Hepatocyte Growth Factor Enhances Alternative Splicing of the Krüppel-like Factor 6 (KLF6) Tumor Suppressor to Promote Growth Though SRSF1

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Abstract

Alternative splicing of the Krüppel-like factor 6 (KLF6) tumor suppressor into an antagonistic splice variant 1 (SV1) is a pathogenic event in several cancers including hepatocellular carcinoma (HCC) because elevated SV1 is associated with increased tumor metastasis and mortality. Ras activation is one factor that can enhance KLF6 splicing in cancer cells, however pathways driving KLF6 splicing are unknown. Splice site selection is regulated by splice factors that include serine/arginine-rich (SR) proteins such as SRSF1 (ASF-SF2), which in turn is controlled by phosphoinositide 3-kinase (PI3K)/Akt and the mitogen-activated protein kinase (MAPK) signaling pathway. Because signaling pathways downstream of the liver mitogen hepatocyte growth factor (HGF) include Akt, we explored whether HGF induces KLF6 alternative splicing. In HepG2 cells, HGF (25 ng/mL) significantly increases the ratio of SV1/KLF6 full by 40% through phosphorylation of Akt and subsequent downregulation of two splicing regulators, SRSF3 (SRp20) and SRSF1. Decreased SRSF3 levels regulate SRSF1 levels by alternative splicing associated with the nonsense-mediated mRNA decay pathway (AS-NMD), which stimulates cell growth by decreasing p21 levels. Enhanced cell replication through increased KLF6 alternative splicing is a novel growth-promoting pathway of HGF that could contribute to the molecule’s mitogenic activity in physiologic liver growth and hepatocellular carcinoma.

Introduction

Krüppel-like factor 6 (KLF6) is a zinc finger protein that belongs to the family of Sp1-like/KLF transcription factors, which are composed of an N-terminal activation domain and 3 C2H2 zinc fingers. KLF6 is ubiquitously expressed in human tissues and regulates genes controlling cell cycle, apoptosis, and differentiation (1). Moreover, KLF6 has been identified as a tumor suppressor gene that is inactivated in a number of human cancers by mutation, allelic loss, and/or promoter methylation (2, 3).

Functional inactivation of KLF6 may also occur through enhanced splicing into antagonistic splice isoforms. Three alternative splice variants of KLF6 termed SV1, SV2, and SV3 have been identified (4). Overexpression of SV1 is a common feature of human malignancy, which typically confers a more aggressive behavior and poorer survival. For example, increased expression of SV1 accelerates prostate cancer progression and metastasis (5). Similarly, an increased SV1/KLF6 full mRNA ratio correlates with a more aggressive behavior and/or drug-resistant tumors in glioblastoma (6), hepatocellular carcinoma (HCC), ovarian, and pancreatic cancers (7, 8). Knowledge about KLF6 alternative splicing regulation is incomplete in these tumors, particularly in HCCs.

Human cancers can display alternatively spliced gene products in the absence of genomic mutations, pinpointing the splicing machinery as a cause of disease (9–11). In general, affected proteins include transcription factors, cell signal transducers, and components of the extracellular matrix. Although the role of alternative splicing in cancer is not well characterized, in normal tissues it is regulated by specific signaling pathways, yielding temporally and spatially appropriate patterns of gene expression (12–15). Alternative splicing can regulate gene expression by generating nonproductive isoforms, such as mRNAs that are retained in the nucleus or are subject to nonsense-mediated mRNA decay (NMD), or by encoding proteins with different functions (9, 16).

Alternative splicing events can also be modulated by changes in the protein levels of individual splicing factors, proteins that bind to specific sequence elements to affect splice site selection include SR protein, hnRNPs, and other related RNA-binding proteins (17, 18). Altered expression of splicing factors has been described in various tumor types (19–21).
Recent reports describe ultraconserved (UCR) elements in every member of the SR protein family (22–25). UCRs are present in regions that undergo alternative splicing events that introduce premature translation termination codons (PTC), such that some of the resulting mRNAs are NMD. In mammals, a termination codon is recognized as premature when located more than 50 to 55 nt upstream of the exon–exon junction marked by the exon junction complex (EJC). EJCs downstream of PTCS are no longer removed during the “pioneer” round of translation and recruit essential NMD factors, including Upf1/Rent1, that in turn promote mRNA degradation (26). For example, the mammalian SR mRNA SC35, and the polypyrimidine tract–binding protein (PTB) autoregulate by promoting the expression of unstable alternatively spliced mRNA isoforms that undergo NMD (27, 28). SRSF3, another SR protein, promotes expression from its own gene of a splicing isoform encoding a truncated protein, and SRSF1 antagonizes this regulation (29). SRSF1, a prototypical SR protein that participates in both constitutive and alternative splicing (9), is regulated by multiple posttranscriptional and translational mechanisms that fine-tune its expression (30). Thus, unproductive splicing can regulate SR protein expression (22, 23).

