitative RT-PCR

Total RNA was extracted with Tri reagent (Sigma), and 2 μg of total RNA was reverse transcribed using the M-MLV reverse transcriptase (Promega) after DNase treatment (Promega). qPCR was performed on the cDNA using SYBR green (Roche) and the CFX96 (Bio-Rad) real-time PCR machine. mRNA levels of hnRNP A1 and hnRNP A2 were measured in mouse normal liver and tumor tissues. mRNA levels of A-Raf isoforms were measured in liver cell lines PHM-1 and HuH7 and in HeLA Tet-on cell lines. Normalization was performed using either GAPDH for mouse samples and PHM-1 cells or β-actin for human HuH7 and HeLa Tet-on cells. Unknown samples were compared to a standard curve, which was established by serial dilutions of a known concentration of cDNA. The PCR reaction is composed of the following steps: one cycle for 10 sec at 95°C

and 40 cycles of 5 sec at 95°C and 20 sec at 55°C. Primers are listed in Supplemental Table S1.

Xenograft tumor formation in mice

Stable pools of PHM-1 cells expressing hnRNP A1, hnRNP A1b, hnRNP A2, or hnRNP B1were injected (3×10^6 cells/site in 200 µL PBS) subcutaneously into each rear flank of nude mice using a 26-gauge needle. Tumor growth was monitored biweekly. HuH7 cells expressing specific shRNAs were injected (2×10^6 cells/site in 200 µL PBS) subcutaneously near both rear flanks of SCID mice using a 26-gauge needle. Tumor volume was measured twice a week.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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Author contributions: A.S. and R.K. designed the experiments. A.S., V.B.H., P.D., and I.S. performed experiments. L.Z., E.P., J.R., and W.K. contributed reagents and technical help. A.S. and R.K. analyzed the data and wrote the manuscript.

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