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The gene encoding the splicing factor SF2/ASF is a proto-oncogene

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Abstract

Alternative splicing modulates the expression of many oncogene and tumor-suppressor isoforms. We have tested whether some alternative splicing factors are involved in cancer. We found that the splicing factor SF2/ASF is upregulated in various human tumors, in part due to amplification of its gene, *SFRS1*. Moreover, slight overexpression of SF2/ASF is sufficient to transform immortal rodent fibroblasts, which form sarcomas in nude mice. We further show that SF2/ASF controls alternative splicing of the tumor suppressor BIN1 and the kinases MNK2 and S6K1. The resulting BIN1 isoforms lack tumor-suppressor activity; an isoform of MNK2 promotes MAP kinase–independent eIF4E phosphorylation; and an unusual oncogenic isoform of S6K1 recapitulates the transforming activity of SF2/ASF. Knockdown of either SF2/ASF or isoform-2 of S6K1 is sufficient to reverse transformation caused by the overexpression of SF2/ASF *in vitro* and *in vivo*. Thus, SF2/ASF can act as an oncoprotein and is a potential target for cancer therapy.

For about three-quarters of human genes, individual or partial exons can be included in or excluded from different versions of the mature messenger RNA by alternative splicing[1]. Many alternative splicing factors combinatorially affect this process to determine which mRNA isoforms will serve as the templates for protein synthesis in a given cell type, developmental stage or physiological state[2].

SR proteins are a family of RNA-binding proteins that are essential for splicing. They act at multiple steps of spliceosome assembly and function in both constitutive and regulated splicing. Some heterogeneous nuclear ribonucleoprotein (hnRNP) proteins, which rapidly associate with nascent transcripts, have been implicated in the repression of certain alternative splicing events[2]. hnRNP and SR proteins can have antagonistic effects on the alternative splicing of particular exons in several genes[2]. Both types of factor can bind directly to precursor (pre)-mRNA transcripts, eliciting changes in the alternative splicing of various pre-mRNA substrates in a concentration-dependent manner, both *in vitro* and in transfection experiments[3,4]. Thus, changes in the expression of these proteins can affect the

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alternative splicing of an undefined number of cellular transcripts and might account for some of the known splicing changes in cancer[5–7].

There is ample evidence for a connection between alternative splicing and cancer. Mutations that affect the alternative splicing of tumor suppressors, such as *BRCA1/2*, *WT1*, *APC*, *TP73* (p73) and *TSC1/2*, and result in their inactivation, account for some types of inherited and sporadic susceptibility to cancer[7–9]. Alternative splicing also has an important role in programmed cell death: many central components of this process are encoded by alternatively spliced mRNAs, and often the products of alternative splicing have opposing effects on apoptosis[10]. In addition, the activities of many oncogenes and tumor suppressor genes are modulated by alternative splicing[7,11,12]. Many other genes that are involved in proliferation and invasiveness are alternatively spliced, and specific isoforms that contribute to the transformed phenotype are frequently elevated in tumors[13,14]. Some alternative splicing factors, especially from the hnRNP family, are also elevated in cancer cells, and the increased expression of some of these proteins correlates with patient prognosis and can serve as a diagnostic marker for malignancy[15,16]. In particular, the levels of hnRNP A1/A2 proteins are elevated in some tumor samples, especially in breast cancer, and the inhibition of hnRNP A1/A2 expression by RNA interference (RNAi) can selectively induce apoptosis of these cancer cells in culture[17]. In the case of the SR protein family, there are several reports of elevated expression associated with cancer: for SRp20 during the late stages of a mouse mammary tumorigenesis model; for SRp20, SF2/ASF and SC35 in ovarian cancer[18,19]; and for SF2/ASF in gastric carcinoma[20]. Another study reported a reduction in SR protein mRNAs in nonfamilial colon adenocarcinomas[16].

Here we have tested whether an alternative splicing factor can directly affect transformation. We found that SF2/ASF is an oncoprotein with roles in both the establishment and maintenance of transformation, and we describe some of its key targets in the transformation mechanism.

Results

Upregulation of splicing factors in human cancers

To examine the expression of SF2/ASF, SRp55, hnRNPA1 and hnRNPA2/B1 in a panel of normal and tumor-derived tissues, we used a protein microarray and probed the membrane with highly specific monoclonal antibodies to these splicing factors. As controls, we used antibodies to β-catenin and lamin A-C. We detected increased expression of SF2/ASF in many types of tumor, relative to their respective normal controls, including tumors of the colon, thyroid, small intestine, kidney and lung (Fig. 1a). A subset of these tumors also showed increased expression of SRp55, a closely related SR protein, whereas the hnRNP A/B proteins showed decreased expression or no change in the same tumors.

We next measured the expression of these splicing factors at the mRNA level in a large number of lung, colon, kidney, liver, pancreas and breast tumors (50 each) and corresponding normal samples from the same tissues (five each) using real-time reversetranscription (RT)-PCR (Fig. 1b). We found overexpression (greater than two-fold) of SF2/ASF, hnRNP A1 and SRp55 in ∼ 1/4, > 1/3 and ∼ 1/2 of the lung tumor samples, ∼

1/4, ∼ 1/3 and ∼ 1/5 of the colon tumors and ∼1/8, ∼1/6 and 1/5 of the breast tumors, respectively. SRp55 was also overexpressed in 42% of pancreatic tumors (Fig. 1b). We also detected gene amplification of *SFRS1* (SF2/ASF) in four breast tumors, and for three of these, the increased DNA copy number correlated with elevated mRNA levels (Fig. 1c). For the tumor specimen with the highest *SFRS1* copy number (4.4×), surrounding breast tissue from the same patient showed the normal copy number (Fig. 1c), demonstrating that the amplification was tumor-specific. *HNRPA1* (hnRNPA1) was not amplified in any of the 300 tumor samples analyzed, whereas one colon, two lung, two kidney and five breast tumors had a two- to three-fold increase in the DNA copy number of *SFRS6* (SRp55) (data not shown). SF2/ASF is encoded by *SFRS1* on chromosome 17q23, a genomic region that is commonly amplified in breast and other cancers, and whose amplification correlates with poor prognosis[21]. We also found mRNA and protein overexpression of SF2/ASF in three breast cancer cell lines (MCF7, BT474 and ZR751) that have 17q23 amplification[21], the first two of which had elevated *SFRS1* copy numbers (Supplementary Fig. 1 online).

SF2/ASF transforms immortal rodent cells

We then tested whether SR or hnRNP A/B proteins have oncogenic activity in several assays. First, we determined whether their overexpression is sufficient to transform immortal NIH 3T3 and Rat1 fibroblasts. By expressing T7-tagged human complementary DNAs in a retroviral vector, we generated stably transduced pools of cells that slightly overexpress (less than two-fold) selected SR proteins or hnRNP A1 (Fig. 2a and Supplementary Fig. 2 online). These tagged versions of the splicing factors have the same splicing, shuttling and translational activities as their untagged counterparts[4,22,23]. NIH 3T3 cells that overexpressed SF2/ASF formed colonies in soft agar, whereas the overexpression of two other SR proteins, SC35 and SRp55, resulted in fewer colonies (50% and < 10%, respectively; Fig. 2b). Overexpression of SF2/ASF also promoted anchorage-independent growth of Rat1 cells, whereas three other overexpressed SR proteins did not (Supplementary Fig. 2). Finally, overexpression of hnRNPA1 in NIH 3T3 but not Rat1 cells also induced colony formation in soft agar (Fig. 2b and Supplementary Fig. 2). Thus, SF2/ASF is oncogenic in both cell lines, whereas the other splicing factors seem to have context-dependent or no oncogenic activity.

NIH 3T3 cells that stably overexpressed the above factors were then injected into nude mice. The cells that overexpressed SF2/ASF gave rise to large tumors (Fig. 2c,d). Overexpression of hnRNP A1 also resulted in tumors, but they were smaller and developed more slowly (Fig. 2c). In contrast, neither SC35 overexpression nor SRp55 overexpression in NIH 3T3 cells gave rise to tumors (data not shown). Tumors derived from NIH 3T3 cells that overexpressed SF2/ASF or hnRNP A1 showed histological characteristics of high-grade sarcomas with spindleshaped morphology (Fig. 2e). The similar morphology of these tumors indicates that their different growth rates (Fig. 2c) might reflect differences at the molecular level.

Effects on apoptosis and proliferation

Inhibition of apoptosis is an important step in tumorigenesis, so we next investigated whether SF2/ASF overexpression can inhibit this process in primary cells. The

overexpression of SF2/ASF had a protective effect against apoptosis induced by serum starvation or DNA damage in E1A-expressing mouse embryo fibroblast (MEF) cells, which are sensitized to apoptosis[24] (Fig. 3a,b). Overexpression of SC35 was also protective, whereas overexpression of SRp55 or hnRNP A1 had little or no protective effect. Overexpression of SF2/ASF did not prevent E1A-mediated induction of p53 and its target gene *MDM2* (Fig. 3c). In addition to its antiapoptotic effects, SF2/ASF also cooperated with oncogenic H-Ras to enhance proliferation (Fig. 3d).

