

Oncogenic Splicing Factor *SRSF1* Is a Critical Transcriptional Target of MYC

Shipra Das,^{1,2} Olga Anczuków,¹ Martin Akerman,¹ and Adrian R. Krainer^{1,*}

¹Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, USA

²Graduate Program in Genetics, Stony Brook University, NY 11794, USA

*Correspondence: krainer@cshl.edu

DOI 10.1016/j.celrep.2011.12.001

SUMMARY

The SR protein splicing factor *SRSF1* is a potent proto-oncogene that is frequently upregulated in cancer. Here, we show that *SRSF1* is a direct target of the transcription factor oncoprotein MYC. These two oncogenes are significantly coexpressed in lung carcinomas, and MYC knockdown downregulates *SRSF1* expression in lung-cancer cell lines. MYC directly activates transcription of *SRSF1* through two noncanonical E-boxes in its promoter. The resulting increase in *SRSF1* protein is sufficient to modulate alternative splicing of a subset of transcripts. In particular, MYC induction leads to *SRSF1*-mediated alternative splicing of the signaling kinase *MKNK2* and the transcription factor *TEAD1*. *SRSF1* knockdown reduces MYC's oncogenic activity, decreasing proliferation and anchorage-independent growth. These results suggest a mechanism for *SRSF1* upregulation in tumors with elevated MYC and identify *SRSF1* as a critical MYC target that contributes to its oncogenic potential by enabling MYC to regulate the expression of specific protein isoforms through alternative splicing.

INTRODUCTION

SRSF1 (formerly SF2/ASF) is a prototypical member of the SR protein family, a conserved class of splicing regulators. Besides its central roles in constitutive and alternative splicing (Ge and Manley, 1990; Krainer et al., 1990; Mayeda and Krainer, 1992), *SRSF1* regulates other aspects of RNA metabolism, including mRNA stability (Sun et al., 2011; Lemaire et al., 2002), nuclear export (Huang et al., 2003), nonsense-mediated mRNA decay (Zhang and Krainer, 2004), translation (Sanford et al., 2004), and miRNA processing (Wu et al., 2010). The *SRSF1* gene is essential, and depletion of the protein triggers genomic instability, cell-cycle arrest, and apoptosis (Xu et al., 2005; Li et al., 2005), whereas its overexpression drives transformation of immortal rodent fibroblasts (Karni et al., 2007). *SRSF1* negatively autoregulates its expression through various posttranscriptional and translational mechanisms (Sun et al., 2010; Wu et al., 2010), yet despite this stringent homeostatic control, it is frequently up-

regulated in many different cancers (Ezponda et al., 2010; Karni et al., 2007; Thorsen et al., 2011). *SRSF1* resides on Chromosome 17q23, a locus that is amplified in some tumors (Sinclair et al., 2003), accounting for some instances of *SRSF1* overexpression (Karni et al., 2007).

Altered transcriptional regulation might also cause *SRSF1* overexpression in tumors. MYC (alias c-Myc) is a potent oncogenic transcription factor that is frequently overexpressed or hyperactive in many cancers (Adhikary and Eilers, 2005). Consistent with a possible role of MYC in regulating *SRSF1*, the *SRSF1* promoter region directly or indirectly binds MYC, according to a ChIP-on-Chip analysis of CpG island arrays (Mao et al., 2003); in addition, expression microarray analyses reported *SRSF1* among >100 genes upregulated in response to MYC in multiple cell lines (Coller et al., 2000; Schlosser et al., 2005).

RESULTS AND DISCUSSION

Considering that *SRSF1* is markedly overexpressed in lung cancer (Ezponda et al., 2010; Karni et al., 2007), we analyzed public microarray data from a panel of 132 lung tumors, to determine whether *MYC* overexpression correlates with elevated *SRSF1* levels in this context. Indeed, we found a strong positive correlation between *MYC* and *SRSF1* expression at the RNA level (Figure 1A). Among eight other known or putative MYC-regulated splicing factors we analyzed (David et al., 2010; Li et al., 2003; Rauch et al., 2011; Zeller et al., 2003), only *HNRNPH1* and *PTBP1* expression correlated significantly with *MYC* expression in these lung-tumor samples (Table S1 available online). We extended this analysis to a panel of normal and tumor-derived lung cell lines, and also found a significant correlation at the protein level between MYC and *SRSF1* (Figure 1B), with most cancer cell lines overexpressing both proteins, relative to IMR90 primary lung fibroblasts. In contrast, MYC expression did not correlate in these cells with that of other SR proteins, such as *SRSF9* (Figure 1B) and *SRSF6* (data not shown). siRNA-mediated knockdown of MYC in two of these cell lines, the large cell lung cancer cell line NCI.H460 and the bronchoalveolar adenocarcinoma cell line NCI.H1666, resulted in significant decreases in *SRSF1* expression, both at the transcript and protein level, indicating that *SRSF1* expression is under MYC control (Figures 1C and 1D). However, another bronchoalveolar adenocarcinoma cell line, A549, did not show this effect, indicating additional context-dependent levels of control (Figure S1); this may be due to threshold effects, as

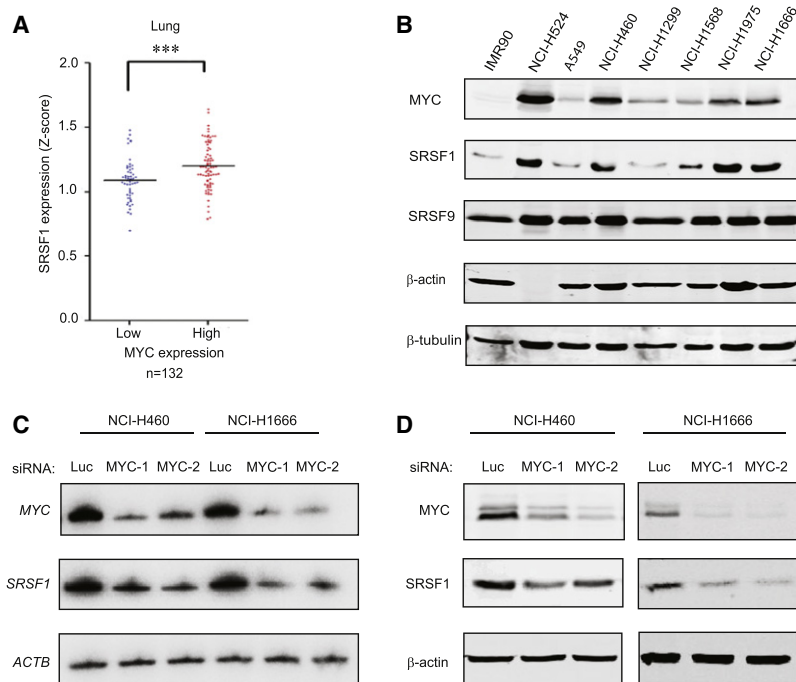


Figure 1. SRSF1 Expression Correlates with MYC Levels in Human Lung Tumors and Cell Lines

