Splicing Factor hnRNP A2/B1 Regulates Tumor Suppressor Gene Splicing and Is an Oncogenic Driver in Glioblastoma

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Abstract

The process of alternative splicing is widely misregulated in cancer, but the contribution of splicing regulators to cancer development is largely unknown. In this study, we found that the splicing factor hnRNP A2/B1 is overexpressed in glioblastomas and is correlated with poor prognosis. Conversely, patients who harbor deletions of the HNRNPA2B1 gene show better prognosis than average. Knockdown of hnRNP A2/B1 in glioblastoma cells inhibited tumor formation in mice. In contrast, overexpression of hnRNP A2/B1 in immortal cells led to malignant transformation, suggesting that HNRNPA2B1 is a putative proto-oncogene. We then identified several tumor suppressors and oncogenes that are regulated by HNRNPA2B1, among them are c-FLIP, BEN1, and WWOX, and the proto-oncogene RON. Knockdown of RON inhibited hnRNP A2/B1 mediated transformation, which implied that RON is one of the mediators of HNRNPA2B1 oncogenic activity. Together, our results indicate that HNRNPA2B1 is a novel oncogene in glioblastoma and a potential new target for glioblastoma therapy. Cancer Res; 71(13): 4464–72. ©2011 AACR

Introduction

The process of alternative splicing is widely misregulated in cancer, and many tumors express new splicing isoforms that are absent in the corresponding normal tissue (1–3). 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 (4–6). However, the contribution of alternative splicing regulators to cancer development has been largely unknown. Only recently the first functional evidence showed that some splicing factors can act as potent oncogenes and are upregulated in human cancers (7, 8). hnRNP proteins are abundant RNA-binding proteins expressed in most human tissues (9, 10). The hnRNP A/B family is a subset of hnRNP proteins with closely related sequences and a conserved modular structure (10). They can affect alternative splicing, frequently by antagonizing SR proteins, in part, through the recognition of exonic splicing silencers elements (11–13). Additional functions of these proteins in postsplicing events, such as mRNA trafficking, and replication and transcription of cytoplasmic RNA viruses, have also been reported (10). Recent studies have shown that hnRNP A1 also affects the maturation of specific miRNAs among them pre-miR-18a, which is part of a cluster of miRNAs with oncogenic activity (onco-miRs; refs. 14–16). Previous studies showed the overexpression of hnRNP A1 and hnRNP A2/B1 in lung and breast cancer (17, 18). Moreover, knockdown of hnRNP A1 and A2/B1 in breast cancer cells induced apoptosis that was specific for cancer cells (19). However, a direct role for HNRNPA2B1 as an oncogene in human cancer has not yet been shown. Recent studies found that hnRNP A1 and A2/B1 modulate alternative splicing of the glycolytic PKM2 enzyme in cancer cells, suggesting a possible role for hnRNP A1 and A2/B1 in the regulation of tumor metabolism (20, 21).

Another unsolved question is the biochemical and biological difference between members of the hnRNP A/B protein family. To date, their splicing activities, both in vitro and in knockdown or transfection assays, showed similar splicing effects on several substrates (11, 20–22). Thus, it is not clear to what extent there is redundancy in their splicing targets and biological activities. We found that hnRNP A2/B1, but not any other hnRNP A/B or SR protein, is a prognostic marker for glioblastoma patient survival and a potent oncogene in glioblastoma development and tumor maintenance (Figs. 1–3; Supplementary Figs. S1–S3). In a search for its alternative splicing targets, we found that hnRNP A2/B1 modulates alternative splicing of the tumor suppressors BIN1, WWOX, the antiapoptotic proteins c-FLIP and caspase-9B, the insulin receptor (IR), and the RON proto-oncogene among others (Fig. 4; Supplementary Table S1). In all of these splicing events, hnRNP A2/B1 enhanced the expression of the oncogenic isoforms of these genes (Fig. 4). We further show that RON knockdown inhibits transformation of glioma cells overexpressing exogenous...
hnRNP A2/B2, suggesting that RON is an important target in hnRNP A2/B1–mediated transformation (Fig. 5).