Effects on alternative splicing of endogenous targets

SF2/ASF is essential for cell viability and embryonic development and has pleiotropic activities, though it has been characterized most extensively as a splicing factor that affects alternative splicing in a concentration-dependent manner[25]. We therefore examined the splicing patterns of transcripts from selected endogenous genes with roles in transformation and apoptosis, for which alternatively spliced isoforms have been characterized (Supplementary Table 1 online). These genes include known or putative tumor suppressors, such as *BIN1*, *BRCA1*, *MGEA6*, *TSC2*, *VHL* and *TP73*, and proto-oncogenes, such as *ERBB2*, *RPS6KB1* (S6K1), *MYBL2* (B-myb) and *MYB* (C-myb)[7,11,12,26]. The BIN1 protein interacts with the product of the *MYC* (c-Myc) proto-oncogene, suppressing its oncogenic activity, and is inactivated or deleted in various cancers[11,27]. The inclusion of exon 12A abolishes the tumor-suppressor activity of *BIN1* by interfering with MYC binding, and transcripts that include this exon are overexpressed in melanoma[11]. Human, mouse and rat cells that stably overexpressed SF2/ASF showed increased inclusion of exon 12A of *BIN1* (Fig. 4 and Supplementary Table 1). By contrast, overexpression of SRp55 or hnRNP A1 did not promote inclusion of exon 12A (Fig. 4a). Transient SF2/ASF knockdown by RNAi in HeLa cells induced skipping of exon 12A—seen as a reduction in the levels of the two *BIN1* mRNA isoforms that carry this exon—without a marked effect on the two isoforms that lack exon 12A, one of which is abundant in these cells (Fig. 4b). Consistent with this, overexpression of SF2/ASF in an inducible NIH 3T3 mouse cell line increased the inclusion of exon 12A in a dose-dependent manner (Fig. 4c). These results indicate that *BIN1* is an *in vivo* target of regulation by SF2/ASF and that SF2/ASF inactivates this putative tumor suppressor through alternative splicing.

The Ras/MAP kinase (MAPK) and phosphatidylinositol-3-OH kinase (PI3K) pathways are hyperactivated in many human tumors[28]. We therefore searched for possible splicing targets of SF2/ASF in these two pathways and identified the pre-mRNAs of MNK2 and S6K1 as SF2/ASF splicing targets. Human *RPS6KB1* encodes S6K1, a kinase that phosphorylates the small-subunit ribosomal protein S6 and regulates translation[29]. Only one human *RPS6KB1* spliced mRNA has been reported, whereas the mouse gene has two isoforms (http://www.ensembl.org/, version 29). However, we detected similar patterns of S6K1 isoforms in human, mouse and rat cells (Fig. 4d, Supplementary Fig. 1 and Supplementary Table 1). In all three species, SF2/ASF overexpression promoted the expression of isoform-2 mRNA, which is identical to isoform-1 up to exon 6, but encodes a protein with a different C terminus (Supplementary Fig. 3 online). By contrast, overexpression of hnRNP A1 and SRp55 had no substantial effect on the S6K1 isoform distribution (Fig. 4d), whereas the

effect of SC35 resembled that of SF2/ASF (Supplementary Table 1). Moreover, knockdown of SF2/ASF increased the expression of isoform-1 and decreased that of isoform-2 (Fig. 4e).

MNK1 and MNK2 are the only known kinases that phosphorylate eIF4E on Ser209 (ref. 30). The MNK2 gene, *MKNK2*, is alternatively spliced, generating two catalytically active isoforms that are differentially regulated and localized[31]. Overexpression of SF2/ASF resulted in increased expression of the MNK2b isoform and a corresponding reduction in the MNK2a isoform (Fig. 4d and Supplementary Fig. 1), whereas SF2/ASF knockdown caused reduced expression of MNK2b (Fig. 4e).

The levels of SF2/ASF also affected the alternative splicing of other oncogenes, including C-myb and B-myb[12] (Supplementary Table 1). In addition, SF2/ASF overexpression promoted the inclusion of exon 5 in the transcription factor gene *TEAD1* (TEF-1) and of exon 2 in the splicing factor gene *HNRPA2B1* (Fig. 4d), whereas knockdown of SF2/ASF promoted skipping of these alternative exons (Fig. 4e). In the case of the tumor antigen gene *MGEA6*, knockdown of SF2/ASF promoted the inclusion of exon 19 (Fig. 4e). Knockdown of SF2/ASF also induced skipping of exons 9 and 10 of the tumor suppressor gene *BRCA1*, although SF2/ASF overexpression had no effect on this gene (Supplementary Table 1). Overexpression or knockdown of SF2/ASF had no effect on various other alternative splicing events. We conclude that changes in the levels of SF2/ASF result in changes in the alternative splicing of endogenous transcripts from many genes, including several important oncogenes and tumor suppressors. The effects are specific, in that several transcripts tested were not affected, and increasing the levels of other SR proteins did not result in the same spectrum of changes.

SF2/ASF induces an oncogenic isoform of *RPS6KB1*

As shown above, the alternative splicing of *RPS6KB1* (S6K1) is regulated by SF2/ASF, which promotes expression of the isoform-2 mRNA (Fig. 4d,e). We investigated whether this change in isoform mRNA ratios is reflected at the protein level. Mouse and human cells overexpressing SF2/ASF showed elevated levels of the isoform-2 protein (dubbed ‘p31’) (Fig. 5a and Supplementary Fig. 1). S6K1 is a substrate of the serine-threonine kinase mTOR and regulates cell growth and apoptosis[32]. Thus, we tested whether alternative splicing of mouse *Rps6kb1* can affect transformation. We transduced NIH 3T3 cells with retroviruses encoding the two mouse S6K1 isoforms and measured their ability to grow in soft agar. Remarkably, cells that overexpressed isoform-2 formed colonies, whereas cells that overexpressed isoform-1 did not, even though isoform-1 was expressed at much higher levels (Fig. 5b,c). The cells that overexpressed isoform-2 formed fewer colonies than SF2/ASF-overexpressing cells, even though the former expressed higher levels of isoform-2 (Fig. 2b, Fig. 5c and data not shown), indicating that SF2/ASF might activate additional targets that also contribute to transformation. Nevertheless, the expression of S6K1 isoform-2 was sufficient to qualitatively recapitulate the oncogenic effects of SF2/ASF overexpression. We also investigated whether SF2/ASF expression correlated with the S6K1 isoforms in our panel of 50 lung tumors and found a positive correlation between SF2/ASF expression and the isoform-2/isoform-1 ratio (Supplementary Fig. 4 online).

SF2/ASF induces phosphorylation of eIF4E

The effect of SF2/ASF on the alternative splicing of *MKNK2* (MNK2) (Fig. 4d) prompted us to investigate whether the induction of isoform MNK2b changes the phosphorylation state of eIF4E[33]. The MAPKs ERK1, ERK2 and p38 are phosphorylated by upstream MAPK kinases, and they in turn phosphorylate MNK1 and MNK2, which is usually required for full activity of MNK1 and MNK2 kinases[31]. Remarkably, although SF2/ASF did not enhance phosphorylation of ERK1/2 or p38, it strongly enhanced phosphorylation of eIF4E on Ser209 (Fig. 5d and data not shown). Consistent with its effect on *MKNK2* splicing (Fig. 4d), SRp55 also enhanced phosphorylation of eIF4E, although to a lesser extent than SF2/ASF (Fig. 5d).

Reversion of SF2/ASF-mediated transformation

Finally, we investigated whether SF2/ASF and isoform-2 of S6K1 are essential for SF2/ASF-mediated transformation and whether they contribute to the maintenance of the transformed phenotype of cancer cells. We used a series of small hairpin RNAs (shRNAs) to knock down SF2/ASF or each of the S6K1 isoforms in NIH 3T3 cells that overexpressed SF2/ASF (Fig. 6); as a human cancer model, we also used the lung-cancer cell line NCI-H460, in which SF2/ASF is upregulated compared with other lung-cancer cells. This upregulation correlated with alternative splicing of the targets of SF2/ASF (*RPS6KB1*, *MKNK2* and *BIN1*) (Fig. 6, Supplementary Fig. 1 and data not shown).

In the NIH 3T3 cells that were transduced with SF2/ASF, two SF2/ASF-specific shRNAs with different potencies, targeting the 3′ untranslated region (UTR) of SF2/ASF, inhibited the expression of the endogenous protein without affecting that of the transduced cDNA, which lacks the natural 3′ UTR (Fig. 6a). Remarkably, these shRNAs reversed the transformed phenotype of these cells (Fig. 6b) and of the NCI-H460 cells (Fig. 6g), inhibiting their ability to form colonies in soft agar. These SF2/ASF shRNAs also inhibited the expression of S6K1 isoform-2, MNK2b and the BIN1 isoforms with exon 12A in the NCI-H460 cells (Fig. 6f).