(A) *SRSF1* expression profile from microarray analysis of 132 lung tumors (expO). The data were normalized to Z-score and divided into two categories: tumors expressing high or low MYC levels. The dot plot shows the distribution and the median (horizontal line). Mann-Whitney test *** $p < 0.0001$.

(B) Immunoblotting of MYC and SRSF1 in lung-cancer cell lines and lung primary fibroblasts, showing significant correlation between the expression of the two oncoproteins ($r = 0.75$, one-tailed t test * $p = 0.05$).

(C) RT-PCR and (D) Immunoblotting of MYC and SRSF1 in NCI.H460 and NCI.H1666 cells transfected with control siRNA (luciferase) or one of two siRNAs against MYC. See also Figure S1.

A549 cells have relatively low levels of both MYC and SRSF1 (Figure 1B). The imperfect correlation between SRSF1 and MYC expression in the lung cancer cell lines (Figure 1B) indicates that though MYC is an important regulator of SRSF1 expression, SRSF1 overexpression in cancer is not solely attributable to MYC expression; additional factors likely affect its expression at the transcriptional, posttranscriptional, translational, or post-translational levels.

To assess more directly whether *SRSF1* expression is regulated by MYC, we used an inducible MYC-Estrogen Receptor (ER) system (Eilers et al., 1989; Littlewood et al., 1995). We generated IMR90 cells stably expressing the MYC protein fused to a modified ER ligand-binding domain, which binds the synthetic estrogen analog 4-hydroxytamoxifen (4-OHT). The ER.MYC protein is held in the cytoplasm through association with the HSP-90 protein. Upon binding 4-OHT, ER.MYC translocates into the nucleus, where it regulates the expression of target genes. 4-OHT treatment of IMR90-ER.MYC cells led to significant accumulation of *SRSF1* mRNA (Figure 2A) and SRSF1 protein (Figure 2B). As a control for ER.MYC induction, we verified the upregulation of a known MYC target gene, *NCL* (Figure S2A). Moreover, IMR90 cells transduced with empty vector did not induce *SRSF1* upon 4-OHT treatment (Figure S2B). A MYC deletion mutant lacking amino acids 106–143, which comprise MYC Box II (MBII) in the transcription-activation domain (TAD) (Oster et al., 2003), failed to induce *SRSF1* expression (Figures 2A and 2B), indicating that MYC requires an intact TAD to upregulate *SRSF1* expression. We also observed increased SRSF1 levels upon MYC induction in two immortal cell lines: MCF-10A mammary epithelial cells and Rat1a fibroblasts (Figure S2C). Two additional SR protein genes, *SRSF5* and *SRSF11*, showed no change in expression upon MYC induction, though both were predicted as MYC target genes by

a genome-wide ChIP-on-Chip analysis (Li et al., 2003; Figure S2D). In addition to showing the specificity of the effect of MYC on *SRSF1*, these results emphasize the need for validation to determine the true MYC targets among those predicted by genome-wide analyses.

We next analyzed the splicing of two previously reported SRSF1 target genes, *MKNK2* and *TEAD1* (Karni et al., 2007). *MKNK2* encodes the eIF4E-kinase MKNK2 and expresses two isoforms by alternative splicing of 3' exons 13A and 13B, whereas *TEAD1* encodes the transcriptional enhancer factor protein TEF-1 and expresses two isoforms by alternative splicing of exon 5. IMR90 cells overexpressing SRSF1 predominantly expressed the +13B isoform of *MKNK2* and the +5 isoform of *TEAD1*, as expected (Karni et al., 2007; Figure 2C, lanes 1 and 2). Another splicing factor, hnRNPA1, which is also positively regulated by MYC (Biamonti et al., 1993; David et al., 2010) and frequently antagonizes SRSF1 (Mayeda and Krainer, 1992), did not alter *MKNK2* or *TEAD1* splicing (Figure 2C, lane 3). Induction of IMR90-ER.MYC cells with 4-OHT promoted a significant switch in *MKNK2* splicing from the +13A to the +13B isoform and promoted inclusion of exon 5 in the *TEAD1* transcript, consistent with the increase in SRSF1 (Figure 2C, lanes 4 and 5). Furthermore, induction of ER.MYC in cells transfected with siRNA against *SRSF1* did not trigger a change in *MKNK2* or *TEAD1* splicing (Figure 2C, lane 6), indicating that MYC alters *MKNK2* and *TEAD1* splicing through upregulation of *SRSF1* expression. We also observed that both *SRSF1* overexpression and MYC induction led to a significant increase in the overall *MKNK2* transcript level (Figure S2E), suggesting that both factors directly or indirectly regulate *MKNK2* expression at the level of transcription or mRNA stability. We also measured alternative splicing of a third SRSF1 target gene, *BIN1*, which encodes the tumor suppressor and pro-apoptotic protein BIN1. *SRSF1* overexpression promotes inclusion of the 12A exon in the *BIN1* transcript (Karni et al., 2007). However, we did not observe changes in alternative splicing of *BIN1* in response to MYC induction (Figure S2F), perhaps due to other splicing factors also being modulated by MYC and counteracting the SRSF1-mediated inclusion of exon 12A.