Materials and Methods

Cells

U87MG and T98G cells were grown in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal calf serum (FCS), penicillin and streptomycin. NIH 3T3 cells were grown in DMEM supplemented with 10% calf serum (CS), penicillin, and streptomycin. To generate stable transductant pools, NIH 3T3 and U87MG cells were infected with pBABE-puro retroviral vectors expressing T7-tagged human hnRNP A2 cDNA. At 24 hours after infection, the medium was replaced, and 24 hours later, infected cells were selected with puromycin (2 μg/mL) for 72 to 96 hours. In the case of
Figure 2. hnRNP A2/B1 is required for glioblastoma transformation. A, U87MG cells were transduced with retroviruses encoding hnRNP A2/B1-specific shRNA or empty vector [MLP(--)] and after selection cells were analyzed by Western blotting for hnRNP A2/B1 protein expression. β-Catenin was used as a loading control. B, quantification of colony formation in soft agar of cells described in A was examined by methylene blue staining. Error bars represent SD (n = 2). C, representative fields of colonies in soft agar described in B. D, cell proliferation of cells described in A was examined by colony formation in soft agar of cells described in A. Error bars represent SD (n = 2). E, U87MG cells transduced with empty vector (pBABE) or hnRNP A2/B1 (A2) were analyzed by Western blotting for hnRNP A2/B1 protein expression, and β-catenin was used as a loading control. F, quantification of colony formation in soft agar of the cells described in E. Colonies were considered “Big colonies” if they contained approximately more than 100 cells or were more than 1 mm in diameter. Error bars indicate SD (n = 6). G, representative fields of soft agar colonies described in F, H, cell proliferation of cells described in E was examined by methylene blue staining. Error bars represent SD (n = 6). I, cells described in A were injected (10^6 cells/site) subcutaneously near both rear flanks of nude/nude mice, and tumor volume was measured biweekly. Error bars indicate SD (n = 8). J, representative mice described in I are shown.

anchorage-independent growth

Colonies were considered “Big colonies” if they contained approximately more than 100 cells or were more than 1 mm in diameter. Error bars indicate SD (n = 2). G, representative fields of soft agar colonies described in F, H, cell proliferation of cells described in E was examined by methylene blue staining. Error bars represent SD (n = 6). I, cells described in A were injected (10^6 cells/site) subcutaneously near both rear flanks of nude/nude mice, and tumor volume was measured biweekly. Error bars indicate SD (n = 8). J, representative mice described in I are shown.

Tumorogenesis assays in nude mice

U87MG cells expressing MLP-puro or MLP-puro-containing hnRNP A2 shRNAs or NIH 3T3 cells overexpressing hnRNP A2 or an empty vector (pBABE) were injected (2 x 10^6 cells per site in 200 μL of PBS) subcutaneously into each rear flank of (Atimic-Nu/Nu) nude mice by using a 26-gauge needle. Tumor growth was monitored twice a week as described (7).

Immunoblotting

Cells were lysed in SDS and analyzed for total protein concentration as described (7). A total of 30 or 20 μg of total protein from each cell lysate was separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were blocked, probed with antibodies, and detected using enhanced chemiluminescence detection. Primary antibodies were as follows: anti-β-catenin (1:2,000; Sigma); anti-β-actin (1:2,000; Santa Cruz Biotechnology); anti-RON (1:1,000 Santa Cruz); anti-hnRNAP A2/B1 (1:1,000; Santa Cruz); and T7 tag (1:5,000; Novagen). Secondary antibody was horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit (1:10,000; Jackson Laboratories).