We also generated shRNAs that were specific for each of the two isoforms of S6K1. An shRNA against exon 7 of isoform-1, which corresponds to part of the 3′ UTR of isoform-2 (Supplementary Fig. 3), unexpectedly knocked down only isoform-2 and inhibited colony formation in NCI-H460 and SF2/ASF-overexpressing NIH 3T3 cells (Fig. 7 and Supplementary Fig. 5 online). By contrast, two shRNAs against the N-terminal region of S6K1, which in practice downregulated only isoform-1, had little effect on transformation (Fig. 7 and Supplementary Fig. 5). Cells that expressed all the test and control SF2/ASF and S6K1 shRNAs remained viable and continued to grow in culture like the control cells (data not shown).

To test whether tumorigenesis can be inhibited *in vivo*, we injected the NCI-H460 cells and NIH 3T3-SF2/ASF transductants expressing SF2/ASF shRNAs into nude mice; in both cases, knockdown of SF2/ASF greatly inhibited tumor growth (Fig. 6c,d,h). We conclude that SF2/ASF is important for tumor maintenance in at least some settings.

Discussion

Evidence that SF2/ASF is a proto-oncogene

We have presented three lines of evidence to address whether *SFRS1*, which codes for the splicing factor SF2/ASF, meets a rigorous definition of a proto-oncogene[28]. First, activation of this gene in cancer can occur—for example, by amplification of the chromosome 17q23 region, as is common in breast tumors[21]. We characterized a breast tumor with 4× amplification of *SFRS1* and upregulation of SF2/ASF, for which the surrounding breast tissue from the same patient had a normal gene copy number, showing that amplification of *SFRS1* is a somatic, tumor-specific event (Fig. 1). Second, we showed that ectopic expression of *SFRS1* cDNA transforms immortal rodent fibroblasts in culture, which can then form high-grade sarcomas in nude mice (Fig. 2). Third, we found that restoring normal *SFRS1* expression in these cells, or in a human lung-cancer cell line with SF2/ASF upregulation, reversed the transformed state of these cells and their tumorigenicity in nude mice (Fig. 6). Our data are thus consistent with *SFRS1* being a proto-oncogene.

Many tumors without amplification of *SFRS1* expressed abnormally high levels of SF2/ASF (Fig. 1); it remains to be determined whether this upregulation involves mutational, transcriptional or epigenetic mechanisms. A mutation in the coding region of *SFRS1* was recently identified in a breast-cancer cell line—together with mutations in many other genes—so it is possible that this oncogene can also be activated by mutation[34].

In addition to its oncogenic activity, SF2/ASF protects E1A-sensi-tized MEF cells against apoptosis (Fig. 3). This antiapoptotic effect seems to be downstream of p53, or independent of it, as SF2/ASF overexpression did not prevent E1A-mediated induction of p53 and its target gene *MDM2*, or induction of other E2F1 targets, such as caspases 2, 7 and 9 (Fig. 3 and data not shown). In addition, SF2/ASF and SRp55 cooperated with oncogenic Ras to enhance the proliferation of p53-null MEF cells (Fig. 3).

Mechanism of SF2/ASF-driven transformation

Our working hypothesis has been that increased levels of SF2/ASF elicit concentration-dependent changes in the alternative splicing of certain key target genes and that the combined effects of the resulting isoforms drive the multistep transformation process.

We therefore investigated the expression of several endogenous genes that are involved in transformation and apoptosis and that are known to be regulated by alternative splicing. Many of these genes were not affected by overexpression or knockdown of individual SR or hnRNP A1 proteins, at least within the transcript regions we probed (Supplementary Table 1). However, we identified several cancerrelated genes whose patterns of isoform expression were strongly affected by SF2/ASF levels, including the putative tumor suppressor *BIN1*, the transcription factor *TEF-1*, the kinase MNK2 and the ribosomal-protein kinase S6K1 (Figs. 4 and 6, Supplementary Fig. 1 and Supplementary Table 1). These observations markedly expand the number of known endogenous alternatively spliced gene targets of SR proteins[20,35–38].

The Ras-MAPK and PI3K-mTOR pathways are deregulated in many cancers, contributing to the establishment and maintenance of the transformed phenotype[28,39]. Therefore, we investigated whether SF2/ASF can modulate these signaling pathways. We found that alternative splicing of two downstream components of these pathways, MNK2 and S6K1, is deregulated by SF2/ASF overexpression, leading to activation of these enzymes independently of upstream signaling activity.

The effects of SF2/ASF on the alternative splicing of *MKNK2* link SF2/ASF to the Ras-MAPK pathway. Notably, activation of eIF4E phosphorylation by SF2/ASF bypasses the proximal steps of this pathway, so that SF2/ASF activates MNK2b splicing and eIF4E phosphorylation downstream of MAPK activation (Fig. 5d). As eIF4E phosphorylation contributes to its oncogenic activity[40], modulation of MNK2 splicing is probably an important branch of SF2/ASF's transforming activity (Fig. 8).

Overexpression of SF2/ASF also led to altered isoform expression of S6K1, which is involved in cell growth and cell death[32] (Fig. 4d,e, Fig. 5a and Supplementary Fig. 1). SF2/ASF had a strong effect on the expression of mouse, human and rat S6K1 isoforms with distinct C termini (Fig. 4d,e and Supplementary Figs. 1 and 3). SF2/ASF increased the expression of isoform-2, which includes three alternative cassette exons between exons 6 and 7 that are skipped in isoform-1, as well as an alternative poly(A) site within intron 7 (Supplementary Fig. 3). This mRNA isoform encodes a 316-residue polypeptide that lacks the regulatory domain at the C terminus of isoform-1, which is phosphorylated by mTOR[29,41], and that has half of its kinase domain altered. The sequences of the two isoforms are identical for the first 5 of 12 conserved kinase subdomains, and they diverge within subdomain VIA and thereafter[42] (Supplementary Fig. 3). Thus, isoform-2 has the same ATP-binding site as isoform-1 but lacks a conserved aspartate in the presumptive active site, so its functional properties probably differ from those of the previously studied isoform-1 of S6K1, and it is probably inactive as a kinase.

Remarkably, we found that isoform-2 transforms NIH 3T3 cells, whereas isoform-1 does not, even when present at much higher levels (Fig. 5b,c). Thus, p31 has potent transforming activity even as a minor isoform. This result provides one possible molecular explanation for SF2/ASF-mediated transformation: the induction of an oncogenic isoform that is a specific splicing target of SF2/ASF (Fig. 8). The fact that SF2/ASF expression in lung tumors correlates with the S6K1 isoform-2/isoform-1 mRNA ratio (Supplementary Fig. 4) further supports the potential relevance of this alternative splicing event in human tumors. A stronger correlation might be precluded by the effect of other splicing factors on S6K1 splicing (Supplementary Table 1), differences in translational efficiency and protein turnover between the two isoforms, stromal cell contamination and tumor-type heterogeneity. However, we found a clear correlation between SF2/ASF levels and S6K1 isoform mRNA and protein levels in human lung and breast cancer cell lines (Supplementary Fig. 1).

In addition, we found that alternative splicing of mouse *Rps6kb1* is essential for SF2/ASF-mediated transformation, as knockdown of isoform-2, but not isoform-1, blocked SF2/ASF-mediated transformation (Figs. 7 and 8 and Supplementary Fig. 5). Although we obtained

only one shRNA that was specific for S6K1 isoform-2, we observed knockdown of this isoform at the mRNA and protein level in human and mouse cells. The phenotypic effects of this knockdown on transformation were consistent with those of SF2/ASF knockdown and opposite to those of isoform-2 or SF2/ASF overexpression, arguing against off-target effects.

The 17q23 genomic region, which is commonly amplified in breast and other tumors, comprises several putative proto-oncogenes (*RPS6KB1*, *PPM1D* and *TBX2*)[7,21]. Our results show that *SFRS1* is a novel proto-oncogene that also resides in this region and modulates the oncogenicity of S6K1, the product of *RPS6KB1*. The physical and functional linkage between *SFRS1* and *RPS6KB1* indicates that when both genes are coamplified, they might synergize to enhance tumorigenicity, similar to oncogenes that are part of other amplicons[43,44].

In addition to the modulation of MNK2 and S6K1 splicing, we found that SF2/ASF regulates the expression of isoforms of *BIN1*, which counteracts the *MYC* proto-oncogene[11,45]. SF2/ASF induces the inactive isoforms of this putative tumor suppressor[11], indicating that SF2/ASF upregulation can modulate the apoptotic activity of MYC and that SF2/ASF and MYC might cooperate.