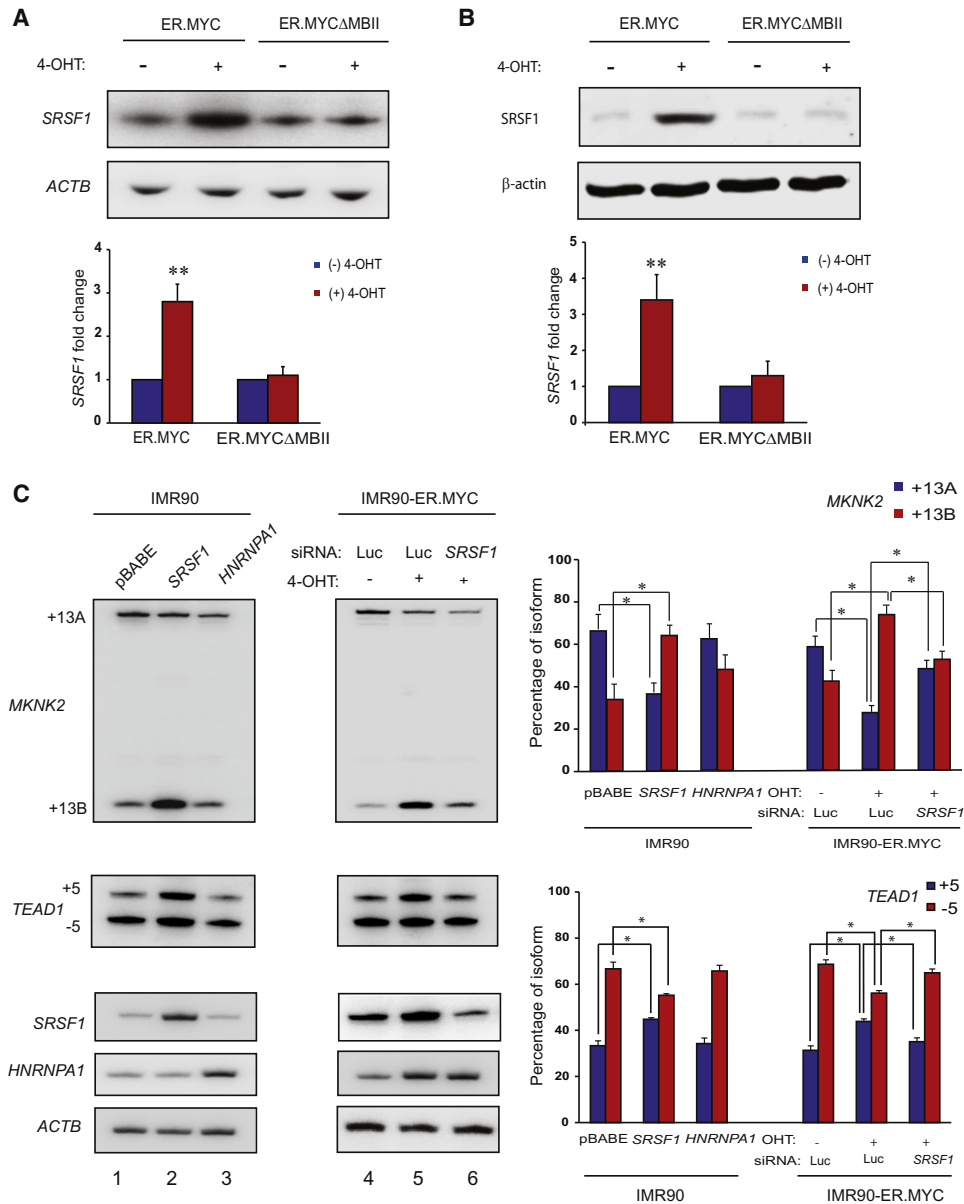


Figure 2. MYC Regulates SRSF1 Expression and Alternative Splicing of SRSF1 Target Genes

(A and B) (A) RT-PCR and (B) Immunoblotting of SRSF1 from IMR90-ER.MYC or IMR90-ER.MYCΔMBII cells induced with 4-OHT. Error bars, SD; n = 3; t test **p < 0.01.

(C) RT-PCR of MKNK2 and TEAD1 mRNA isoforms in IMR90-ER.MYC cells induced with 4-OHT, with or without SRSF1 knockdown. IMR90 cells overexpressing SRSF1 or hnRNPA1 are shown as controls. Error bars, SD; n = 3, *p < 0.05. See also Figure S2.

Because MYC was predicted to bind the *SRSF1* promoter by ChIP-on-Chip analysis of CpG islands (Mao et al., 2003), we investigated whether *SRSF1* is a direct transcriptional target of MYC. Treatment of IMR90-ER.MYC cells with the protein-synthesis inhibitor cycloheximide prior to 4-OHT induction of ER.MYC did not abrogate the upregulation of *SRSF1* mRNA (Figure 3A), indicating that de novo protein synthesis is not required for MYC to activate *SRSF1* expression. Moreover, analysis of the human *SRSF1* promoter sequence revealed

three putative noncanonical MYC binding sites (E-boxes). We therefore used ChIP to assess binding of MYC to the *SRSF1* promoter locus in the lung-carcinoma cell line NCI.H460, which downregulates *SRSF1* expression in response to MYC knockdown (Figures 1C and 1D). Our ChIP analysis revealed significant enrichment of MYC at the *SRSF1* proximal promoter region comprising two E-boxes mapping at -412 and -39, relative to the transcription start site (Figure 3B). We also detected MYC enrichment at a third E-box at position +146,