Growth curves

U87MG or NIH 3T3 cells were infected with the indicated retroviruses. After selection, 5,000 cells per well were seeded in 96-well plates. Cells were fixed and stained with methylene blue as described (7), and the A650 of the acid-extracted stain was measured on a plate reader (Bio-Rad).
of total RNA was reverse transcribed using the AffinityScript
60
were 95
(v/v) DMSO was included in some reactions. PCR conditions
of TaqGold (Invitrogen), and 0.2
Quantitative RT-PCR
Primers are described in Supplementary Table S2.
agarose gels or on 6% nondenaturing polyacrylamide gels.
minutes at 72
10
PCR buffer with 15 mmol/L MgCl2 (Invitrogen), 2.5 units
reactions containing 0.2 mmol/L deoxynucleotide (dNTP) mix,
were analyzed by Western blotting for hnRNP A2/B1 protein expression
staining. Error bars indicate the SD (\( \pm \)).
proliferation of cells described in A was examined by methylene blue
haematoxylin and eosin.
tumors derived from NIH 3T3 cells overexpressing hnRNP A2/B1, stained
micrographs of formalin-fixed, paraffin-embedded tissue sections from
formation in soft agar of cells described in A. Error bars represent SD
\( \pm \). C, representative fields of colonies in soft agar described in B. D,
polation of cells described in A was examined by methylene blue staining. Error bars indicate the SD (\( \pm \)). E, cells described in A were injected (10^6 cells/site) subcutaneously near both rear flanks of nude/nude
mice, and tumor volume was measured biweekly. Error bars indicate SD
2). C, representative fields of colonies in soft agar described in B. D,
were analyzed by Western blotting for hnRNP A2/B1 protein expression
were 95°C for 10 seconds, 40 cycles of 95°C for 10 seconds, and 58°C for 20 seconds.

Results
The splicing factor hnRNP A2/B1 is upregulated in brain cancer
We analyzed the expression of hnRNP A2/B1 in several types of brain cancers, using the Oncomine database (http://www.oncomine.org), and found hnRNP A2/B1 overexpression in tumor samples from glioblastoma, oligodendrogioma, and astrocytoma patients compared with normal brain tissues (Fig. 1A). These results support a recent finding that hnRNP A2/B1, hnRNP A1, and polypyrimidine tract-binding protein (PTB) are overexpressed in glioblastoma (20). We further analyzed 3 normal brain (blue bars) and 22 glioblastoma grade IV tumor samples by quantitative RT-PCR (qRT-PCR) and found that, in 19 of the 22 tumors (red bars), hnRNP A2/B1 was overexpressed more than 2-fold than the average expression in the normal brain samples (Fig. 1B).

The expression and gene copy number of the splicing factor hnRNP A2/B1 are inversely correlated with glioma patient survival
Using the REMBRANDT database of the National Cancer Institute (https://cintegrator.nci.nih.gov), we analyzed the correlation of hnRNP A2/B1 expression and gene copy number and patient survival. We found a very significant inverse correlation (\( P = 2.62E-5 \)) between patient survival and elevated expression or gene copy number of \textit{HNRNPA2B1} (Fig. 1C and D). These results indicate that patients with tumors harboring elevated copy number of \textit{HNRNPA2B1} have poor prognosis of survival. Analysis of the same samples for overexpression of known oncogenes (\textit{MYC} and \textit{EGFR}) or downregulation of known tumor suppressors (\textit{TP53} and \textit{TP73}) did not show significant correlation with patients survival (Supplementary Figs. S1 and S2). Gene copy number of the tumor suppressor \textit{PTEN}, however, showed significant correlation with patient survival, and Akt2 expression and copy numbers were also inversely correlated with patient survival (Supplementary Fig. S3) corroborating previous findings (24). It is important to mention that hnRNP A2/B1 prognostic value was
statistically better than AKT2 (Fig. 1C and D) and thus it might be a better biomarker for glioma prognosis. Interestingly, neither gene copy number nor expression of the other splicing factors from the hnRNP A/B protein family (hnRNP A1, hnRNP A0, and hnRNP A3) or SR protein family showed significant inverse correlation with patient survival (Supplementary Fig. S4).