It is possible that the effects we observed on alternative splicing of endogenous genes are indirect. However, these effects are consistent with the known properties of SF2/ASF, based on previous biochemical and transfection experiments, including the concentration dependence, shown here by the reciprocal effects of overexpression and RNAi. In some cases, overexpression of one splicing factor might affect expression of another splicing factor through various feedback and cross-regulatory loops[46], potentially initiating a regulatory cascade. For example, overexpression of SF2/ASF partially abrogated expression of endogenous SF2/ASF (Fig. 5d and Supplementary Fig. 2) and affected the alternative splicing of hnRNP A2/B1 (Fig. 4). Some of the phenotypic effects of SF2/ASF and the other splicing factors could involve other aspects of gene expression besides alternative splicing, given their reported effects on post-transcriptional processes such as mRNA turnover, export and translation[25].

Knockdown of SF2/ASF can induce genomic instability, including double-stranded DNA breaks and a high rate of mutations[47]. However, it is unlikely that SF2/ASF overexpression triggers a similar response, as we did not observe induction of p53—reflecting DNA damage—although the p53 pathway remained intact, as shown by the ability of E1A to induce p53 and its target gene, *MDM2* (Fig. 3c and data not shown). In addition, we did not observe mutations in several cDNAs from cells overexpressing SF2/ASF (data not shown).

Potential roles of other splicing factors in cancer

Although SF2/ASF is the first splicing factor shown to be directly involved in cancer, the effects we observed for some of the other splicing factors in various oncogenic assays, together with their expression patterns, indicate that they might also contribute to cancer development in appropriate contexts. SR proteins share a high degree of homology and have similar splicing activities, although the degree of overlap in their *in vivo* targets is

unknown[2]. Our analysis of endogenous transcripts in cells that stably overexpressed individual SR proteins showed only limited overlap in the alternative splicing events affected by each protein (Fig. 4 and Supplementary Table 1). In some of the functional assays, particular SR proteins had effects similar to those of SF2/ASF: for example, SC35 enhanced colony formation in NIH 3T3 and Rat1 cells and slightly inhibited apoptosis (Fig. 2b, Fig. 3b and Supplementary Fig. 2); and SRp55, which is overexpressed and in some cases amplified in certain cancers (Fig. 1 and data not shown), cooperated with Ras to enhance proliferation (Fig. 3) and anchorage-independent growth (data not shown) and enhanced eIF4E phosphorylation (Fig. 5d). Although each of these oncogenic assays reflects effects on apoptosis and cell proliferation that contribute to transformation, multiple changes are required to achieve tumorigenicity *in vivo*[28]. Of the splicing factors we tested, only SF2/ASF had strong transforming activity, and it was far more tumorigenic in nude mice than hnRNP A1 (Fig. 2), indicating that only SF2/ASF affects alternative splicing of the appropriate set of targets in NIH 3T3 cells in such a way as to promote rapid tumorigenesis. However, some of the related splicing factors might be tumorigenic in other cell types or genetic contexts.

Role of SF2/ASF in tumor maintenance

The ability of SF2/ASF to transform cells demonstrates its role in tumor establishment. However, a separate question is whether it is also required during the late stages of tumorigenesis, when many other events—for example, accumulation of other mutations—have taken place. We found that knockdown of SF2/ASF in NIH 3T3 cells that had been transduced with SF2/ASF, and in lung carcinoma cells with high SF2/ASF expression, inhibited tumor formation in nude mice (Fig. 6), showing that SF2/ASF can also contribute to tumor maintenance *in vivo*. Thus, partial inhibition of upregulated SF2/ASF might have therapeutic utility, even in advanced tumors. Although these results represent a proof of principle, other tumors that were not initially driven by SF2/ASF upregulation are unlikely to revert through its inhibition.

Together, our results show that the splicing factor SF2/ASF is a powerful proto-oncogene whose upregulation results in the simultaneous modulation of alternative splicing of key target genes. These post-transcriptional changes in gene expression result, on one hand, in the inactivation of putative tumor suppressors such as BIN1, and, on the other hand in the generation of oncogenic isoforms—for example, S6K1 isoform-2 (Fig. 8). In addition, by modulating the alternative splicing of MNK2, SF2/ASF promotes eIF4E phosphorylation, bypassing upstream Ras-MAPK signaling. We do not expect just one or two genes to mediate the transforming effects of SF2/ASF; rather, several target genes are likely to be involved. However, we determined that alternative splicing of *RPS6KB1* is a key target for SF2/ASF-mediated transformation. Finally, our results imply that splicing factors, and specifically SF2/ASF, are potentially useful targets for the development of cancer therapies.

Methods

Cells

NIH 3T3, Rat1, HeLa, MEF and IMR90 cells were grown in DMEM, and NCI-H460 cells in RPMI 1640, supplemented with 10% (v/v) FBS, penicillin and streptomycin. To generate stable cell pools, NIH 3T3, Rat1, MEF and IMR90 cells were infected with pBABE-puro or pWZL-hygro retroviral vectors[48] expressing T7-tagged human splicing factor cDNAs. We replaced the medium 24 h after infection, and 24 h later, infected cells were selected with puromycin (2 μg ml^{-1}) or hygromycin (200 μg ml^{-1}) for 72 h. In the case of double infection with pWZL-hygro-E1A or pWZL-hygro-Ras[48], cells were treated with hygromycin for 72 h after selection with puromycin. For expression of SF2/ASF in a retroviral-based ecdysone-inducible system[49], we infected NIH 3T3 inducible host cells with T7-SF2/ASF in a PI-hygro vector. After selection in 200 μg ml^{-1} hygromycin, cells were analyzed for inducibility by western blotting. Induction was carried out with ponasterone A (A.G. Scientific, Inc.). Mouse *Rps6kb1* isoform-1 and isoform-2 (Ensembl transcripts ENSMUST00000020824 and ENSMUST00000058286, respectively) were amplified by RT-PCR from MEF-cell total RNA using specific primers to introduce an N-terminal T7 tag (Supplementary Table 2 online). The cDNAs were cloned in pBABE and verified by sequencing, and NIH 3T3 and MEF cells stably expressing these isoforms were generated as above.

RNA interference

For inhibition of SF2/ASF and PP2Cγ expression, HeLa cells were seeded (7×10^4 cells per well) in six-well plates in antibiotic-free medium. After 24 h, cells were transfected with 200 pmol short interfering RNA (siRNA) per well (Dharmacon), using Oligofectamine (Invitrogen). After a further 72 h, cells were lysed, and protein and RNA were extracted as described below. siRNA target sequences were 5′-TTGACCACTGAAGAAGTCA-3′ (PP2Cγ) and 5′-ACGATTGCCGCATCTACGT-3′ (SF2/ASF); both siRNA strands had 3′ dTdT tails. shRNAs against mouse and human SF2/ASF (SF2-sh1, SF2-sh2), a control shRNA with two mismatches (SF2-$sh1^m$) and shRNAs against mouse and human S6K1 isoforms 1 and 2 (p70-sh1, p70-sh2, p31-sh1) (Supplementary Table 2) were cloned in the LMP retroviral vector. NCI-H460 and NIH 3T3 cells were transduced and selected as described above.

Immunoblotting

Cells were lysed in SDS and analyzed for total protein concentration as described[50]. We separated 30 μg of total protein from each cell lysate by SDS-PAGE and transferred it onto a nitrocellulose membrane. The membranes were blocked and probed with antibodies, using enhanced chemiluminescence detection. Quantification was done with NIH-Image software (http://rsb.info.nih.gov/nih-image/download.html). Primary antibodies were β-catenin (1:5,000, Transduction Laboratories; or 1:2,000, Sigma); SF2/ASF (mAb AK96 culture supernatant, 1:100)[22]; hnRNP A1 (mAb 4B10, 1:1,000); hnRNP A2 (mAb DP3, 1:1,000); SRp55 (mAb 8-1-28 culture supernatant); lamin A-C (1:1,000, Santa Cruz Biotechnology); T7 tag (1:5,000, Novagen); MDM2 (mAb 2A10 culture supernatant, 1:100); p53 (1:1,000 CM5, NovoCastra); S6K1 (N terminus; 1:200, Pharmingen); and phosphor–eIF4E Ser209,

eIF4E, phospho-ERK (T202/Y204), ERK1/2 (1:1,000, Cell Signaling Technology). Secondary antibodies were HRP-conjugated goat anti-mouse or anti-rabbit IgG (H+L) (1:10,000, Pierce).

Immunoprecipitation and western blotting

Cells were lysed with RIPA buffer, the lysates were cleared by centrifugation at 12,000g for 15 min, and the supernatant was passed through a 0.45-μm filter. For each cell line, 50 μl of 50% (v/v) protein G–Sepharose beads was incubated with 2 μg of antibody against S6K1 (Pharmingen) for 1 h at 4 °C. The beads were then incubated with 1 mg of total protein from each lysate for 2–3 h. The beads were washed three times with RIPA buffer and boiled for 5 min in 50 μl of 2 × SDS sample buffer. After SDS-PAGE and transfer as above, the membranes were probed with antibodies against S6K1. In some experiments, TrueBlot secondary antibody (1:1,000; eBioscience) was used to avoid detection of the heavy and light chains.