In addition, splicing factors can be regulated through posttranslational modifications in response to cellular signaling pathways (31–33). We have previously reported that the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway can increase KLF6 alternative splicing, mediated through the splice factor SRSF1 in HCCs (7).

Hepatocyte growth factor (HGF) can activate the PI3K/Akt signaling pathway (34, 35), which is frequently altered in the splice factor SRSF1 in HCCs (7).

Herein, we have explored the mechanisms of HGF–HepG2 cells were cultured in Dulbecco’s modified Eagles’ medium (DMEM) and HCT116 cells in McCoy’s 5A medium (modified) containing 10% FBS, 100 μg penicillin/mL and 100 μg streptomycin/mL, in a humidified incubator containing 5% CO₂, at 37°C. Cells were passed every 2 days using 0.05% Trypsin-EDTA solution, to maintain subconfluency. Chemicals used in studies included LY294002, triciribine U0126, and PD98059 (from Calbiochem) and HGF (Sigma). Double-stranded, pre-annealed siRNA oligonucleotides directed against SRSF1 (sc-38320), SRSF3 (sc-38338), and control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology, and siRNA directed against Akt (Cat. #6211) was purchased from Cell Signaling. The siRNAs were transfected with siPORT NeoFX Transfection Agent (Applied Biosystems) for 24 to 48 hours in HepG2 cells according to the manufacturer’s protocol.

Transfections were conducted using Lipofectamine 2000 reagent (Invitrogen). Polyclonal pools of stable cell lines were generated by retroviral infection of pBabe-Ctrl and pBabe-SV1 plasmids. Infected cells were selected with 2 μg/mL of puromycin. For each construct, at least 2 independent polyclonal pools of stable cell lines were generated and analyzed.

RNA, real-time PCR (RT-PCR), and quantitative RT-PCR analysis

RNA from cell lines was extracted using the RNeasy Mini kit (Qiagen). RNA was treated with DNase (Roche). One microgram of total RNA was reverse-transcribed per reaction using first-strand complementary DNA synthesis with random primers (Clontech).

RT-PCR. Two hundred nanograms of cDNA was then PCR amplified using AccuPrime Taq DNA (40) Polymerase System (Invitrogen). For analysis of AS-NMD of SRSF1 RNA, we used primers ASF-A and ASF-B (41). Experiments were carried out in triplicate at least three independent times. All values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), using previously described primers (42). Statistical significance was determined by ANOVA.

Quantitative RT-PCR. Quantitative RT-PCR (qRT-PCR) was carried out using a Roche LightCycler 480. Total RNA was treated with DNase (Qiagen). RNA (1 μg) was reverse transcribed for each reaction using first-strand complementary cDNA synthesis with random primers (Promega). mRNA levels were quantified by qRT-PCR using the following PCR primers:

Full KLF6 forward: 5'-CGGACGCACACAGGAGAAAA-3' Full KLF6 reverse: 5'-CGGTGTGCTTTCCGAAGTG-3' SV1 forward: 5'-CCTCGGCAAGGAAAGGAA-3' SV1 reverse: 5'-TCCACAGATCCTTCCTGCTTC-3' GAPDH forward: 5'-CAATGACCCTTCTATTTGACC-3' GAPDH reverse: 5'-GATCTGCTCTCTGGAAATG-3'
mRNA levels and then used to calculate fold change compared with control (without treatment; refs. 2, 4, 40). Alternative splicing of KLF6 was expressed as the ratio between SV1 and KLF6 full mRNA expression levels.