hnRNP A2/B1 is required for glioblastoma transformation and tumorigenicity

To examine the importance of hnRNP A2/B1 to glioma tumor maintenance, hnRNP A2/B1 was knocked down in U87MG and T98G cells or overexpressed in the U87MG glioblastoma cell lines and anchorage-independent growth

Figure 4. hnRNP A2/B1 regulates the alternative splicing of tumor suppressors and oncogenes. U87MG glioblastoma cancer cells were transduced with retroviruses encoding shRNA empty vector (MLP), or hnRNP A2/B1–specific shRNA, empty pBABE vector, or pBABE containing hnRNP A2/B1. After selection cells were lysed, RNA isolated, and the alternative splicing patterns of RON, INSR, ENAH, CFLAR, BIN, CASP9, and WWOX were examined by RT-PCR, using the indicated isoform-specific primers (arrowheads). The splice variants are indicated by boxes at the right side of each gel. A, RON exon 11 skipping/inclusion. B, INSR exon 11 skipping/inclusion. C, ENAH exon 11a skipping/inclusion. D, CFLAR exon 7 skipping/inclusion. E, BIN1 exons 12a and 13 skipping/inclusion. F, CASP9 exons 3 to 6 skipping/inclusion. G, WWOX exons 6 to 8 skipping/inclusion. H, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control. Numbers under each panel represent the average and SD of fold change in the inclusion of the indicated exon compared with the empty vectors (normalized as 1) in 2 to 4 independent experiments.

Figure 5. Knockdown of RON reverses transformation of U87MG cells overexpressing hnRNP A2. U87MG cells transduced with hnRNP A2 cDNA were cotransduced with the indicated lentiviruses containing shRNAs against RON or an empty vector. After selection cells were plated into soft agar and 14 days later colonies were counted. A, Western blots showing RON levels in the different cell populations. B, quantitation of colonies formed in soft agar from cells described in A. C, representative fields of cells described in A (magnification ×10).

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was analyzed by colony formation in soft agar. Colony formation of U87MG and T98G cells with hnRNP A2/B1 knockdown was reduced in contrast to increased colony number and size in glioblastoma cells overexpressing hnRNP A2/B1 (Fig. 2A–C and E–G; Supplementary Fig. S5). One of the features that can contribute to colony formation is the proliferation rate of the cells. Notably, for tumor development and progression, other aspects such as motility, invasiveness, the ability to grow in an anchorage-independent manner and to resist apoptotic cues can be more prominent. To examine whether hnRNP A2/B1 plays a role in proliferation, we measured proliferation rates of U87MG glioblastoma cells with up- and downregulation of hnRNP A2/B1. The knockdown of hnRNP A2/B1 did not reduce the viability of the glioblastoma cells and slightly inhibited their ability to proliferate on plastic in 10% serum but inhibited their proliferation in lower serum concentrations (Fig. 2D; Supplementary Fig. S6), suggesting that hnRNP A2/B1 contributes to cell proliferation in low growth factor conditions. Importantly, in hnRNP A2/B1 knockdown and overexpression cell pools, hnRNP A2/B1 silencing and overexpression were sustained over the growth curve period, eliminating the possibility that cell density or an aberrant clone could overcome hnRNP A2/B1 silencing and overexpression (Supplementary Fig. S6). Overexpression of hnRNP A2/B1 upregulated the proliferation rate of U87MG and NIH 3T3 cells in all growth factor concentrations (Figs. 2H and 3D; Supplementary Fig. S6A–C), indicating that it can contribute to enhanced proliferation at both low and high growth factor conditions when overexpressed. To determine the importance of hnRNP A2/B1 for glioma tumor formation in vivo, nude mice were injected with U87MG cells with and without knockdown of hnRNP A2/B1. Cells with empty vector gave rise to fast-growing large tumors whereas mice injected with U87MG cells, in which hnRNP A2/B1 was knocked down, developed either small tumors in some of the injection sites or no tumors at all (Fig. 2I and J). The importance of hnRNP A2/B1 to brain cancer maintenance was also confirmed in the T98G glioma cell line (Supplementary Fig. S5). T98G cells with hnRNP A2/B1 knockdown showed reduced colony formation in soft agar, similar to results in U87MG cells (Supplementary Fig. S5).