Anchorage-independent growth

Colony formation in soft agar was assayed as described[50]. Plates were incubated at 37 °C and 6% CO_2. After 10–18 d, colonies from ten different fields in each of two wells were counted for each transductant pool, and the average number of colonies per well was calculated. The colonies were stained as described and photographed under a light microscope at 100 × magnification.

Tumors in nude mice

NIH 3T3 stable pools expressing the indicated splicing factors or lacZ, were injected (2×10^6 cells per site in 200 μl) subcutaneously into each rear flank of (NIH nu/nu) nude mice, using a 26-gauge needle. Tumor growth was monitored as described[48]. For NCI-H460 cells expressing SF2/ASF shRNA, nude mice were γ-irradiated (400 rad) before subcutaneous injection with 10^6 cells per site as above.

Apoptosis experiments

Primary MEFs were infected with the indicated retroviruses, with or without hygromycin-resistant E1A retrovirus. After 72 h of puromycin and 72 h of hygromycin selection, cells were allowed to recover for 24 h and seeded (4×10^4 cells per well) on 12-well plates (Nunc). After 24 h, the medium was replaced with fresh medium containing the indicated adriamycin concentrations, and the cells were incubated for 24 h. For serum starvation, cells were washed with PBS and grown for 24 h in medium containing 0.1% (v/v) FBS. Cells were then trypsinized, and dead cells were stained with trypan blue. These assay conditions induce apoptotic cell death[24].

Growth curves

Primary MEFs from wild-type or $p53^{-/-}$ mice 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[50], and the A_{650} of the acid-extracted stain was measured on a plate reader (BioRad).

RT-PCR

Total RNA was extracted with Trizol reagent (Invitrogen) and 2 μg of total RNA was reverse transcribed with SuperScript II (Invitrogen). PCR was performed on 1/10 (2 μl) of the cDNA, in 50-μl reactions containing 0.2 mM dNTP mix, 10× PCR buffer with 15 mM $MgCl_2$ (Invitrogen), 2.5 units of TaqGold (Invitrogen) and 0.2 μM of each primer; 5% (v/v) DMSO was included in some reactions. For semiquantitative PCR, primers were end-labeled with [γ-^{32}P]ATP and polynucleotide kinase (NEB). PCR conditions were 95 °C for 5 min, then 35 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 45 s, followed by 10 min at 72 °C. For radioactive PCR, 30 cycles were used. PCR products were separated on 1.5% or 2% agarose gels or on 6% nondenaturing polyacrylamide gels.

Quantitative PCR

DNA copy number was quantified using ABI 7700 or ABI 7900 Sequence Detection Systems (Applied Biosystems). We determined the mRNA levels of SF2/ASF, hnRNPA1, SRp55 and S6K1 isoforms in tumors and in the corresponding normal tissues (Clontech) by performing quantitative PCR with fluorogenic TaqMan probes, directly after reverse transcription. Absolute mRNA levels for SF2/ASF, hnRNP A1 and SRp55 were within 50% for five samples of each normal tissue. Actin was used as a reference probe.

Primers

For primers used, see Supplementary Table 2.

Statistical analysis

Where appropriate, the data are presented as the means ± s.d. Data points were compared by the unpaired two-tailed Student's t test, and the calculated *P*-values are indicated in the figure legends. For soft-agar colony assays, means ± s.d. and *P*-values were calculated for ten fields per well in duplicate wells for each transductant pool. For the analysis of the correlation between SF2/ASF expression and S6K1 isoform ratios in 50 lung tumors, the correlation coefficient *r* was calculated by nonparametric Spearman rank correlation, and a bootstrap technique was used to determine a 95% confidence interval.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Johnson JM, et al. Genome-wide survey of human alternative pre-mRNA splicing with exon junction microarrays. Science. 2003; 302:2141–2144. [PubMed: 14684825]

2. Black DL. Mechanisms of alternative pre-messenger RNA splicing. Annu Rev Biochem. 2003; 72:291–336. [PubMed: 12626338]

3. Mayeda A, Krainer AR. Regulation of alternative pre-mRNA splicing by hnRNP A1 and splicing factor SF2. Cell. 1992; 68:365–375. [PubMed: 1531115]

4. Cáceres JF, Stamm S, Helfman DM, Krainer AR. Regulation of alternative splicing *in vivo* by overexpression of antagonistic splicing factors. Science. 1994; 265:1706–1709. [PubMed: 8085156]

5. Xu Q, Lee C. Discovery of novel splice forms and functional analysis of cancerspecific alternative splicing in human expressed sequences. Nucleic Acids Res. 2003; 31:5635–5643. [PubMed: 14500827]

6. Wang Z, et al. Computational analysis and experimental validation of tumor-associated alternative RNA splicing in human cancer. Cancer Res. 2003; 63:655–657. [PubMed: 12566310]

7. Venables JP. Aberrant and alternative splicing in cancer. Cancer Res. 2004; 64:7647–7654. [PubMed: 15520162]

8. Venkitaraman AR. Cancer susceptibility and the functions of BRCA1 and BRCA2. Cell. 2002; 108:171–182. [PubMed: 11832208]

9. Hammes A, et al. Two splice variants of the Wilms' tumor 1 gene have distinct functions during sex determination and nephron formation. Cell. 2001; 106:319–329. [PubMed: 11509181]

10. Wu JY, Tang H, Havlioglu N. Alternative pre-mRNA splicing and regulation of programmed cell death. Prog Mol Subcell Biol. 2003; 31:153–185. [PubMed: 12494766]

11. Ge K, et al. Mechanism for elimination of a tumor suppressor: aberrant splicing of a brain-specific exon causes loss of function of Bin1 in melanoma. Proc Natl Acad Sci USA. 1999; 96:9689–9694. [PubMed: 10449755]

12. Kumar A, Baker SJ, Lee CM, Reddy EP. Molecular mechanisms associated with the regulation of apoptosis by the two alternatively spliced products of c-Myb. Mol Cell Biol. 2003; 23:6631–6645. [PubMed: 12944488]

13. Tennenbaum T, et al. A splice variant of alpha 6 integrin is associated with malignant conversion in mouse skin tumorigenesis. Proc Natl Acad Sci USA. 1995; 92:7041–7045. [PubMed: 7624366]

14. Steinman HA, et al. An alternative splice form of Mdm2 induces p53-independent cell growth and tumorigenesis. J Biol Chem. 2004; 279:4877–4886. [PubMed: 14612455]

15. Tockman MS, et al. Prospective detection of preclinical lung cancer: results from two studies of heterogeneous nuclear ribonucleoprotein A2/B1 overexpression. Clin Cancer Res. 1997; 3:2237–2246. [PubMed: 9815620]

16. Ghigna C, Moroni M, Porta C, Riva S, Biamonti G. Altered expression of heterogenous nuclear ribonucleoproteins and SR factors in human colon adenocarcinomas. Cancer Res. 1998; 58:5818–5824. [PubMed: 9865741]

17. Patry C, et al. Small interfering RNA-mediated reduction in heterogeneous nuclear ribonucleoparticule A1/A2 proteins induces apoptosis in human cancer cells but not in normal mortal cell lines. Cancer Res. 2003; 63:7679–7688. [PubMed: 14633690]

18. He X, Ee PL, Coon JS, Beck WT. Alternative splicing of the multidrug resistance protein 1/ATP binding cassette transporter subfamily gene in ovarian cancer creates functional splice variants and is associated with increased expression of the splicing factors PTB and SRp20. Clin Cancer Res. 2004; 10:4652–4660. [PubMed: 15269137]

19. Fischer DC, et al. Expression of splicing factors in human ovarian cancer. Oncol Rep. 2004; 11:1085–1090. [PubMed: 15069551]

20. Ghigna C, et al. Cell motility is controlled by SF2/ASF through alternative splicing of the Ron protooncogene. Mol Cell. 2005; 20:881–890. [PubMed: 16364913]

21. Sinclair CS, Rowley M, Naderi A, Couch FJ. The 17q23 amplicon and breast cancer. Breast Cancer Res Treat. 2003; 78:313–322. [PubMed: 12755490]

22. Càceres JF, Screaton GR, Krainer AR. A specific subset of SR proteins shuttles continuously between the nucleus and the cytoplasm. Genes Dev. 1998; 12:55–66. [PubMed: 9420331]

23. Sanford JR, Gray NK, Beckmann K, Càceres JF. A novel role for shuttling SR proteins in mRNA translation. Genes Dev. 2004; 18:755–768. [PubMed: 15082528]