To detect total SRSF1, transcripts, we used ASF_cost_for and ASF_cost_rev (41). Statistical significance was determined by ANOVA.

**Protein extracts, immunoprecipitation, and Western blots**

**Protein extracts.** To prepare whole-proteins extracts, cells were harvested, washed in PBS, and then lysed in ice-cold buffer (50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 50 mmol/L NaF, 1% Nonidet P-40), containing 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl-fluoride, 1 mmol/L sodium pyrophosphate, and protease inhibitor Complete Mini Mixture (Roche). The protein content of the extracts was determined by the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories).

**Immunoprecipitation.** Protein extracts (500 μg) from HepG2 cells, untreated or treated with HGF for 12 hours, were incubated with 2 μg of specific antibody against SRSF1 (Santa Cruz # sc-10255) overnight at 4°C, followed by an incubation with 20 μL of protein A/G sepharose for 2 hours at 4°C. Samples were washed with lysis buffer. The immune complexes were subsequently boiled off the beads in 2× SDS protein loading buffer. After boiling, the samples were resolved in a 12% SDS-PAGE gel, and the amounts of phosphorylated SRSF1 were detected using a specific phospho-SPR antibody mAb1H4 (Zymed).

**Western blot.** Protein (30–100 μg) from whole-cell extracts were fractionated on a SDS-PAGE and transferred to polyvinylidene difluoride (PVDI) membranes (Immobilion-P). The amount of protein and the integrity of transfer were verified by staining with Ponceau-S solution (Sigma). The filters were then blocked with non-fat milk and incubated overnight at 4°C, with primary antibodies at the following dilutions: 1:500 anti-SRSF1 (Santa Cruz # sc-10255), 1:500 anti-SRSF3 (Santa Cruz # sc-365772), 1:500 anti-p21 (Santa Cruz # sc-397), 1:500 anti-KLF6 (Santa Cruz # sc-7158), 1:1,000 anti-Akt (Cell Signaling # 9272), 1:500 anti-phospho-Akt (Thr308; Cell Signaling # 9625), 1:1,000 anti-Erk1/2 (Cell Signaling # 13779), 1:500 anti-phospho-Erk1/2 (Thr202/Tyr204; Cell Signaling # 20G11). Of note, the anti-KLF6 antibody (Santa Cruz # sc-7158) is a rabbit polyclonal antibody raised against amino acids 28 to 201 of KLF6 of rat origin, which includes most of the unique activation domain but not the zinc finger region. This antibody recognizes both KLF6 full-length and the SV1 isoform. Signals from the primary antibodies were amplified using species-specific antisera conjugated with horseradish peroxidase (Sigma) and detected with a chemiluminescent substrate detection system ECL (Amersham Biosciences). The specificity of the antibodies used in this work was confirmed by omitting the primary antibodies in the incubation medium. Blots were stripped and reprobed with anti-GAPDH (1:5,000) as a protein loading control. Enhanced chemiluminescence images were analyzed and quantified with a Bioquant Nova (Bioquant Lifesciences). Values were normalized to control and expressed as relative fold changes.

**Analysis of proliferation**

As previously described (2), proliferation was determined by assaying [3H]-thymidine incorporation. Cells were plated 8 × 10^4 cells/mL onto 12-well culture plates, in complete media incubated for 24 hours, and subsequently serum-starved for 24 hours in media supplemented with 0.5% FBS and then treated with 25 ng/mL HGF. At appropriate time points after stimulation, 1 μCi/mL [3H]-thymidine (Amersham) was added. After 4 hours, cells were washed 3 times with ice-cold PBS and fixed in methanol for 30 minutes at 4°C. After methanol removal and cell drying, cells were solubilized in 0.25% sodium hydroxide/0.25% SDS. After neutralization with hydrochloric acid (1 N), disintegrations per minute were estimated by liquid scintillation counting.

**Statistical analysis**

Unless otherwise stated, all data represent means ± SEM. Statistical analysis was conducted on SPSS 17 (Statistical Package for Social Sciences) licensed to Mount Sinai School of Medicine (New York, NY). Statistical significance was estimated by the Student t test or, when appropriated, by ANOVA followed by the Fischer least significant difference (LSD) test for multiple comparisons. Differences were considered significant at a level of P ≤ 0.05.

