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Regulation and Function of Oncogenic Splicing Factor SRSF1

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Abstract of the Dissertation

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SRSF1 is a prototypical member of the Ser/Arg-rich family of splicing factors. In addition to its involvement in constitutive and alternative splicing, SRSF1 regulates many other aspects of RNA metabolism, including transcription, mRNA export, stability and decay, translation, and miRNA processing. In addition, SRSF1 contributes to maintaining genomic stability, and is an important regulator of the cell cycle and cell viability. In spite of multiple post-transcriptional and translational mechanisms for SRSF1 auto-regulation, it is frequently upregulated in cancer. Here we focus on studying the regulation of SRSF1 expression and function.

We have found *SRSF1* to be a direct transcriptional target of the oncoprotein MYC, suggesting a mechanism for *SRSF1* upregulation in a subset of tumors with elevated MYC levels. MYC activates transcription of *SRSF1* through two non-canonical E-boxes in the *SRSF1* promoter. MYC activity alters the splicing profile of some but not all of SRSF1's splicing targets. Furthermore, *SRSF1* induction by MYC is important for MYC-mediated transformation.

While investigating the SRSF1 protein interactome using quantitative mass spectrometry, we identified a novel function of SRSF1. SRSF1 interacts with the ribosomal protein RPL5 and the E3 ubiquitin ligase MDM2 upon induction of ribosomal stress. This results in decreased

ubiquitylation and increased stability of the tumor-suppressor protein p53, which then activates the cellular stress response.

We previously showed that upregulation of *SRSF1* leads to transformation of immortalized cells. However, upon overexpression in primary fibroblasts, SRSF1 recruits the RPL5-MDM2 complex and stabilizes p53, resulting in oncogene-induced senescence, a tumor-protective response. Our studies therefore identify a transcription factor responsible for *SRSF1* overexpression in certain tumors, and an anti-tumorigenic mechanism through which cells initially respond to *SRSF1* overexpression.

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Publications and Ongoing Work

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Chapter 2 describes the work on MYC-mediated transcriptional regulation of SRSF1, published mainly in *Cell Reports* and as a part of a paper in *Nature Structural and Molecular Biology*. Olga Anczuków and Martin Akerman performed the analysis of the microarray data. Adrian Krainer and I wrote the manuscript.

Chapter 3 describes the work on SRSF1's role in ribosomal stress and OIS, published in *Molecular Cell* and outlined as a review in *Cell Cycle*. Oliver Fregoso initiated the project, and I continued it when he moved to Fred Hutchinson Cancer Research Center, Washington. Oliver performed the mass spectrometry analysis and the siRNA-mediated knockdown of RPL5. All other experiments were designed and performed by me either on my own or in collaboration with Oliver. Martin Akerman performed the analysis of the microarray data from human tumors. Oliver, Adrian, and I wrote the manuscript, with Oliver and I as co-first authors.

Chapter 1. Background

1.1 Split Genes and RNA Splicing

In the latter half of the 1970s, more than two decades after the discovery of the DNA double helix, two independent studies described the existence of 'split genes' and RNA splicing (Berget et al., 1977; Chow et al., 1977). The formation of R-loop structures (region of unhybridized, single-stranded DNA) in mRNA:DNA hybridization experiments revealed the presence of intragenic sequences that are removed from the primary mRNA transcript, prior to its export from the nucleus into the cytoplasm. Though initially discovered in adenovirus, it quickly became apparent that such 'split genes' are a characteristic feature of the majority of eukaryotic genomes, and their existence adds a further layer of complexity to the central dogma of molecular biology (Crick, FH., 1958).

The eukaryotic split genes, unlike the prokaryotic genes, contain protein-coding sequences called exons, interspersed with non-coding sequences called introns. The human genome reportedly contains approximately 230,000 exons and 210,000 introns, or an average of 9 exons and 8 introns per gene. (Sakharkar et al., 2004). Expression of protein-coding genes, which initiates with transcription by RNA Pol II, leads to generation of the pre-mRNA, which contains both exons and introns. This pre-mRNA then undergoes RNA splicing, wherein the introns are excised and the exons are precisely ligated to generate a functional mRNA with an uninterrupted coding sequence and the potential to code for a functional protein. Thus pre-mRNA splicing, in addition to 5'-capping and 3'-end cleavage and polyadenylation is a crucial and highly regulated event in gene expression (Crick, FH., 1979).