**hnRNP A2/B1 overexpression transformed NIH 3T3 and converted them to tumorigenic**

On the basis of our results that hnRNP A2/B1 is required for the glioblastoma-transformed phenotype, we examined the ability of this factor to transform normal immortal cells. Upregulation of hnRNP A2/B1 in NIH 3T3 cells induced colony formation in soft agar in contrast to control cells that did not form colonies (Fig. 3A–C). Moreover, these cells formed fast-growing tumors when injected subcutaneously into nude mice (Fig. 3E and F). Pathologic analysis of the tumors formed by cells overexpressing hnRNP A2/B1 showed that these tumors look like high-grade invasive sarcoma with high mitotic index (Fig. 3G). These results suggest that **HNRNPA2B1** is a driving oncogene on its own and probably directly contributes to glioblastoma development.

**hnRNP A2/B1 levels affect the alternative splicing of several tumor suppressors and oncogenes**

To examine the effect of **HNRNPA2B1** on alternative splicing in glioma tumor cells, we analyzed the alternative splicing pattern of several genes for which alternatively spliced isoforms have been characterized and for some of them shown to contribute to transformation, invasion, and apoptosis or to be affected by hnRNP A2/B1 (Fig. 4; Supplementary Fig. S7 and Table S1). Knockdown of hnRNP A2/B1 in U87MG cells increased the inclusion, whereas upregulation of hnRNP A2/B1 increased the skipping of exon 11 of **RON**, a tyrosine kinase receptor involved in the invasiveness and motility of tumor cells (refs. 25, 26; Fig. 4A). These results suggest that similar to the splicing factor oncprotein **SRSF1** (**SF2/ASF**), hnRNP A2/B1 upregulation contributes to cellular transformation by increased skipping and upregulation of the ΔRON oncogenic splicing isoform (25). Importantly, the levels of **SRSF1** (**SF2/ASF**) or **SRSF6** (**SRp55**), another SR protein, and the levels of hnRNP A1, another hnRNP A/B family member, did not change upon hnRNP A2/B1 down- or upregulation (Supplementary Fig. S8). In NIH 3T3 cells overexpressing hnRNP A2, we could detect only one isoform of RON, suggesting that either this splicing event occurs only in humans or that it hardly occurs in fibroblasts. The IR is the main mediator of insulin metabolism and glucose levels in the body and is expressed in most tissues. Skipping of exon 11 from the **INSR** transcript generates the splicing variant IR-A that binds the growth factor insulin-like growth factor II (**IGF-II**), in addition to insulin. This splicing variant is overproduced in many cancers and has been implicated in an autocrine loop in cancer cells (27, 28). hnRNP A2/B1 knockdown increased the inclusion of exon 11 whereas its overexpression increased exon 11 skipping generating the mitogenic isoform (Fig. 4B). The **EVAH** gene has been shown to play a role in the epithelial-to-mesenchymal transition process and to affect cellular motility and invasion. Overexpression did not affect the inclusion of exon 11a, suggesting that it is not a splicing target of hnRNP A2/B1 in U87MG cells (Fig. 4C). **CFLAR** (**c-FLIP**) is an antiapoptotic protein that is alternatively spliced and has been shown not only to inhibit TNF and TNF-related apoptosis-inducing ligand (**TRAIL**)–induced apoptosis (29, 30) but also to enhance motility and invasion through activation of the mitogen-activated protein kinase (**MAPK**)–signal regulated kinase (**MAPK-ERK**) pathway (31, 32). We found that hnRNP A2/B1 downregulation decreased the levels of the long isoform of **c-FLIP** whereas overexpression of hnRNP A2/B1 increased the level of the long isoform, raising the possibility that upon hnRNP A2/B1 knockdown, glioma cells might become more sensitive to apoptotic stimuli and at the same time be less motile and less invasive (ref. 31; Fig. 4D). The tumor suppressor **BIN1** has been shown to be regulated by alternative splicing and inclusion of exon 12a of **BIN1** inactivates its tumor suppressor activity (33, 34). **BIN1** exon 12a was also identified as a target of the SR protein **SRSF1** (**SF2/ASF**; ref. 7). We found that similar to the effect of **SRSF1** overexpression, hnRNP A2/B1 overexpression in both U87MG and NIH 3T3 cells enhanced exon 12a inclusion generating the antiapoptotic isoform of **BIN1** whereas its knockdown