**Results**

**KLF6 alternative splicing is regulated by HGF signaling**

Because our previous studies highlighted a key role of KLF6 alternative splicing in human cancer, especially HCCs, we sought to identify potential signals that promote KLF6 splicing. As noted, a recent study using global gene expression analysis to identify HGF-regulated targets found that HGF inhibition significantly upregulates the KLF6 tumor suppressor (43). This finding indicated that HGF might exert its growth-promoting activity, in part, by inhibiting the growth-suppressive KLF6. On the basis of this link, we explored the possibility that HGF might also regulate KLF6-mediated growth effects through induction of KLF6 alternative splicing to generate more SV1. A dose–response study identified an optimal concentration of 25 ng/mL of HGF in inducing cell proliferation, initially over a 24-hour interval (not shown). Next, HepG2 cells were incubated with HGF at 25 ng/mL for progressive intervals (0–24 hours). Increased KLF6 alternative splicing, expressed as a ratio of SV1 to KLF6 full, was evident 12 hours after HGF stimulation (Fig. 1A, left), with increasing SV1 and decreasing KLF6 full at both the mRNA and protein levels, as determined by qRT-PCR and Western blotting (Figs. 1A, right, and B respectively). To confirm that this impact of HGF affected known transcriptional targets of KLF6, we also assessed p21 expression, which is normally induced by KLF6 full and antagonized by SV1. As expected, increased KLF6 alternative splicing by HGF was associated with a significant decrease in p21 expression (Fig. 1C).
Next, we examined whether stimulation of \textit{KLF6} splicing by HGF led to increased cellular proliferation and to what extent an increase could be tied directly to levels of SV1. We attempted to use siRNA to stably knock down SV1 in HepG2 but were unable to generate a stable cell line because cells underwent apoptosis—the same was true for Huh7 and Hep3B lines (data not shown). This effect is consistent with the previously reported anti-apoptotic activity of SV1 in ovarian cancer cells (40). Because we previously showed in HCT116 colon cancer cells that the growth-promoting...
effect of Ras is partially mediated by SV1 (7), we used this cell type for these experiments, as unlike HCC lines, HCT116 remain viable despite stable knockdown of SV1. As shown in Fig. 1D, approximately 94% knockdown of HGF was achieved in mRNA levels. In cells with stable SV1 knockdown, HGF failed to increase cell growth, indicating that a substantial fraction of the growth-promoting effect of HGF in HCT116 is mediated by SV1. In contrast, stable expression of an empty vector had no impact on HGF-mediated cellular growth (Fig. 1E).

**HGF increases KLF6 alternative splicing through the PI3K pathway**

HGF signaling normally activates 2 well-characterized intracellular cascades, mitogen-activated protein kinase and PI3K (35). Accordingly, we examined whether these pathways contribute to HGF-mediated KLF6 splicing in HepG2 cells. First, we monitored PI3K and Erk after 12 hours of HGF stimulation by Akt and Erk phosphorylation, respectively (Fig. 2A). For that purpose, we used Erk-specific inhibitors U0126 and PD98059, the PI3K inhibitor LY294002, and the Akt inhibitor triciribine. Whereas U0126 and PD98059 had no effect on the SV1/KLF6 full ratio based on qRT-PCR, treatment with 10 µmol/L LY294002 and 10 µmol/L triciribine both significantly reduced the SV1/KLF6 mRNA ratio within 12 hours of HGF stimulation (Fig. 2B). These data highlight the role of PI3K signaling in the stimulation of KLF6 alternative splicing, leading to increased generation of SV1.

**HGF increases KLF6 alternative splicing through a decrease in SRSF1 levels**

We previously reported that Ras/Akt signaling can increase KLF6 alternative splicing, mediated by the splice factor SRSF1 (7). Normally, SRSF1 activity is regulated either through its phosphorylation and/or the absolute levels of SRSF1 protein. In our experiments, there was no change in SRSF1 phosphorylation after HGF, based on
IgMInput

To link SRSF1 directly to the regulation of splicing, we transiently knocked down SRSF1 levels using siRNA oligonucleotides and then assessed the splicing, we transiently knocked down SRSF1 levels using siRNA oligonucleotides and then assessed the splicing, we transiently knocked down SRSF1 levels using siRNA oligonucleotides and then assessed the splicing, we transiently knocked down SRSF1 levels using siRNA oligonucleotides and then assessed the splicing, we transiently knocked down SRSF1 levels using siRNA oligonucleotides and then assessed the splicing, we transiently knocked down SRSF1 levels using siRNA oligonucleotides and then assessed the splicing, we transiently knocked down SRSF1 levels using siRNA oligonucleotides and then assessed the splicing, we transiently knocked down SRSF1 levels using siRNA oligonucleotides and then assessed the splicing.