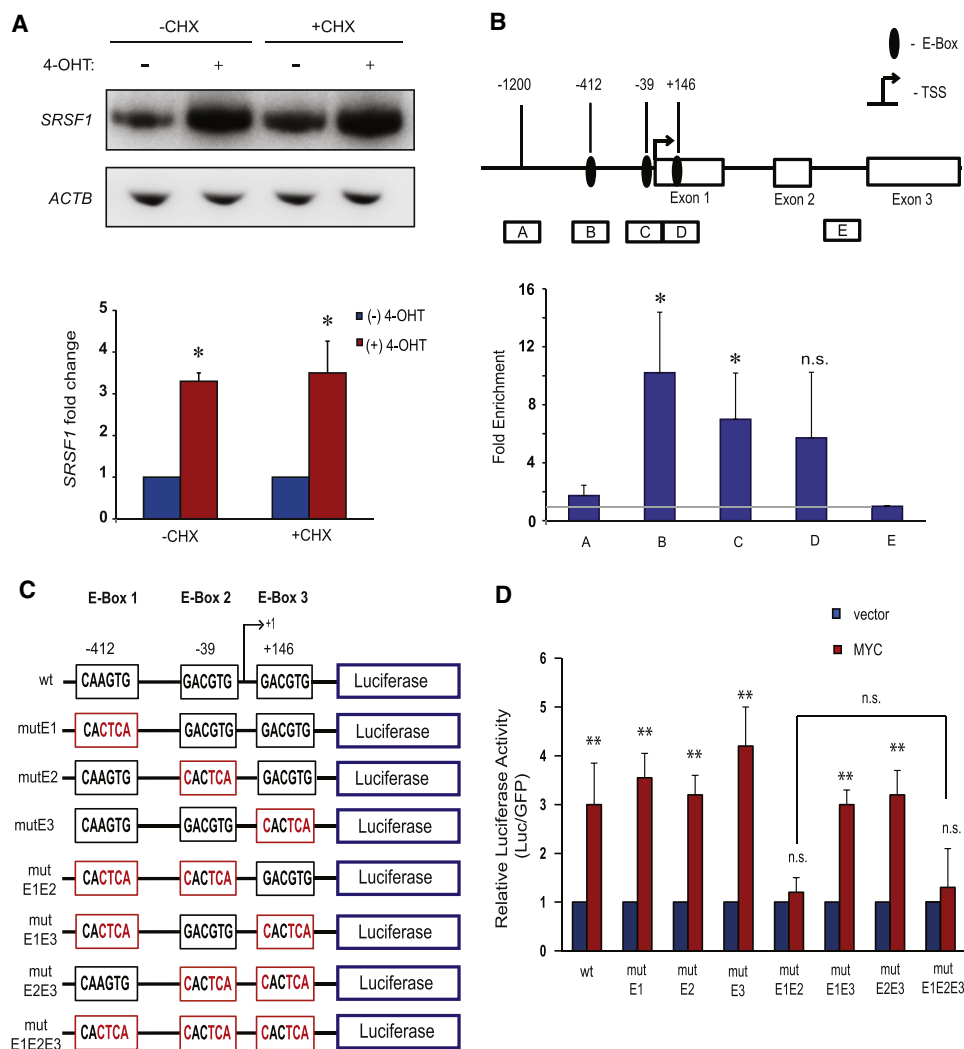


Figure 3. MYC Binds to and Activates the Human *SRSF1* Promoter

(A) RT-PCR of IMR90-ER.MYC cells treated with 4-OHT, with or without cycloheximide. Error bars, SD; n = 3; **p < 0.01.

(B) MYC chromatin immunoprecipitation analysis at the *SRSF1* promoter locus in the lung carcinoma NCI-H460 cell line. Diagram of the *SRSF1* gene indicating the E-boxes and amplicons (A-E) used for ChIP assays. The results are expressed as DNA enrichment in fragmented chromatin immunoprecipitated with anti-MYC antibody (relative to anti-rabbit IgG immunoprecipitation) and normalized to the amplicon E signal, as measured by quantitative PCR. The horizontal gray line represents no change in MYC-specific enrichment. Error bars, SD; n = 3; t test *p < 0.05; n.s., not significant.

(C) Diagram of the wild-type *SRSF1* promoter, comprising three noncanonical E-boxes, and the E-Box mutants generated for reporter assays. Mutant E-boxes and residues are indicated in red. (D) Luciferase assay of reporter constructs in (C) cotransfected with MYC cDNA or vector control into NIH 3T3 cells. Luciferase activity was normalized to cotransfected GFP, and the relative activity is plotted. Error bars, SD; n = 3; t test **p < 0.01; n.s., not significant.

relative to the transcription start site, but this was not significant and likely corresponds to chromatin fragments that overlap the E-box at -39 (Figure 3B). MYC binding to the *SRSF1* proximal promoter region is also evident in genome-wide data from HeLa and K562 cells obtained by the ENCODE genome-wide ChIP sequencing project (UCSC genome browser, assembly NCBI36/hg18, Yale/UC Davis/Harvard study). The same study also reported the binding of MYC's obligate heterodimerization partner MAX (Amati and Land, 1994) to the *SRSF1* proximal promoter region, suggesting MYC activity at the locus.

To determine whether these are functional MYC binding sites, we amplified a 1,500 bp genomic fragment of the *SRSF1* promoter, comprising these putative E-boxes (from -1,200 to +300 relative to the transcription-start site [TSS]), inserted it upstream of a luciferase reporter gene, and assayed for its MYC responsiveness in transfected NIH 3T3 cells. We also generated constructs with mutations in the three E-boxes, either individually or together, to an inactive CACTCA sequence (Figure 3C). MYC overexpression resulted in ~3-fold induction of luciferase activity for the wild-type construct (Figure 3D), relative to the vector control. The double, but not the individual,