24. de Stanchina E, et al. E1A signaling to p53 involves the p19(ARF) tumor suppressor. Genes Dev. 1998; 12:2434–2442. [PubMed: 9694807]
25. Huang Y, Steitz JA. SRprises along a messenger's journey. Mol Cell. 2005; 17:613–615. [PubMed: 15749011]
26. Mixon M, Kittrell F, Medina D. Expression of Brca1 and splice variant Brca1-delta11 RNA levels in mouse mammary gland during normal development and tumorigenesis. Oncogene. 2000; 19:5237–5243. [PubMed: 11077440]
27. Sakamuro D, Elliott KJ, Wechsler-Reya R, Prendergast GC. BIN1 is a novel MYC-interacting protein with features of a tumour suppressor. Nat Genet. 1996; 14:69–77. [PubMed: 8782822]
28. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000; 100:57–70. [PubMed: 10647931]
29. Holz MK, Ballif BA, Gygi SP, Blenis J. mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. Cell. 2005; 123:569–580. [PubMed: 16286006]
30. Waskiewicz AJ, et al. Phosphorylation of the cap-binding protein eukaryotic translation initiation factor 4E by protein kinase Mnk1 *in vivo*. Mol Cell Biol. 1999; 19:1871–1880. [PubMed: 10022874]
31. Scheper GC, et al. The N and C termini of the splice variants of the human mitogenactivated protein kinase-interacting kinase Mnk2 determine activity and localization. Mol Cell Biol. 2003; 23:5692–5705. [PubMed: 12897141]
32. Harada H, Andersen JS, Mann M, Terada N, Korsmeyer SJ. p70S6 kinase signals cell survival as well as growth, inactivating the pro-apoptotic molecule BAD. Proc Natl Acad Sci USA. 2001; 98:9666–9670. [PubMed: 11493700]
33. Scheper GC, Morrice NA, Kleijn M, Proud CG. The mitogen-activated protein kinase signal-integrating kinase Mnk2 is a eukaryotic initiation factor 4E kinase with high levels of basal activity in mammalian cells. Mol Cell Biol. 2001; 21:743–754. [PubMed: 11154262]
34. Sjöblom T, et al. The consensus coding sequences of human breast and colorectal cancers. Science. 2006; 314:268–274. [PubMed: 16959974]
35. Kim S, Shi H, Lee DK, Lis JT. Specific SR protein-dependent splicing substrates identified through genomic SELEX. Nucleic Acids Res. 2003; 31:1955–1961. [PubMed: 12655012]
36. Xu X, et al. ASF/SF2-regulated CaMKIIdelta alternative splicing temporally reprograms excitation-contraction coupling in cardiac muscle. Cell. 2005; 120:59–72. [PubMed: 15652482]
37. Wang HY, Xu X, Ding JH, Bermingham JR Jr, Fu XD. SC35 plays a role in T cell development and alternative splicing of CD45. Mol Cell. 2001; 7:331–342. [PubMed: 11239462]
38. Blanchette M, Green RE, Brenner SE, Rio DC. Global analysis of positive and negative pre-mRNA splicing regulators in *Drosophila*. Genes Dev. 2005; 19:1306–1314. [PubMed: 15937219]
39. Bjornsti MA, Houghton PJ. Lost in translation: dysregulation of cap-dependent translation and cancer. Cancer Cell. 2004; 5:519–523. [PubMed: 15193254]
40. Topisirovic I, Ruiz-Gutierrez M, Borden KL. Phosphorylation of the eukaryotic translation initiation factor eIF4E contributes to its transformation and mRNA transport activities. Cancer Res. 2004; 64:8639–8642. [PubMed: 15574771]
41. Schalm SS, Blenis J. Identification of a conserved motif required for mTOR signaling. Curr Biol. 2002; 12:632–639. [PubMed: 11967149]
42. Hanks SK, Hunter T. Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. FASEB J. 1995; 9:576–596. [PubMed: 7768349]
43. Zender L, et al. Identification and validation of oncogenes in liver cancer using an integrative oncogenomic approach. Cell. 2006; 125:1253–1267. [PubMed: 16814713]
44. Kim M, et al. Comparative oncogenomics identifies NEDD9 as a melanoma metastasis gene. Cell. 2006; 125:1269–1281. [PubMed: 16814714]
45. DuHadaway JB, Sakamuro D, Ewert DL, Prendergast GC. Bin1 mediates apoptosis by c-Myc in transformed primary cells. Cancer Res. 2001; 61:3151–3156. [PubMed: 11306501]
46. Bourgeois CF, Lejeune F, Steévenin J. Broad specificity of SR (serine/arginine) proteins in the regulation of alternative splicing of pre-messenger RNA. Prog Nucleic Acid Res Mol Biol. 2004; 78:37–88. [PubMed: 15210328]

47. Li X, Manley JL. Inactivation of the SR protein splicing factor ASF/SF2 results in genomic instability. Cell. 2005; 122:365–378. [PubMed: 16096057]

48. McCurrach ME, Lowe SW. Methods for studying pro- and antiapoptotic genes in nonimmortal cells. Methods Cell Biol. 2001; 66:197–227. [PubMed: 11396004]

49. Stolarov J, et al. Design of a retroviral-mediated ecdysone-inducible system and its application to the expression profiling of the PTEN tumor suppressor. Proc Natl Acad Sci USA. 2001; 98:13043–13048. [PubMed: 11687610]

50. Karni R, Jove R, Levitzki A. Inhibition of pp60c-Src reduces Bcl-XL expression and reverses the transformed phenotype of cells overexpressing EGF and HER-2 receptors. Oncogene. 1999; 18:4654–4662. [PubMed: 10467412]

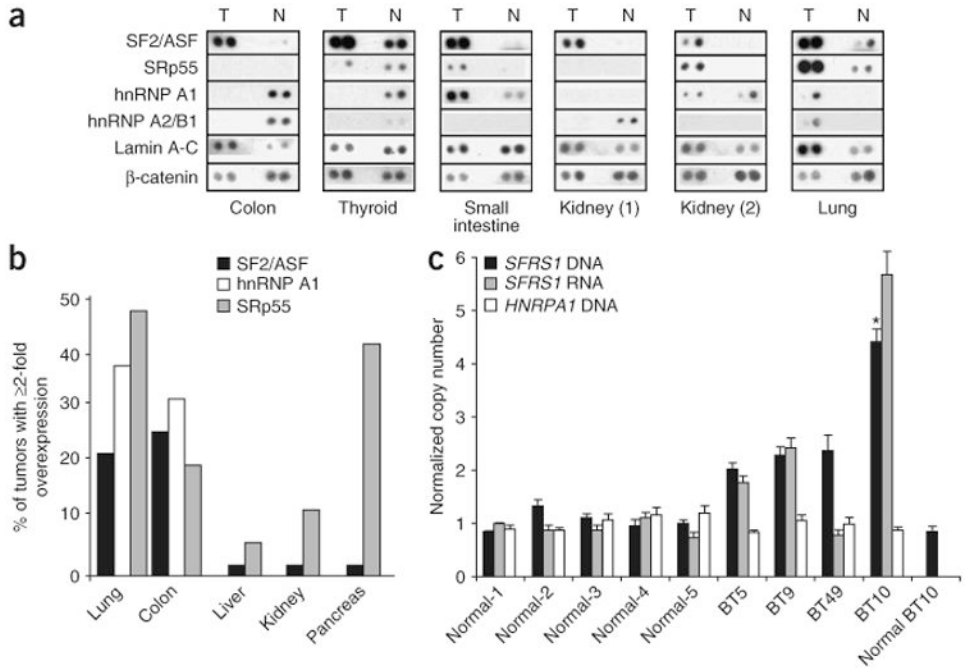

Figure 1.

Upregulation and amplification of SF2/ASF in human tumors. (**a**) A protein microarray (Biochain) consisting of 47 pairs of tumor samples (T) and their normal tissue counterparts (N), spotted as duplicates, was probed with the indicated antibodies. Representative tumor samples overexpressing SF2/ASF and corresponding controls: colon, adenocarcinoma; thyroid, follicular carcinoma; small intestine, leiomyoma; kidney (1), clear cell carcinoma; kidney (2), granular cell carcinoma. (**b**) Expression of splicing factor mRNA was measured by reverse-transcription quantitative PCR (RT-qPCR) in 50 tumor samples and five normal samples from each indicated tissue and normalized to β-actin mRNA. Bars show the proportion of tumors overexpressing the mRNA more than two-fold over the mean of the five normal samples. Expression of hnRNP A1 was determined only in lung and colon tumors. (**c**) DNA and RNA were measured by qPCR and RT-qPCR in 50 breast tumor samples and five normal samples. Data for the four tumors (infiltrating ductal carcinomas) with elevated DNA copy numbers of SF2/ASF are shown relative to the mean of the normal samples. Normal BT10, surrounding normal breast tissue from the patient with the BT10 tumor. Means ± s.d. from triplicate qPCR measurements are shown. *P = 4.9 times; 10^{-10} for the BT10 versus normal BT10 comparison; n = 6.