**HGF regulates Akt-dependent SRSF1 alternative splicing**

Because the level of SRSF1 protein regulates HGF-mediated splicing of KLF6, we further explored potential mechanisms regulating SRSF1 levels. Recently, it has been found that alternative splicing coupled to nonsense-mediated decay (AS-NMD) can regulate the expression of all SR splicing factors and several other RNA-binding proteins (22, 23). In particular, the 3′-untranslated region (UTR) of the SRSF1 gene contains an intron (3′-UTR intron) that is normally retained in a mature full-length transcript. Splicing of this intron produces an RNA molecule ("NMD-" transcript) in which the natural termination codon is recognized as a premature stop codon and the mRNA is degraded by the NMD pathway (Fig. 4A). We examined whether this mechanism could be responsible for the lower level of SRSF1 in HepG2 cells in response to HGF. RT-PCR analysis confirmed that splicing of the 3′-UTR intron occurs in HepG2 cells after stimulation with HGF; untreated cells express more SRSF1 full isofrom, whereas the NMD+ transcript is more predominant in treated cells (Fig. 4B). Moreover, full/NMD+ ratio decreased after the HGF stimulation (Fig. 4C). As predicted by the AS-NMD model, this splicing switch is accompanied by lower levels SRSF1 (Fig. 4D).

As noted, we previously identified Ras/Akt signaling as driving KLF6 alternative splicing through SRSF1 (7), and here we additionally show that the PI3K signaling pathway affects the expression of SRSF1 levels. Accordingly, we further investigated the contribution of the Akt pathway to the regulation of SRSF1 splicing in HepG2 cells in response to HGF. To directly verify this possibility, we specifically
HGF Increases Alternative Splicing of KLF6

**Figure 4.** HGF regulates PI3K/Akt-dependent SRSF1 and alternative splicing (AS). A, schematic representation of AS in the 3′-UTR of SRSF1 transcripts. Intron retention generates the stable full transcript of SRSF1, whereas splicing leads to the assembly of EJC downstream of the natural stop codon (NMD+ transcript). The positions of the primers ASF-A and -B are indicated. B and C, HepG2 cells were serum-deprived and then stimulated by adding 25 ng/mL HGF in the absence or presence of an Akt inhibitor (LY294002, 10 µmol/L). Cells were harvested at 0 and 12 hours thereafter, and the splicing profile of SRSF1 transcripts was identified by RT-PCR with primers A and B. The densitometric data were measured in arbitrary units, and represent the mean ± SEM for 5 different experiments. *P < 0.05 significantly different from control cells; †, P < 0.05 significantly different versus 25 ng/mL HGF treatment cells without PI3K inhibition. Values shown are the mean ± SEM for 5 different experiments.

HGF inhibited the PI3K activity in HGF-treated cells with the PI3K inhibitor, LY294002 (10 µmol/L). As shown in Fig. 4B and C, LY294002 altered the splicing profile of SRSF1 mRNA in HGF-treated cells to favor the production of the full transcript of SRSF1 mRNA and protein increasing the production of the NMD+ transcript (Fig. 5C).

As predicted by AS-NMD model, this splicing switch was accompanied by a lower level of SRSF1 mRNA and protein (Fig. 5D and E). This finding implicates SRSF3 in SRSF1 splicing regulation.

**SRSF3 regulates alternative splicing of SRSF1**

To understand the molecular mechanism regulating AS-NMD of the SRSF1 transcript, we analyzed the SRSF1 3′-UTR sequence. This region contains one putative binding site (ATACCAT) for SRSF3, a splicing regulator († in Fig. 5A). SRSF3, which is also a SR protein, promotes expression from its own gene of a splicing isoform encoding a truncated protein, and SRSF1 antagonizes this regulation (29). On the basis of this property, we hypothesized a role of SRSF3 in controlling SRSF1 splicing. To address whether endogenous SRSF3 protein affects the alternative splicing of SRSF1, we depleted the expression of SRSF3, using siRNA oligonucleotides in HepG2 cells. As shown in Fig. 5B, knocking down SRSF3 changed the splicing profile of SRSF1 mRNA by increasing the production of the NMD+ transcript (Fig. 5C).