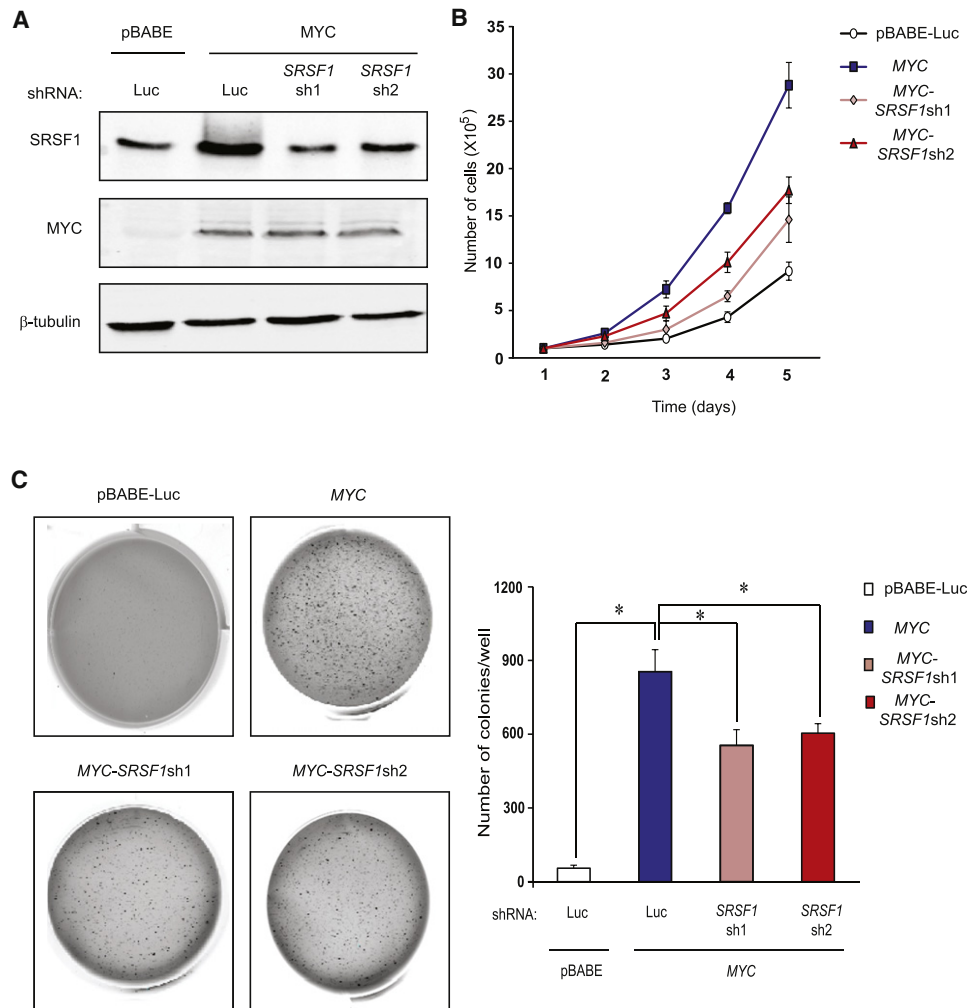


Figure 4. SRSF1 Knockdown Impairs Anchorage-Independent Growth of MYC-Transformed Cells

(A) Immunoblotting of MYC and SRSF1 in the Rat1a-pBabe-Luc control cell line, Rat1a-MYC, and Rat1a-MYC cells transduced with one of two shRNAs against SRSF1.

(B) Growth curves of the four cell lines from (A). Error bars, SD; n = 3.

(C) Anchorage-independent growth of cell lines from (A) in soft-agar colony-formation assays. Error bars, SD; n = 3; t test *p < 0.05.

mutations of E-boxes 1 and 2 abrogated this MYC-induced activation, suggesting functional redundancy between the two elements (Figure 3D). Mutation of the third putative noncanonical E-box (E-box 3), downstream of the TSS, either alone or in combination with the other E-boxes, did not abrogate, or further reduce, luciferase activity, indicating that this site is nonfunctional (Figure 3D). We conclude that SRSF1 is a direct transcriptional target of MYC, with two functional noncanonical E-boxes in its promoter.

Both MYC and SRSF1 are strong oncogenes that control cell proliferation, cell-cycle progression, and apoptosis. We therefore asked whether SRSF1 induction is required for MYC-induced transformation. We generated MYC-overexpressing Rat1a fibroblasts transduced with either a control luciferase shRNA or two different shRNAs against SRSF1. SRSF1 knockdown was carefully modulated by optimizing the retroviral

MOI so as to cancel out the MYC-induced increase in SRSF1 protein, but without completely depleting it from the cells (Figure 4A). As expected, Rat1a-MYC cells showed elevated SRSF1 expression and increased proliferation, compared to Rat1a-pBabe-Luc control cells (Figure 4B). SRSF1 knockdown resulted in a significant decrease in the proliferation rate of the MYC-overexpressing cells, though it remained significantly higher than the control. In accordance with this result, DNA-content analysis by flow cytometry revealed a higher percentage of the MYC-overexpressing cells in the S-G2-M phase, compared to the vector control (Figure S3). SRSF1 knockdown significantly decreased the proportion of dividing cells, with more cells accumulating in the G0-G1 phases. Moreover, SRSF1 knockdown did not promote cell death, ruling out apoptosis as a cause of the observed decrease in growth rate (Figure S3). Rat1a-MYC-SRSF1sh cells showed significantly

decreased anchorage-independent growth, compared to Rat1a-MYC cells (Figure 4C). We conclude that *SRSF1* is a critical MYC target gene, required for MYC's full activity in tumorigenesis.

Recently, MYC was shown to regulate the expression of other splicing factors—hnRNPA1, hnRNPA2, PTB1, and hnRNPH—and through them to regulate alternative splicing of pyruvate kinase M and oncogenic A-Raf kinase pre-mRNAs (David et al., 2010; Rauch et al., 2011). In the present study, we show MYC-mediated positive regulation of the oncogenic splicing factor SRSF1. MYC activates transcription from the *SRSF1* promoter, and the resulting increase in SRSF1 leads to altered splicing of some but not all of its target genes. Furthermore, we found *SRSF1* to be a critical MYC target, necessary for MYC's oncogenic activity. We have also found that *SRSF1* and MYC cooperate in transforming mammary epithelial cells, and their expression correlates in human breast tumors (Anczuków et al., 2012). The SRSF1 target genes that do undergo a splicing change upon MYC induction are therefore likely to be important mediators of MYC activity. Furthermore, considering the role of SRSF1 in multiple processes other than splicing, such as translation and mTOR signaling, there are likely several additional downstream effectors of SRSF1 that contribute to MYC function. The overall picture that emerges from these studies is that, in addition to regulating transcription of its target genes, MYC also indirectly regulates the expression of protein isoforms through regulation of alternative splicing of a subset of transcripts, and these changes contribute to MYC's biological functions.

EXPERIMENTAL PROCEDURES

Plasmids

T7-tagged *SRSF1* and *HNRNPA1* cDNAs cloned in the pBabe-Puro retroviral vector were described previously (Karni et al., 2007). pBabe-Puro-ER.MYC (Littlewood et al., 1995) was used to generate the pBabe-Puro-ER.MYCΔMBII construct by Quick-change site-directed mutagenesis (Stratagene). The Transcriptional Regulatory Element Database (TRED) (Zhao et al., 2005) was used to obtain the *SRSF1* promoter sequence (Promoter ID 18315). The *SRSF1* promoter from −1,200 to +300 (relative to the TSS) was amplified from human genomic DNA (Promega) and cloned into the pGL3 vector (Promega). MYC-binding sites in the wild-type *SRSF1* promoter were mutated by Quick-change site-directed mutagenesis.