1.2 The Splicing Reaction and its cis-Regulators

The process of splicing is catalyzed in the nucleus and consists of two sequential trans-esterification reactions (Burge et al., 1999; Wachtel & Manley, 2009). In the first reaction, the 2'-OH of an adenosine nucleotide at an intronic conserved sequence element called the branch point (BPS), nucleophilically attacks the first nucleotide of the intron, leading to the cleavage of the phosphodiester bond between the upstream exon and the intron. In the next reaction, the now liberated 3'-OH end of the upstream exon makes a nucleophilic attack at the phosphodiester bond between the intron and the downstream exon, leading to the ligation of the upstream and

downstream exons and the removal of the intervening intron in the form of a lariat. The nucleotide at the branch-point and the upstream and downstream exon-intron boundaries are all part of conserved elements that conform to degenerate consensus sequence motifs called the 5' splice site (5'ss), the 3' splice site (3'ss), the branch point sequence (BPS) which is located 18-40 nucleotides upstream of the 3'ss, and the Poly-pyrimidine tract (PPT) between the BPS and the 3'ss. While these degenerate sequence motifs are necessary, they are not always sufficient for definition of the exon/intron boundaries and require additional cis-regulatory sequences known as intronic and exonic splicing enhancers (ISE, ESE) and silencers (ISS, ESS) and the corresponding trans-acting proteins that bind to them (Fu, 2004) (**Figure 1.1**).

1.3 The Splicing Machinery

The splicing reaction is catalyzed by a large macromolecular complex called the spliceosome, which comprises five snRNAs (small nuclear RNA) and more than 150 associated spliceosomal proteins (Black, 2003; Jurica and Moore, 2003). There are two different spliceosome complexes in metazoans and some plants—the major (also called U2-type) and the minor spliceosome (also called U12-type). The major spliceosome, which is composed of the U1, U2, U4/U6 and U5 small nuclear ribonucleoproteins (snRNP), carrying the U1, U2, U4/U6 and U5 snRNAs, respectively, catalyzes the excision of the majority of eukaryotic introns-defined by GU-AG boundaries. The minor spliceosome, on the other hand, typically removes a minor class of introns in some cases defined by AT-AC boundaries and is composed of the U11, U12, U4atac/U6atac and the U5 snRNPs (Patel and Steitz, 2003).

The spliceosome is a highly dynamic complex and its specific components recognize and bind the 5'-ss, 3'-ss, the PPT and the BPS in a step-wise fashion. The assembly of the spliceosome and its subsequent catalytic activity can be divided into the following ordered, sequential series of events (Brow, 2002; Burge et al., 1999; Wahl et al., 2009):

The E complex. The assembly of the spliceosome on the intron initiates with the ATP-independent binding of the U1 snRNP, mediated through base-pairing between the U1 snRNA and the pre-mRNA sequence at the 5'ss, and stabilized by the proteins in the snRNP.

Concomitantly, the BPS is bound by Splicing Factor 1/Branchpoint Binding Protein (SF1/BBP). SF1/BBP interacts with the U2 auxiliary factor (U2AF), which is a heterodimer of the PPT-binding U2AF65 subunit and the U2AF35 subunit, which binds the conserved AG dinucleotide at the 3'-ss. Together, the assembly of these proteins on the pre-mRNA forms the E complex and defines the sites at which splicing will occur.

The A complex. The next step is the formation of the A complex, wherein U2 snRNP displaces SF1 and binds to the BPS in an ATP-dependent manner. The binding of U2 snRNP to the pre-mRNA is through complementary base-pairing and the interaction is stabilized by the U2 snRNP-associated SF3a and SF3b complex (Gozani et al., 2006).

The B complex. Subsequently, a preassembled U4/U6/U5 tri-snRNP is recruited to the A complex, to form the B complex. U5 snRNP interacts with the 5′ and 3′ splice site sequences by weak base pairing between the uridine-rich loop of U5 snRNA and the borders of the two exons. The 3′ end of the U6 snRNA base pairs with the 5′ end of U2 snRNA. At this point, all snRNPs are present on the intron, but the complex is catalytically inactive.

The B* and C complex. Extensive conformational and compositional rearrangements including U1 and U4 destabilization and release result in the formation of the catalytically active B* complex, which catalyzes the first trans-esterification step in the splicing reaction. Subsequent rearrangements result in formation of the C complex, which catalyzes the second trans-esterification reaction, resulting in the excision of the intron as a lariat and the ligation of the upstream and downstream exons (Konarska et al., 2006).