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**HNRNPA2B1 Is a Driving Oncogene in Glioblastoma**

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enhanced exon 12a skipping (Fig. 4E; Supplementary Fig. S7). CASP9, the gene coding for caspase-9 has been previously shown to be alternatively spliced by skipping of exons 3 to 6 to generate a truncated dominant negative isoform that inhibits apoptosis and is overexpressed in several cancers (35, 36). We found that hnRNP A2/B1 knockdown enhanced the production of full-length caspase-9 whereas its overexpression enhanced skipping of exons 3 to 6 generating the antiapoptotic isoform caspase-9B (Fig. 4F). WWOX is a known tumor suppressor that resides at a common fragile site (37) and is frequently inactivated in several types of cancer including glioblastoma (38). Skipping of exons 6 to 8 of WWOX has been reported in breast cancer (39). We found that hnRNP A2/B1 knockdown enhanced inclusion of alternatively spliced exons 6 to 8 whereas its overexpression induced skipping of these exons (Fig. 4G). Skipping of these exons causes deletion of 180 amino acids including its substrate binding domain and its alcohol dehydrogenase domain probably inactivating its catalytic activity (39). However, the functional role of these skipped isoforms requires further examination. WWOX tumor-suppressive activity is related to its anti-invasive and antiapoptotic functions (37, 39), which are a common theme shared with the other targets of hnRNP A2/B1 we identified. hnRNP A2/B1 also affected the alternative splicing of exon 7B of hnRNP A1, another member of the hnRNP splicing factor family (Supplementary Figs. S7 and S8A). Skipping of this exon has been shown to be affected by hnRNP A1 and hnRNP A2/B1 levels in an autoregulatory fashion and thus our results corroborate these previous findings (40). The levels of hnRNP A2/B1 also affected the alternative splicing of other genes, including known HNRNPAP2B1 targets such as exon 7 of the SMN gene and other splicing events (Supplementary Fig. S7 and Table S1). Importantly, many splicing events we examined were not affected by HNRNPAP2B1 depletion or overexpression (Supplementary Table S1).

Knockdown of RON reverses transformation of glioblastoma cells overexpressing hnRNP A2/B1

To examine whether RON contributes to hnRNP A2/B1-mediated transformation, we knocked down the expression of the RON proto-oncogene in U87MG cells overexpressing ectopic hnRNP A2 (Fig. 5A). We found that stable knockdown by 2 different shRNAs reduced RON levels and inhibited colony formation in soft agar of U87MG cells (Fig. 5B and C). These results indicate that RON is one of the important mediators of hnRNP A2/B1 oncogenic activity in glioblastoma cells, even though other targets can probably contribute as well.