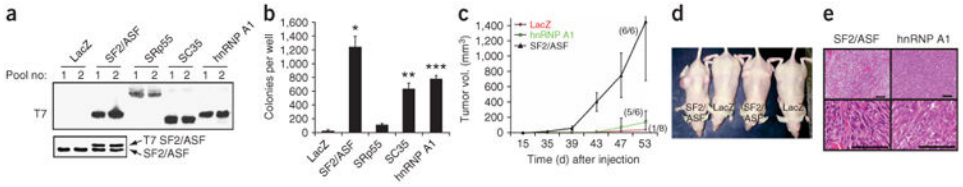

Figure 2.

SF2/ASF transforms immortal cells and is tumorigenic in nude mice. (**a**) Total proteins from duplicate pools of NIH 3T3 stable cell lines transduced with retroviruses expressing LacZ or T7-tagged SF2/ASF, SRp55, SC35 or hnRNP A1 were analyzed by western blotting with anti-T7. The first four samples were also analyzed with anti-SF2/ASF (below) to compare the expression of endogenous and transduced SF2/ASF (**b**) Quantification of soft-agar colony formation by the stably transduced cell lines. The mean ± s.d. for each pair of pooled lines is shown. *P*-values in pairwise comparisons to the LacZ control: **P*=4 × 10^{-8}; ***P* = 10^{-7}; ****P* = 7 × 10^{-8}. (**c**) Tumor growth curve in mice injected with 2 × 10^{6} cells from the indicated NIH 3T3 pooled lines. The number of tumors formed per number of injections is shown in parentheses. SRp55 and SC35 cell lines (six each) did not form tumors during the same time course (data not shown). Error bars, s.d. (**d**) Representative mice injected with LacZ-expressing control cells, or with SF2/ASF-overexpressing cells. (**e**) Light micrographs of formalin-fixed, paraffin-embedded tissue sections from tumors derived from NIH 3T3 cells overexpressing SF2/ASF of hnRNP A1, stained with hematoxylin and eosin. Scale bars, 100 μm.

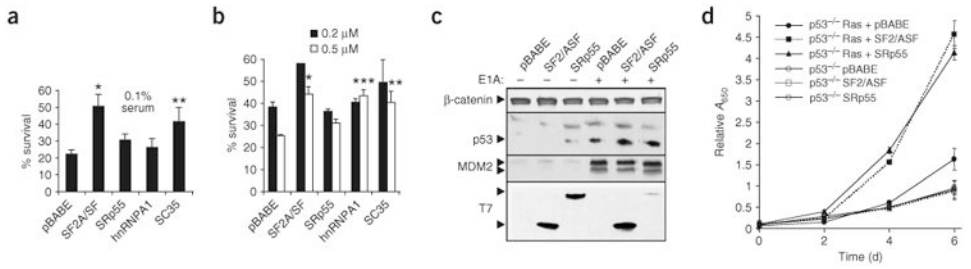

Figure 3.

SF2/ASF overexpression protects E1A-sensitized MEF cells against apoptosis and enhances the proliferation of Ras-transformed cells. Primary wild-type MEF cells were transduced with retroviruses expressing the indicated human splicing factor cDNAs, or with the empty vector; some cells were cotransduced with adenovirus E1A or activated Ras, as indicated. Error bars indicate s.d. (**a**) After drug selection, cells coinfected with E1A were plated and serum-starved, and cell death was measured by trypan blue staining. *P*-values in pairwise comparisons to the pBABE control: $*P = 5\times 10^{-8}$; $**P = 5 \times 10^{-5}$; $n = 3$. (**b**) Cells coinfected with E1A were plated and treated with the indicated adriamycin concentrations after 24 h, and cell death was measured as in **a**. $*P = 0.003$, $**P = 0.01$, $***P = 0.006$; $n = 3$; for SF2/ASF at low adriamycin, $n = 1$. (**c**) MEF cells were infected with the indicated retroviruses, plated after single or double selection (10^6 cells per 10-cm plate) and lysed in SDS after 24 h. Western blots were carried out using the indicated primary antibodies. The reduction in SRp55 in the presence of E1A was not observed with other cell lines (data not shown). (**d**) $p53^{-/-}$ MEF cells were transduced with the empty vector or with retroviruses expressing SF2/ASF or SRp55, either alone or with oncogenic Ras. Cells were fixed at 48-h intervals and stained with methylene blue. Each point represents the mean relative absorbance from six wells.

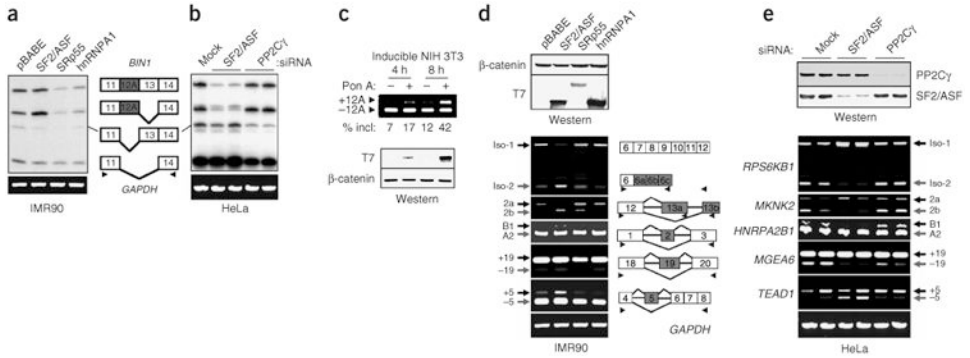

Figure 4.

Specific alternative splicing changes induced by overexpression of splicing factors or knockdown of SF2/ASF. (**a**) Human primary cells (IMR90) were infected with the indicated retroviruses, and stable pools were selected. Total RNA was analyzed by radioactive RT-PCR, nondenaturing PAGE and autoradiography using the indicated primers (arrowheads). *GAPDH* mRNA was analyzed as a control. The exon structure of each *BIN1* isoform is indicated. (**b**) HeLa cells were transfected in duplicate with siRNAs specific for SF2/ASF or PP2Cγ, or mock-transfected. After 72 h, total RNA was analyzed as in **a**. (**c**) NIH 3T3 cell lines with inducible expression of SF2/ASF were plated and induced 24 h later with 5 μM ponasterone A for the indicated times. RNA was analyzed by radioactive RT-PCR; quantification of the proportion of exon inclusion (% incl) by phosphorimaging analysis is shown below the ethidium bromide–stained agarose gel. Only two isoforms were detected in these mouse cells. Expressed T7-tagged SF2/ASF and endogenous β-catenin were analyzed by western blotting. (**d**) Total RNA and protein were extracted from IMR90 primary cells overexpressing the indicated splicing factors. For both panels, total RNA was analyzed by RT-PCR using the indicated isoform-specific primers (arrowheads). Alternatively spliced exons are shaded. Western blotting was carried out as in **c**. (**e**) Total RNA and protein were extracted from duplicate samples of HeLa cells treated with siRNAs targeting SF2/ASF or PP2Cγ, or mock-treated. PP2Cγ and SF2/ASF protein levels were analyzed by western blotting. RNA samples were analyzed as in **d**.

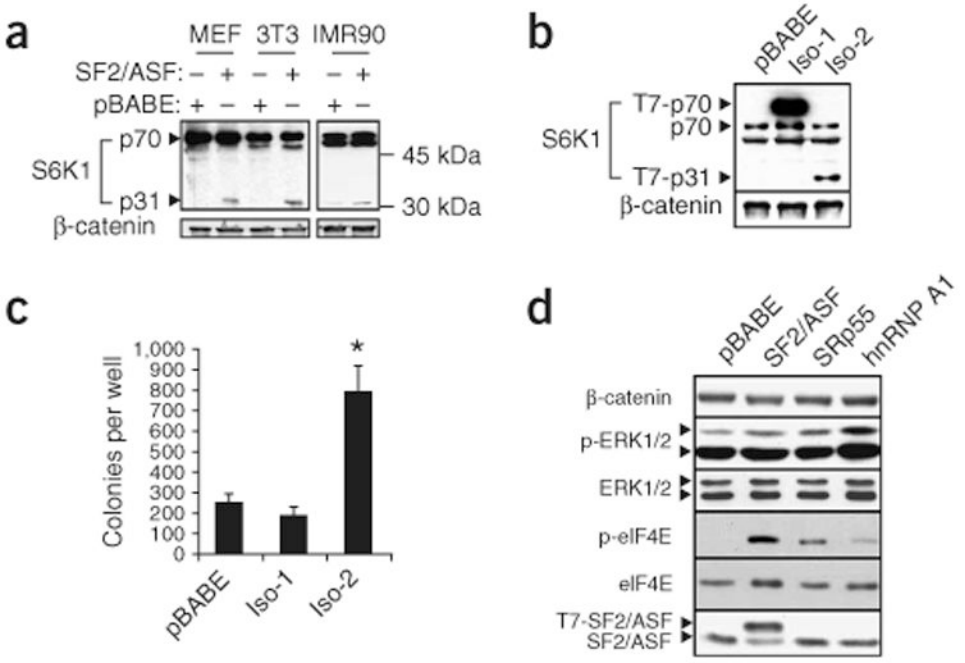

Figure 5.