Finally, the remaining components of the spliceosome dissociate and the snRNPs are recycled for the next round of splicing.

1.4 Alternative Splicing

Alternative splicing is the process by which a single gene can produce multiple mRNA transcripts through the differential inclusion of exons and exon-segments (Black, 2003; Maitlin, 2005). It was first discovered about 30 years ago, when it was found that the membrane-bound

and secreted forms of the immunoglobin M protein are produced by two different mRNA transcripts, but both transcripts are generated from the same gene (Earlya et al., 1980). Alternative splicing can hence allow a single gene to code for multiple protein isoforms with varying localization or functions and contributes enormously to protein diversity and phenotypic complexity in mammals. Recent mRNA-sequencing studies have shown that about 95% of multi-exon genes in humans undergo alternative splicing, and there are about 100,000 intermediate to high abundance alternative splicing events in major human tissues (Pan et al., 2008; Wang et al., 2008). The process of alternative splicing proceeds by much the same mechanism as constitutive splicing. However, alternatively spliced exons tend to have weaker 5' and 3' splice sites, as compared to constitutively spliced exons (Lear et al., 1990; Roca et al., 2005). They are also reported to contain fewer ESE elements on average (Black et al. 2003).

Alternatively spliced isoforms are either co-expressed or expressed in a cell-or tissue-specific manner and different isoforms can be expressed at different stages of development or in response to extra-cellular signals. In addition, alternative splicing regulates the expression and function of certain genes at the post-transcriptional level by targeting their alternatively spliced isoforms to degradation by nonsense mediated mRNA decay (NMD), nuclear retention, or generation of proteins that are mis-localized or non-functional. Accordingly, alternative splicing is a highly regulated process-both spatially and temporally.

1.5 Trans-acting Splicing Regulators

Splice-site recognition and assembly of the E complex at the splice sites is an important and carefully regulated step of pre-mRNA splicing, and to a large extent depends on the sequence complementarity of the 5'ss to U1 snRNP, the BPS to U2 snRNP and PPT length. As mentioned previously, while the 5'ss, 3'ss, BPS and PPT loosely conform to degenerate consensus sequences, they have a low degree of sequence conservation. For instance, the consensus sequence of the 5'ss alone is a compilation of >9000 sequence variants (Roca et al., 2012). They therefore are not sufficient for definition of the exon-intron boundary and spliceosome assembly. This is especially true for splicing of alternative exons, which have been shown to have weaker 5' and 3' ss, i.e., they have low complementarity to the spliceosomal snRNAs and thus require additional cues for the recruitment of the spliceosomal components. The problem is made even

more complex by the existence of pseudo-splice sites-sequences that resemble the bona fide splice sites and vastly outnumber them in the genome (Sun et al., 2000).

Thus, splice-site recognition and intron excision by the spliceosome further depend on the interplay between cis-acting regulatory elements (ESE, ESS, ISE and ISS), clustered in the vicinity of true splice sites, and the trans-acting RNA-binding proteins that specifically bind to them (Dreyfuss et al., 2002). These regulatory proteins can act as splicing activators or repressors, and generally function by regulating recruitment of the spliceosomal components to the intron. While activators promote splicing at the splice site and exon inclusion by facilitating spliceosome assembly, repressors inhibit splicing and lead to exon exclusion by interfering with binding of the spliceosomal components at the splice sites (Long and Cáceres, 2009).

Since alternate exons contain weak splice sites, the functions of the splicing regulators become even more important in alternative splicing. An individual splice site is influenced by multiple splicing enhancer and silencer elements and the splicing outcome at the site is a result of the combinatorial effect of the corresponding splicing activators and repressors. Alternative splicing events are thus regulated spatially and temporally by a delicate balance maintained between the expression and activity of splicing regulators. While many splicing regulators are ubiquitously expressed, they do show variation in levels of expression in different tissues or at different points in development. Furthermore, there are plicing regulators, such as NOVA, nPTB, FOX1 and FOX2, ESRP1 and ESRP2, which are expressed in a tissue-specific manner.

Two extensively studied classes of splicing regulators are the SR proteins and a subset of the hnRNPs (Busche et al., 2012).