Discussion

An emerging body of data shows that alternative splicing misregulation plays an important role in cancer development and tumor maintenance (5–8). Alternative splicing regulators from the SR and hnRNP A/B protein families are overexpressed or downregulated in various cancers and can probably account for many of the cancer-specific alternative splicing changes. Some alternative splicing regulators such as the SR protein SRSF1 (SF2/ASF) have been shown to be upregulated in many cancers and act as potent oncogenes when slightly overexpressed (7).

hnRNP A/B proteins are important regulators of alternative splicing and in several examples of in vitro and in vivo splicing assays seemed to act antagonistically to SR proteins (11–13). Even though hnRNP A/B proteins can alter alternative splicing in an opposite manner to oncogenic splicing factors such as SRSF1 (SF2/ASF), there is no evidence that they act as tumor suppressors, and, on the contrary, members of the hnRNP A/B protein family are overexpressed in some cancers (7, 17–20) and change the splicing pattern of genes that contribute to the transformed phenotype (20, 21, 41). Moreover, recently, it has been shown that the splicing factors hnRNP A1 and A2/B1 as well as PTB are under the transcriptional control of the myc proto-oncogene and can modulate the splicing of PKM2, activating a metabolic switch to aerobic glycolysis that is a hallmark of cancer cells (the “Warburg effect”; refs. 20, 21). However, to date, there is no direct evidence that any member of the hnRNP A/B family is an oncogene that can transform cells and is genetically upregulated (amplified, translocated, or mutated) in cancer. We show here that the splicing factor hnRNP A2/B1 is overexpressed in gliomas compared with normal brain samples in many cases because of the amplification of the HNRNPAP2B1 gene (Fig. 1A–D). Moreover, overexpression and amplification of hnRNP A2/B1 correlate with poor prognosis of glioma patients whereas depletion of the HNRNPAP2B1 gene correlates with better prognosis than average (Fig. 1C and D). Importantly, none of the other hnRNP A/B proteins, as well as the SR proteins or some other known oncogenes, showed significant inverse correlation with survival of glioma patients (Supplementary Figs. S1 and S2). In accordance with previous findings, deletion of the tumor suppressor PTEN and overexpression of the proto-oncogene Aki2 showed significant inverse correlation with glioma patient survival (24; Supplementary Fig. S3). Taken together, these findings suggest that hnRNP A2/B1 is a valuable prognostic marker for glioblastoma development and patient survival. To examine whether hnRNP A2/B1 is causatively involved in glioblastoma tumor development, we downregulated the expression of hnRNP A2/B1. We found that knockdown of hnRNP A2/B1 in U87MG glioblastoma cells partially inhibited their proliferation, especially in low-serum condition (Fig. 2D; Supplementary Fig. S6). This result suggests that the cell cycle and viability may not be the major antioncogenic effectors in cells with hnRNP A2/B1 knockdown. However, knockdown of hnRNP A2/B1 inhibited colony formation in soft agar as well as tumor formation in nude mice of glioblastoma cells (Fig. 2A–C, I, and J; Supplementary Fig. S5), suggesting that hnRNP A2/B1 is important for glioblastoma tumor development and maintenance. Moreover, overexpression of hnRNP A2/B1 in the U87MG glioblastoma cells enhanced their proliferation in all serum concentrations and increased colony size and number in soft agar (Fig. 2E–H; Supplementary Fig. S6), indicating that hnRNP A2/B1 may not be a limiting factor when growth factors are present but plays a positive role in glioblastoma transformation when upregulated. To examine whether HNRNPAP2B1 acts as an oncogene, we slightly overexpressed (2-fold) hnRNP A2/B1...
B1 in immortal mouse fibroblasts (NIH 3T3 cells) and examined their oncogetic properties. NIH 3T3 cells overexpressing hnRNP A2/B1 became transformed, formed colonies in soft agar, and were tumorigenic in nude mice forming tumors with hallmarks of high-grade sarcomas (Fig. 3). These data suggest that HNRNPA2B1 acts as a proto-oncogene when slightly upregulated and thus it is not only a marker but also a driving oncogene in glioblastoma development. To identify possible alternative splicing targets of hnRNP A2/B1 that might mediate its oncogetic activity, we examined the alternative splicing pattern of several genes known to undergo alternative splicing in cancer and to contribute to the transformed phenotype. We identified several alternative splicing events modulated by hnRNP A2/B1, among them the tumor suppressor BINI, the antiapoptotic gene CFLAR (c-FLIPI), and CASP9 all regulators of the apoptotic process (29–36). The tumor suppressor gene WWOX, the INSR gene, the long isoform of CFLAR, and the RON tyrosine kinase receptor are all involved in motility and invasion (25–28, 31, 32, 37–39). Our results suggest that hnRNP A2/B1 activates an alternative splicing program that enhances the production of antiapoptotic isoforms of genes such as BINI, CASP9, and CFLAR, as well as invasion-promoting isoforms such as ΔRON, CFLAR-long, and IR-A, which contribute to transformation and invasion.