Cell Culture and Stable Cell Line Generation

IMR90, NIH 3T3, and Rat1a cells were grown in DMEM medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin, and streptomycin. NCI-H524, NCI-H460, NCI-H1299, NCI-H1568, and NCI-H1975 cells were grown in RPMI 1640 medium (Invitrogen) supplemented with 10% (v/v) FBS, penicillin, and streptomycin. A549 cells were grown in F12K medium (Invitrogen) supplemented with 10% (v/v) FBS. NCI-H1666 cells were grown in DMEM/F12 medium (Invitrogen) supplemented with 5% (v/v) FBS, penicillin, and streptomycin. MCF10A cells were grown in DMEM/F12 medium, supplemented with 5% (v/v) horse serum, 20 ng/ml EGF, 100 μg/ml hydrocortisone, 10 ng/ml cholera toxin, penicillin, and streptomycin. To generate stable pools, IMR-90 and Rat1a cells were infected with pBabe-Puro or pBabe-hygro retroviral vectors expressing ER.MYC or MYC cDNAs, respectively, followed by selection with puromycin (2 μg/ml) or hygromycin (200 μg/ml) for 72 hr. For MYC induction studies, ER.MYC-expressing cells were grown to confluence and treated with 2 μM 4-OHT for 8 hr for RT-PCR, and 48 hr for immunoblotting and splicing analysis.

RNA Interference

For inhibition of MYC or *SRSF1* expression, cells were seeded (2×10^5 cells per well) in 6-well plates in antibiotic-free medium. After 24 hr, cells were transfected with 200 pmol short interfering RNA against MYC (Cell Signaling, Catalog No. 6553) or SRSF1 (target sequence 5'-ACGAUUGCCGCAU CUACGU-3') using Lipofectamine RNAiMAX (Invitrogen). After a further 48 hr, cells were lysed, and protein and RNA were extracted as described below. For stable knockdown of SRSF1, Rat1a cells were separately transduced with each of two SRSF1 shRNAs cloned in the retroviral vector LMP9, and selected with 2 μg/ml puromycin for 4 days.

Immunoblotting

Cells were lysed in RIPA buffer and protein was quantitated using a Bradford Protein Assay kit (Bio-Rad). Total protein (25 μg) from each lysate was separated by SDS-PAGE and transferred onto a nitrocellulose membrane (Whatman), followed by blocking with 5% (w/v) dry milk in Tris-buffered saline with 0.05% (v/v) Tween-20, probing with the indicated antibodies, and quantitation using an Odyssey infrared-imaging system (LI-COR Biosciences). Primary antibodies used were: MYC (Cell Signaling rAb, 1:500); SRSF1 (mAb AK96 culture supernatant (Hanamura et al., 1998), 1:500); SRSF9 (mAb culture supernatant, 1:50); β-actin (Sigma mAb, 1:10,000), and β-tubulin (Genscript rAb, 1:10,000). Secondary antibodies were IRdye 800 or 680 anti-rabbit or anti-mouse (LI-COR Biosciences, 1:10,000).

RT-PCR Analysis

Cells were lysed with Trizol reagent (Invitrogen) and total RNA was extracted. Following DNase I digestion (Promega), 2 μg of total RNA was reverse-transcribed with Improm-II reverse transcriptase (Promega). Radioactive PCR (25 cycles) with [α - 32 P]-dCTP was used to amplify endogenous transcripts. The products were run on a 5% native polyacrylamide gel, visualized by autoradiography, and quantitated on a FUJIFILM FLA-5100 phosphorimager (Fuji Medical Systems) using Multi Gauge software Version 2.3 (Fujifilm). The PCR primers used were as follows:

h*SRSF1*F: 5'-ATGTCGGGAGGTGGTGTGATTTC-3'
 h*SRSF1*R: 5'-TGTTCCACGGCCGCTTCGAG-3'
 r*SRSF1*F: 5'-CGCGACATCGACCTGAAGAAC-3'
 r*SRSF1*R: 5'-CCACGACACAGTGCCATCTCG-3'
*HNRNPA1*F: 5'-AAAGACCAGGTGCCACTTA-3'
*HNRNPA1*R: 5'-AATCTTATCCACGAGCATGG-3'
MYCF: 5'-GGTACAAGCTGGAGGTGGAG-3'
MYCR: 5'-AATCTTATCCACGAGTCATGG-3'
NCLF: 5'-TTTCTTTCTTTGGCTGGT-3'
NCLR: 5'-ATGGCAAGAATGCCAAGAAG-3'
*MKNK2*Ex11F: 5'-CCAAGTCTGCAGCACCCCTG-3'
*MKNK2*Ex13aR: 5'-GATGGGAGGGTCAGGCGTGGTC-3'
*MKNK2*Ex13bR: 5'-GAGGAGGAAGTACTGTCCCAC-3'
*TEAD1*Ex3, 4F: 5'-AGACGAAGGCAAATGTATGG-3'
*TEAD1*Ex9, 8R: 5'-CGTAGGCTCAAACCCTGGAAT-3'
*BIN1*Ex11F: 5'-CCTCCAGATGGCTCCCCTGC-3'
*BIN1*Ex15R: 5'-CCCGGGGGCAGGTCCAAGCG-3'
 β-actinF: 5'-GTGCCATTATGAGGGCTA-3'
 β-actinR: 5'-CTGGCAGCTCGTAGCTCTTT-3'

Chromatin Immunoprecipitation

ChIP assays were performed as described (Steger et al., 2008). Crosslinking was performed with sequential 15 mM EGS (Pierce) and 1% (v/v) formaldehyde treatment. Antibodies used for immunoprecipitation were rabbit anti-myc (Cell Signaling, 9402) and rabbit IgG (Cell Signaling). Immunoprecipitated DNA was analyzed by quantitative PCR using SYBR green (ABI) on an ABI 7900HT instrument. PCR primers were as follows:

Amplicon A:
 F: 5'-CCCAGCCTGATTTGAATTTT-3'
 R: 5'-GAAAATACCGGTCTCTCAGG-3'
 Amplicon B:
 F: 5'-GGATTAGACGCACCTACGA-3'

R: 5'-CGATTCTCCAGGAATGAGG-3'

Amplicon C:

F: 5'-ACGTAGCCCTCGCAGCAC-3'

R: 5'-GGACTCGAGAACAGGCCTTC-3'

Amplicon D:

F: 5'-CTTTTCGTCAACGCCATGT-3'

R: 5'-GTCCTCGAACTCAACGAAGG-3'

Amplicon E:

F: 5'-GGATTGATGTGAAGGGACGA-3'

R: 5'-TGGAATCCAGAGTCCAAAAT-3'

Luciferase Reporter Assay

MYC expression vector (500 ng), 100 ng of pGL3-Luciferase reporter comprising nucleotides -1,200 to +300 of the *SRSF1* promoter—with or without E-box mutations—and 100 ng of pEGFP vector were cotransfected into NIH 3T3 cells using Fugene 6 (Roche). Thirty-six hours after transfection, the cells were lysed, and luciferase activity was measured using a Dual Luciferase Reporter Assay kit (Promega). RNA was extracted from the remaining cell lysate, and the GFP level was measured by radioactive RT-PCR and used as a transfection control to normalize luciferase activity.

Growth Curves and Proliferation Assay

Rat1a cells transduced with pBABE-hygro, pBABE-MYC, LMP-Puro, LMP-SRSF1sh1, or LMP-SRSF1sh2 were seeded at 1×10^5 cells per 60 mm dish. At the indicated times, triplicate plates of cells were trypsinized, stained with Trypan blue, and unstained cells were counted using a hemocytometer.

Anchorage-Independent Growth

Rat1a cells from each transductant pool were plated (20,000 cells per well) in triplicate in 0.35% (w/v) agar in DMEM supplemented with 10% (v/v) FBS on a layer of 0.7% (w/v) agar. Cells were incubated at 37°C and 5% (v/v) CO₂ for 14 days. Colonies were stained with 0.005% (w/v) Crystal Violet, and whole-well images were taken using the Odyssey Imaging System. The images were analyzed using Image-J software, and the average number of colonies per well for each transductant pool was determined.

Microarray Analysis

The GEO GSE2109 data set from the Expression Project for Oncology (<http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE2109>) was used to obtain expression profile data from 132 clinically annotated human lung tumors. Each sample was standardized by calculating Z-scores based on the sample average and SD across the entire set of genes. Expression profiles of *SRSF1* and *MYC* were extracted for all the samples. A contingency table was built showing the number of samples with high expression of both *SRSF1* and *MYC*, only *SRSF1*, only *MYC*, or neither (Z-score >1.29, corresponding to a p value of 0.1). A Mann-Whitney test was used to compare *SRSF1* expression in lung tumors containing high versus low *MYC* levels (above and below the median).

Statistical Analysis

All histograms were plotted using mean \pm SD. Data points were compared using unpaired two-tailed Student's t tests, and p values are indicated in the figure legends. Pearson correlation was used to evaluate the association between *MYC* and *SRSF1* expression detected by quantitative immunoblotting.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, three figures, and one table and can be found with this article online at [doi:10.1016/j.celrep.2011.12.001](https://doi.org/10.1016/j.celrep.2011.12.001).

LICENSING INFORMATION

This is an open-access article distributed under the terms of the Creative Commons Attribution 3.0 Unported License (CC-BY; <http://creativecommons.org/licenses/by/3.0/legalcode>).

ACKNOWLEDGMENTS

We thank John Kurland, Eric Wang, and Chris Vakoc for reagents and useful discussions; Rafaella Sordella for lung cancer cell lines; and Chaolin Zhang for *SRSF1* promoter-sequence analysis. This work was supported by grant CA13106 from the National Cancer Institute.

Received: October 30, 2011

Revised: December 4, 2011

Accepted: December 7, 2011

Published online: February 9, 2012

REFERENCES

- Adhikary, S., and Eilers, M. (2005). Transcriptional regulation and transformation by Myc proteins. *Nat. Rev. Mol. Cell Biol.* 6, 635–645.
- Amati, B., and Land, H. (1994). Myc-Max: a transcription factor network controlling cell cycle progression, differentiation and death. *Curr. Opin. Genet. Dev.* 4, 102–108.
- Anczuków, O., Rosenberg, A.Z., Akerman, M., Das, S., Zhan, L., Karni, R., Muthuswamy, S., and Krainer, A.R. (2012). The splicing-factor SRSF1 regulates apoptosis and proliferation to promote mammary epithelial cell transformation. *Nat. Struct. Mol. Biol.* 19, 220–228.
- Biamonti, G., Bassi, M.T., Cartegni, L., Mechta, F., Buvoli, M., Cobiainchi, F., and Riva, S. (1993). Human hnRNP protein A1 gene expression. Structural and functional characterization of the promoter. *J. Mol. Biol.* 230, 77–89.
- Coller, H.A., Grandori, C., Tamayo, P., Colbert, T., Lander, E.S., Eisenman, R.N., and Golub, T.R. (2000). Expression analysis with oligonucleotide microarrays reveals that MYC regulates genes involved in growth, cell cycle, signaling, and adhesion. *Proc. Natl. Acad. Sci. USA* 97, 3260–3265.
- David, C.J., Chen, M., Assanah, M., Canoll, P., and Manley, J.L. (2010). HnRNP proteins controlled by c-Myc deregulate pyruvate kinase mRNA splicing in cancer. *Nature* 463, 364–368.
- Eilers, M., Picard, D., Yamamoto, K.R., and Bishop, J.M. (1989). Chimaeras of Myc oncoprotein and steroid receptors cause hormone-dependent transformation of cells. *Nature* 340, 66–68.
- Ezponda, T., Pajares, M.J., Agorreta, J., Echeveste, J.I., López-Picazo, J.M., Torre, W., Pio, R., and Montuenga, L.M. (2010). The oncoprotein SF2/ASF promotes non-small cell lung cancer survival by enhancing survivin expression. *Clin. Cancer Res.* 16, 4113–4125.
- Ge, H., and Manley, J.L. (1990). A protein factor, ASF, controls cell-specific alternative splicing of SV40 early pre-mRNA in vitro. *Cell* 62, 25–34.
- Hanamura, A., Cáceres, J.F., Mayeda, A., Franza, B.R., Jr., and Krainer, A.R. (1998). Regulated tissue-specific expression of antagonistic pre-mRNA splicing factors. *RNA* 4, 430–444.
- Huang, Y., Gattoni, R., Stévenin, J., and Steitz, J.A. (2003). SR splicing factors serve as adapter proteins for TAP-dependent mRNA export. *Mol. Cell* 11, 837–843.
- Karni, R., de Stanchina, E., Lowe, S.W., Sinha, R., Mu, D., and Krainer, A.R. (2007). The gene encoding the splicing factor SF2/ASF is a proto-oncogene. *Nat. Struct. Mol. Biol.* 14, 185–193.
- Krainer, A.R., Conway, G.C., and Kozak, D. (1990). Purification and characterization of pre-mRNA splicing factor SF2 from HeLa cells. *Genes Dev.* 4, 1158–1171.
- Lemaire, R., Prasad, J., Kashima, T., Gustafson, J., Manley, J.L., and Lafyatis, R. (2002). Stability of a PKC δ -1-related mRNA is controlled by the splicing factor ASF/SF2: a novel function for SR proteins. *Genes Dev.* 16, 594–607.
- Li, X., Wang, J., and Manley, J.L. (2005). Loss of splicing factor ASF/SF2 induces G2 cell cycle arrest and apoptosis, but inhibits internucleosomal DNA fragmentation. *Genes Dev.* 19, 2705–2714.
- Li, Z., Van Calcar, S., Qu, C., Cavenee, W.K., Zhang, M.Q., and Ren, B. (2003). A global transcriptional regulatory role for c-Myc in Burkitt's lymphoma cells. *Proc. Natl. Acad. Sci. USA* 100, 8164–8169.