As predicted by AS-NMD model, this splicing switch was accompanied by a lower level of SRSF1 mRNA and protein (Fig. 5D and E). This finding implicates SRSF3 in SRSF1 splicing regulation.

**HGF regulates proliferation through KLF6 alternative splicing by SRSF3 downregulation**

We found that HGF/PI3K/Akt regulates SRSF1 levels through AS-NMD. Because we showed that SRSF3 was also able to regulate SRSF1 AS-NMD, we investigated whether the HGF/PI3K/Akt pathway may regulate SRSF3 levels. As shown in Fig. 6A, HGF stimulation for 12 hours provoked a significant decrease in total SRSF3 protein levels which was abrogated using the PI3K inhibitor, LY294002 (10 µmol/L). To link Akt directly to the regulation of SRSF3 levels, we transiently knocked down Akt levels using siRNA oligonucleotides and then assessed SRSF3 levels by Western
blotting. As shown in Fig. 6B, under these conditions SRSF3 levels were significantly increased, indicating that the level of Akt regulates SRSF3.

To link SRSF3 directly to the regulation of KLF6 alternative splicing and consequently to p21 protein levels, we transiently knocked down SRSF3 using siRNA and then assessed the SV1/KLF6 full ratio and p21 levels by qRT-PCR and Western blotting, respectively. As shown in Fig. 6C, under these conditions, the SV1/KLF6 full ratio was significantly increased, followed by a decrease in the p21 levels, indicating that the level of SRSF3 protein regulates KLF6 alternative splicing.

Because p21 might be regulated by HGF through mechanisms apart from its impact on KLF6 splicing, we also altered the SV1/KLF6 full ratio by inhibiting PI3K following HGF treatment (as previously shown in the first 3 lines of Fig. 2B) and then assayed p21 expression. As shown Fig. 6D, p21 expression was restored when PI3K was inhibiting HGF-mediated splicing, further supporting the conclusion that HGF decreases p21 levels by altering KLF6 alternative splicing. Next, we explored whether HGF regulated proliferation through PI3K/Akt by specifically increasing SV1 levels. To do so, we used HCT116 colon cancer cells, which, as noted above, remained viable despite stable knockdown of SV1. As shown in Fig. 6E, HGF stimulation for 24 hours provoked a significant increase in cell proliferation, which was abrogated using the PI3K inhibitor, LY294002 (10 μmol/L) in HCT116 cells with stable expression of empty vector. In contrast, stable SV1 knockdown had no impact on HGF/PI3K/Akt-mediated cellular growth. Together, our results implicate the HGF/PI3K/Akt pathway in regulating proliferation through KLF6.
alternative splicing, which is coupled to changes in the expression levels of 2 splicing regulators, SRSF3 and SRSF1.

**Discussion**

Here, we have identified a novel mechanism of tumor suppressor inactivation in liver cancer cells via HGF-mediated, enhanced alternative splicing of KLF6 into its growth-promoting isoform. SV1 expression is increased in an oncogenic HGF/Pi3K/Akt-dependent manner, thereby altering the relative ratio of SV1 to KLF6 full, which leads to enhanced cellular proliferation. HGF transduces its signal through the splice regulatory protein SRSF1, which, when abrogated, leads to significantly increased SV1 mRNA, indicating a requirement for SRSF1 to allow KLF6 full
mRNA expression. Moreover, our data also show that HGF/PI3K/Akt-mediated changes in KLF6 alternative splicing are coupled to changes in the expression levels of 2 splicing regulators, SRSF3 and SRSF1. A model incorporating these observations is depicted in Fig. 7, in which c-Met activation by its ligand, HGF, induces PI3K/Akt and downregulates SRSF1 levels through AS-NMD. Decreased SRSF1 enhances KLF6 alternative splicing, altering the SV1/KLF6 full ratio.