Surprisingly, in several cases of alternative spliced exons shown in this article (BINI exon 12a and RON exon 11), hnRNP A2/B1 showed similar splicing effects to the oncogetic SR protein SRSF1 (SF2/ASF), raising the possibility that in many cases in vitro, these splicing factor are not antagonistic as previously thought from in vitro and transient-transfection splicing assays (11–13). hnRNP A2/B1 is a general splicing factor and it is expected to change the splicing of many (currently unknown) transcripts. We know that the alternative splicing of several transcripts did not change upon up- or downregulation of hnRNP A2/B1 (Fig. 4; Supplementary Table S1). We are limited by the small number of transcripts tested, and only a genome-wide analysis will reveal how many transcripts are regulated directly and indirectly by hnRNP A2/B1.

To examine whether RON is an important target that mediated hnRNP A2–induced transformation, we stably knocked down RON in U87MG glioblastoma cells overexpressing exogenous hnRNP A2 cDNA and found that RON knockdown significantly inhibited transformation of these cells, similar to the effect of hnRNP A2/B1 knockdown (Fig. 5). We conclude that RON is one of the important mediators of hnRNP A2–induced transformation and contributes to the transformed phenotype of glioblastoma cells.

Because hnRNP A2/B1 is a general alternative splicing factor and participates in RNA processing steps other than splicing (10, 12), we assume that it has many splicing targets other than RON and that it targets other RNA processing steps (such as miRNA maturation; refs. 14, 15) that contribute to its transforming activity. For example, hnRNP A1 has been shown to promote the maturation of onco-miRNAs such as miR-18a and to downregulate tumor-suppressive miRNAs such as Let-7a (14, 42). hnRNP A2/B1 has also been linked to tumor metabolism (20, 21, 43). It has been shown that hnRNP A2/B1 is downregulated posttranscriptionally by the tumor suppressor gene VHL, which is lost in many tumors and induces aerobic glycolysis (the “Warburg effect”) through hypoxia-inducible factor 1-alpha (HIF-1α) stabilization (43). It will be intriguing to examine whether hnRNP A2/B1 can affect HIF-1α stabilization by competitive binding to VHL when overexpressed. In recent studies, hnRNP A2/B1 has been shown to be transcriptionally controlled by the myc proto-oncogene and to regulate the splicing of the glycolytic enzyme PKM2 contributing to the induction of aerobic glycolysis by another mechanism (20, 21).

Taken together, our data suggest that HNRNPA2B1 is a new biomarker for glioblastoma patient survival and a new proto-oncogene that regulates the splicing and other RNA processing steps of several tumor suppressors and oncogenes. Furthermore, downregulating hnRNP A2/B1 levels in glioblastoma cells should be considered as a new strategy for glioblastoma therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Splicing Factor hnRNP A2/B1 Regulates Tumor Suppressor Gene Splicing and Is an Oncogenic Driver in Glioblastoma

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