SF2/ASF promotes expression of an oncogenic isoform of S6K1 and induces eIF4E phosphorylation. (**a**) MEF, NIH 3T3 and IMR90 cells were transduced with pBABE or SF2/ASF retroviruses and lysed in SDS. Western blotting was carried out using a monoclonal antibody against the N terminus of S6K1 to detect the endogenous isoforms and one against p-catenin as a loading control. (**b**) NIH 3T3 cells were transduced with pBABE or S6K1 isoform-1 or isoform-2 retroviruses, and lysates were analyzed by western blotting as in **a**. The S6K1 antibody reacts with both the endogenous and T7-tagged S6K1 isoforms, but the endogenous isoform-2 is not detected in this exposure. (**c**) Aliquots of cells in **b** were seeded in soft agar, and colonies were counted 14 d later. Means ± s.d. are shown; **P* = 6×10^{-7} compared to the pBABE control. (**d**) MEF cells were transduced with the indicated retroviruses and analyzed by western blotting with the indicated antibodies.

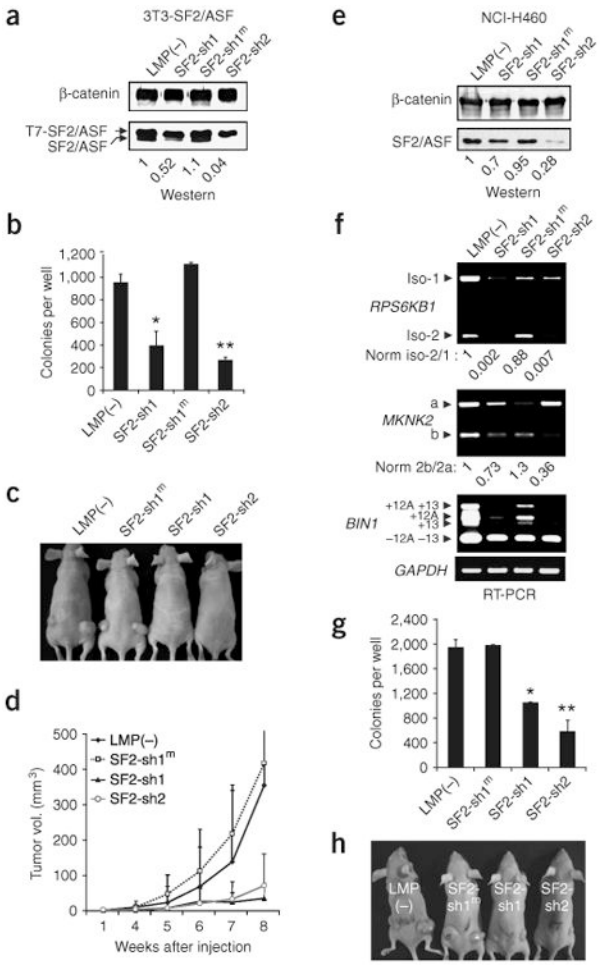

Figure 6.

Knockdown of SF2/ASF reverses transformation of NCI-H460 cells and SF2/ASF-overexpressing NIH 3T3 cells. (**a**) Western blot analysis of SF2/ASF in NIH 3T3 cells overexpressing SF2/ASF after transduction with viruses expressing the indicated SF2/ASF shRNAs. The SF2/ASF-specific shRNAs target the 3′ UTR of SF2/ASF and therefore affect only the endogenous SF2/ASF (lower band), for which normalized protein levels are shown under the blot. SF2-sh1m is a control shRNA with two mismatches. (**b**) Cells described in **a** were seeded in soft agar, and colonies were counted 14 d later; means ± s.d. are shown. *P*-values for comparisons to the LMP(–) control: $*P = 2 \times 10^{-7}$; $**P = 2 \times 10^{-9}$. (**c,d**) Cell lines described in a were injected into nude mice ($n = 8$ injections) and tumor volume was measured weekly; error bars indicate s.d. Representative mice are shown. (**e**) Western blot of total protein from NCI-H460 cells as in a. (**f**) RNA from the cells in e was analyzed by RT-PCR and RT-qPCR to detect *MKNK2, RPS6KB1* and *BIN1* isoform levels, with *GAPDH* as a control. Normalized ratios of *RPS6KB1* isoform-2 to isoform-1 and *MKNK2* 2b to 2a calculated from the RT-qPCR data are shown under the corresponding gels. (**g**) Soft agar colony formation by cells described in **e.** Means ± s.d. are shown. P-values calculated as in **b:** $*P = 3 \times 10^{-11}$; $**P = 2 \times 10^{-15}$. (**h**) Cell lines described in **a** were injected into nude mice ($n = 8$ injections). Representative mice are shown.

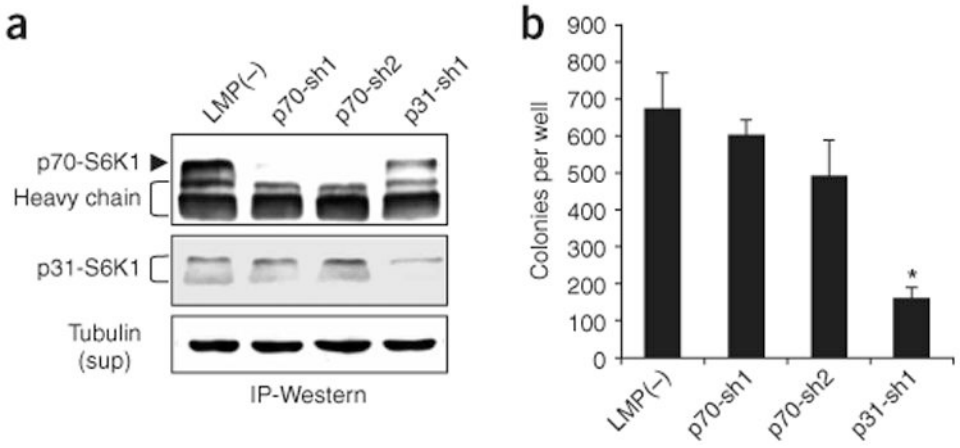

Figure 7.

Knockdown of S6K1 isoform-2 blocks SF2/ASF-mediated transformation. (**a**) Immunoprecipitation (IP)-western blot analysis of S6K1 isoforms in NIH 3T3 cells overexpressing SF2/ASF, after transduction with retroviruses expressing the indicated S6K1 isoform-specific shRNAs. (**b**) Cells described in **a** were seeded in soft agar and colonies were counted as in Figure 6b; means ± s.d. are shown. $*P = 6\times10^{-8}$.

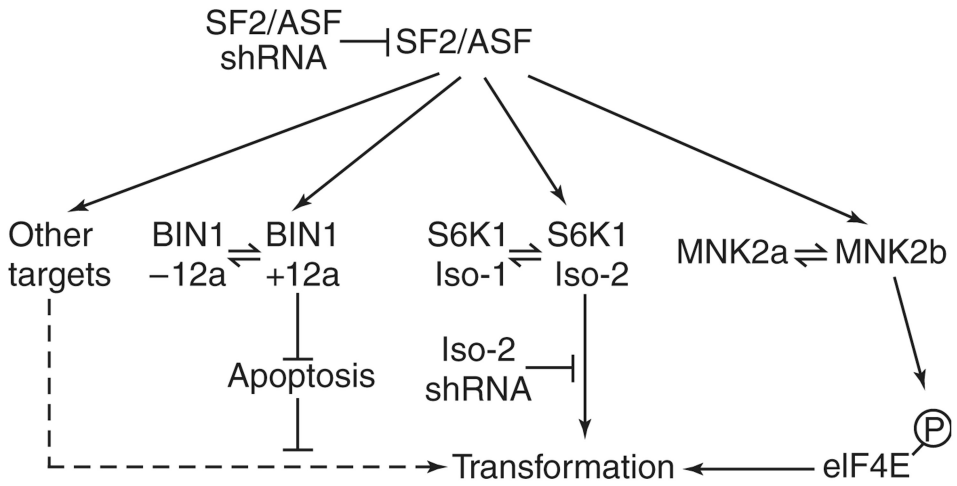

Figure 8.

A model for transformation by SF2/ASF. SF2/ASF (which can be blocked by specific shRNAs) activates several key targets that contribute to the transformed phenotype: induction of MNK2b promotes phosphorylation of eIF4E, which enhances cap-dependent translation; induction of the oncogenic S6K1 isoform-2 directly leads to transformation; and induction of inactive isoforms of the putative tumor suppressor BIN1 inhibits apoptosis. Other SF2/ASF targets are also likely to contribute to transformation.