1.5.1 heterogeneous nuclear Ribonucleoproteins (hnRNPs)

Heterogeneous nuclear ribonuclear proteins (hnRNPs) are a class of RNA-binding proteins, known to be involved in almost every aspect of RNA metabolism, including pre-mRNA splicing, mRNA nuclear export, localization, translation and stability (Dreyfuss et al., 2002). The hnRNP protein family is composed of at least 20 abundant major hnRNPs—designated hnRNPA1 through to hnRNPU—and a few other minor hnRNPs. They have a modular structure with an RNA recognition motif (RRM) domain that facilitates RNA-binding, RGG boxes (Arg-Gly-Gly tri-amino acids repeats) and additional glycine-rich, acidic or proline-rich domains to facilitate

protein-protein interaction (Chaudhury et al., 2010). The members of the hnRNP family that regulate splicing, such as the hnRNP A/B proteins, predominantly act as splicing repressors by binding to ESS or ISS elements and interfering with spliceosome assembly by either cooperatively binding along the exon, blocking recruitment of the snRNPs or SR proteins or by looping out the entire exon, thus making it inaccessible to the spliceosome (He and Smith, 2008; Zheng, 2004).

1.5.2 SR and SR-related Proteins

The classical SR proteins are Ser/Arg-rich splicing regulators. Like the hnRNPs, SR proteins also have a modular structure with one or two RRMs that facilitate sequence-specific binding to RNA and a C-terminal Arg/Ser-rich (RS) domain (Graveley, 2000).

SR protein activity is essential for constitutive splicing, as shown by *in vitro* complementation of splicing deficient HeLa S100 extract, and one SR protein can be interchanged for another, with at least one being required for splicing competence. However, every SR protein also shows a degree of substrate specificity, especially with regards to splicing regulation of alternative exons. They are found to bind to specific ESE sequence elements and activate splicing by promoting spliceosome assembly. The non-redundant role of the different SR protein family members is emphasized by the fact that SR-null mice for *SRSF1, SRSF2* or *SRSF3* are embryonic lethal (Jumaa et al., 1999; Moroy et al., 2007; Wang et al., 2001; Xu et al., 2005).

SR-related proteins are an additional class of splicing regulators which also contain the RS domain but have a different domain organization or lack the RRM. Some of the prominent SR-related splicing regulators are Tra (transformer), Tra-2β1 (transformer-2), SON and SR45.

In addition to splicing regulation, it is becoming increasingly apparent that SR proteins are involved in coupling splicing to other steps in RNA metabolism, such as RNA Pol II mediated-transcription, 3’-end processing, nuclear-export of the mature mRNA, as well as NMD and translation. Furthermore, many SR proteins also possess multiple splicing-independent functions

(Zhong et al., 2009; Twyfells, et al., 2011). This is exemplified by one of the best characterized and extensively studied SR proteins, SRSF1, whose various functions are detailed below.

1.5.3 Nuclear-cytoplasmic Shuttling of SR Proteins

While all 12 members of the SR protein family are nuclear, at least 6 (SRSF1, SRSF3, SRSF4, SRSF6, SRSF7 and SRSF10) shuttle between the nucleus and the cytoplasm (Cáceres et al., 1998). The RS domain of SR proteins has been implicated in regulating their cellular localization. The Ser residues in the RS domain are subject to extensive phosphorylation, which regulates RNA binding, splicing activity, as well as sub-cellular localization (Xiao et al., 1997; Xiao et al., 1998). Phosphorylation of the RS domain is mediated by various kinases, such as SRPK1 and 2 in the cytoplasm (Gui et al., 1994; Koizumi et al., 1997), Clk/Sty 1, 2, 3 and 4 in the nucleus (Colwill et al., 1996; Nayler et al., 1997), topoisomerase I (Rossi et al., 1996) and in fission yeast the kinase DSK1 (Tang et al., 1998), as well as the phosphatases PP1 and PP2A (Misteli et al., 1996; Novoyatleva et al., 2008)

When in the nucleus, the SR proteins localize and concentrate in Inter-chromatin Granule Clusters (IGCs) or nuclear speckles, from where they are recruited to active sites of RNA pol II-mediated transcription to facilitate the splicing process (Misteli et al., 1997). In the IGC, the RS domain is believed to be hypo-phosphorylated, allowing the SR and SR-related proteins to concentrate together (Misteli et al., 1998). Phosphorylation of the RS domain releases the SR proteins from the speckles and triggers their localization to active sites of transcription, through association with the RNA Pol II C-terminal domain (CTD) (Misteli & Spector, 1999). Thus, during splicing initiation, the SR proteins are in a hyper-phosphorylated state, but during the course of the reaction they transition to a partially dephosphorylated state. Some of the SR proteins, including SRSF1, remain associated with the processed mRNA and in their partially dephosphorylated state are exported out of the nucleus into the cytoplasm. Re-phosphorylation of the RS domain in the cytoplasm by SRPK1 & 2 triggers SR protein nuclear import through the β-karyopherin protein transportin-SR.