- Littlewood, T.D., Hancock, D.C., Danielian, P.S., Parker, M.G., and Evan, G.I. (1995). A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. *Nucleic Acids Res.* *23*, 1686–1690.
- Mao, D.Y., Watson, J.D., Yan, P.S., Barsyte-Lovejoy, D., Khosravi, F., Wong, W.W., Farnham, P.J., Huang, T.H., and Penn, L.Z. (2003). Analysis of Myc bound loci identified by CpG island arrays shows that Max is essential for Myc-dependent repression. *Curr. Biol.* *13*, 882–886.
- Mayeda, A., and Krainer, A.R. (1992). Regulation of alternative pre-mRNA splicing by hnRNP A1 and splicing factor SF2. *Cell* *68*, 365–375.
- Oster, S.K., Mao, D.Y.L., Kennedy, J., and Penn, L.Z. (2003). Functional analysis of the N-terminal domain of the Myc oncoprotein. *Oncogene* *22*, 1998–2010.
- Rauch, J., Moran-Jones, K., Albrecht, V., Schwarzl, T., Hunter, K., Gires, O., and Kolch, W. (2011). c-Myc regulates RNA splicing of the A-Raf kinase and its activation of the ERK pathway. *Cancer Res.* *71*, 4664–4674.
- Sanford, J.R., Gray, N.K., Beckmann, K., and Cáceres, J.F. (2004). A novel role for shuttling SR proteins in mRNA translation. *Genes Dev.* *18*, 755–768.
- Schlosser, I., Hölzel, M., Hoffmann, R., Burtscher, H., Kohlhuber, F., Schuhmacher, M., Chapman, R., Weidle, U.H., and Eick, D. (2005). Dissection of transcriptional programmes in response to serum and c-Myc in a human B-cell line. *Oncogene* *24*, 520–524.
- Sinclair, C.S., Rowley, M., Naderi, A., and Couch, F.J. (2003). The 17q23 amplicon and breast cancer. *Breast Cancer Res. Treat.* *78*, 313–322.
- Steger, D.J., Lefterova, M.I., Ying, L., Stonestrom, A.J., Schupp, M., Zhuo, D., Vakoc, A.L., Kim, J.E., Chen, J., Lazar, M.A., et al. (2008). DOT1L/KMT4 recruitment and H3K79 methylation are ubiquitously coupled with gene transcription in mammalian cells. *Mol. Cell Biol.* *28*, 2825–2839.
- Sun, D., Novotny, M., Bulek, K., Liu, C., Li, X., and Hamilton, T. (2011). Treatment with IL-17 prolongs the half-life of chemokine CXCL1 mRNA via the adaptor TRAF5 and the splicing-regulatory factor SF2 (ASF). *Nat. Immunol.* *12*, 853–860.
- Sun, S., Zhang, Z., Sinha, R., Karni, R., and Krainer, A.R. (2010). SF2/ASF autoregulation involves multiple layers of post-transcriptional and translational control. *Nat. Struct. Mol. Biol.* *17*, 306–312.
- Thorsen, K., Mansilla, F., Schepeler, T., Øster, B., Rasmussen, M.H., Dyrskjøt, L., Karni, R., Akerman, M., Krainer, A.R., Laurberg, S., et al. (2011). Alternative splicing of SLC39A14 in colorectal cancer is regulated by the Wnt pathway. *Mol. Cell Proteomics* *10*, M110.002998.
- Wu, H., Sun, S., Tu, K., Gao, Y., Xie, B., Krainer, A.R., and Zhu, J. (2010). A splicing-independent function of SF2/ASF in microRNA processing. *Mol. Cell* *38*, 67–77.
- Xu, X., Yang, D., Ding, J.H., Wang, W., Chu, P.H., Dalton, N.D., Wang, H.Y., Birmingham, J.R., Jr., Ye, Z., Liu, F., et al. (2005). ASF/SF2-regulated CaMKII δ alternative splicing temporally reprograms excitation-contraction coupling in cardiac muscle. *Cell* *120*, 59–72.
- Zeller, K.I., Jegga, A.G., Aronow, B.J., O'Donnell, K.A., and Dang, C.V. (2003). An integrated database of genes responsive to the Myc oncogenic transcription factor: identification of direct genomic targets. *Genome Biol.* *4*, R69.
- Zhang, Z., and Krainer, A.R. (2004). Involvement of SR proteins in mRNA surveillance. *Mol. Cell* *16*, 597–607.
- Zhao, F., Xuan, Z., Liu, L., and Zhang, M.Q. (2005). TRED: a Transcriptional Regulatory Element Database and a platform for in silico gene regulation studies. *Nucleic Acids Res.* *33*(Database issue), D103–D107.