The primary function of SRSF1 and other SR protein family members is splice site selection (44), a role that can potentially affect KLF6 splicing selection. SR proteins interact with pre-mRNAs through their arginine- and serine-rich domains, which also promote protein–protein interactions with core splicing machinery proteins (45). SRSF1 has been identified as a proto-oncogene that is amplified in human tumors and can transform immortalized mouse fibroblasts (46). SRSF1 can alter splicing of the tumor suppressor BIN1 (exon inclusion results in abolished inhibition of c-myc), MNK2 (increased expression of catalytically active splice isoform leading to phosphorylation of eIF4E), and the translation regulatory kinase S6K1 (increased expression of oncogenic isoform), resulting in altered functions of these proteins, all of which contribute to the oncogenic potential of SRSF1 (47). Because SRSF1 can regulate alternative splicing in a concentration-dependent manner, this protein might affect splicing of different sets of target pre-mRNAs, including KLF6. These data highlight the complex network of targets downstream of SRSF1, which when alternatively spliced, can lead to context-dependent and possibly divergent biologic outcomes.

A recent study of SRSF1 has shown that multiple post-transcriptional and translational mechanisms operate to...
fine-tune the level of this protein (30). AS-NMD is one of the mechanisms that control the expression of a large number of pre-mRNA processing factors, many of which directly modulate AS-NMD of their own transcript, thus maintaining their homeostasis through a feedback mechanism (26). Splicing regulators may also modulate AS-NMD in transcripts of RNA-binding proteins. This is the case with 2 pairs of closely related hnRNP proteins—polypyrimidine tract–binding protein (PTB)/neural PTB (nPTB) and hnRNP L/hnRNP LL—that control their relative abundance during cell differentiation through AS-NMD (48). Also, SRSF1 regulates its levels in cell differentiation programs through AS-NMD (41). We have identified another example in HCCs of regulated AS-NMD, in which the SRSF3 affects the level of SRSF1 by regulating unproductive splicing in the 3′-UTR of SRSF1 transcripts after HGF stimulation. The identification of a regulatory circuit in which a splicing regulator, SRSF3, controls the level of SRSF1, reveals a new layer of complexity in splicing regulation. In particular, the findings unveil a hierarchy of actions between splicing factors in which SRSF3 links the signaling pathway to downstream effectors such as SRSF1. Thus far, we have identified only one target of this circuit, that is, the KLF6 transcription factor, which, through alternative splicing, produces the SV1 isoform that leads to enhanced cellular proliferation after HGF stimulation.

The mechanisms exploited by oncogenes or tumor suppressors to either promote or antagonize malignant transformation are being progressively clarified. Our understanding of critical events during tumor progression is expanding, as many molecular and epigenetic changes cooperate in altering tumor cell behavior. Despite its growing importance (49), however, very little is known about the role of alternative splicing in tumorigenesis.

A remarkable finding of our analysis is that changes in the expression levels of the tumor suppressors KLF6 and AS-NMD of SRSF1 in HepG2 cells are controlled by HGF. We have shown that this factor increases the activity of PI3K/Akt and decreases SRSF3 levels, thus leading to a switch in the splicing profile of the SRSF1 transcript. SRSF3 promotes expression from its own gene of a splicing isoform encoding a truncated protein and SRSF1 antagonizes this regulation (30). Further experiments will be required to clarify whether and how the PI3K pathway affects SRSF3 expression at the transcriptional or the posttranscriptional levels and what is the role of phosphorylation by one of the Akt kinases. It is possible that the PI3K pathway regulates other SR protein regulators besides Akt, such as the SRPK and Clik/Sty family of protein kinases. In addition, these operate in the cytosol (50) and it may antagonize the effect of Akt on SR proteins (32).

Collectively, these data suggest a novel growth-promoting pathway of HGF by decreasing the levels of the tumor suppressor KLF6 through downregulation of SRSF1 by AS-NMD. These effects of HGF could contribute to the molecule’s mitogenic activity in physiologic growth regulation of liver, as well as in HCCs.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: U. Muñoz, R. Hännissoo, U.E. Lang, M. Cohen-Naftaly, S.L. Friedman
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Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): U. Muñoz, J.E. Puche, R. Hännissoo, U.E. Lang, M. Cohen-Naftaly
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