1.6 The prototypical SR Protein SRSF1

SRSF1, the founding member of the SR protein family, was originally isolated independently by virtue of two of its activities: promoting spliceosome assembly and constitutive pre-mRNA splicing in splicing-inactive cytoplasmic S100 HeLa cell extract (Krainer et al, 1990); and regulating alternative splicing of the SV40 early pre-mRNA in vitro (Ge at al., 1990). The modular domain structure of SRSF1 comprises two RRMs (RRM1 and RRM2) and a C-terminal RS domain, which is subject to phosphorylation. As a regulator of alternative splicing, SRSF1 promotes alternative exon inclusion and use of proximal alternative 5'-ss or 3'-ss in a concentration-dependent manner, in part through recognition of a seven-nucleotide degenerate ESE sequence element on its pre-mRNA substrate (Cartegni et al., 2002).

1.6.1 The Many Roles of SRSF1

Though it was originally identified as a splicing regulator, SRSF1 has since been found to regulate many other aspects of RNA metabolism and cellular functions (**Figure 1.2**).

a. SRSF1 and mRNA Transcription.

RNA splicing occurs largely co-transcriptionally, wherein the elongation-specific phosphorylated CTD of RNA Pol II associates with the U1 snRNP and splicing regulators, such as the SR proteins (Misteli and Spector, 1999; Bentley et al., 2005; Kornblihtt et al., 2004; Kornblihtt, 2005; Das & Reed et al). This association is thought to enable efficient splice-site recognition and spliceosome assembly along the nascent transcript as it is transcribed by Pol II.

It is becoming evident that a complex interplay exists between the transcription and splicing machineries, with splicing playing a significant role in transcription regulation as well. The presence of promoter-proximal splice sites has been reported to increase transcription of Pol-II transcribed genes, in part through enhancing the initiation step (Furger et al., 2002). Furthermore, depletion of *SRSF1* and *SRSF2* in mouse embryonic fibroblasts leads to an overall decrease in nascent mRNA levels (Lin et al., 2008). SRSF1 was also reported to be recruited to the cytokine IL-2 promoter, where it regulates initiation of transcription (Moulton et al., 2013). Interestingly,

SRSF1 was found to associate with chromatin in a cell-cycle dependent manner and may play a role in regulation of transcription initiation (Loomis et al., 2009).

b. SRSF1 and Nuclear-Export of mRNA.

Following processing, the functional mRNA transcript is bound by export adaptors, which in turn interact with specific receptors for nuclear export. SRSF1, along with two other SR proteins,SRSF3 and SRSF7, acts as one such export adaptor, which interacts with the TAP/NXF1 receptor to mediate mRNA export (Huang and Steitz, 2005). SRSF1 binds to TAP/NXF1 through the linker region between RRM1 & 2 and its activity in nuclear export requires partial dephosphorylation of its RS domain (Kohler and Hurt, 2007; Tintaru et al., 2007). Thus, differential phosphorylation of the shuttling SR proteins can serve as a regulatory mechanism for nuclear export of mRNPs. Rephosphorylation of the RS domain in the cytoplasm destabilizes the interaction of the SR proteins with their mRNA cargo, facilitating release of the mRNA in the cytosol. The phosphorylated SR protein now interacts with the nuclear-import apparatus and shuttles back into the nucleus (Lai et al., 2001).

c. SRSF1 and Non-sense Mediated mRNA Decay (NMD).

NMD is an mRNA quality control and surveillance mechanism by which mRNA transcripts containing a premature termination codon (PTC) are subjected to degradation, so as to prevent formation of toxic, truncated proteins and futile rounds of translation (Nagy and Maquat, 1998). NMD occurs in the cytoplasm, following the pioneer round of translation. Following splicing, the exon-exon junctions formed are bound by a protein complex called the Exon-Junction Complex (EJC). During the pioneer round of translation, the ribosome displaces each EJC as it moves along the mRNA. Translation terminates at the natural stop codon, which is typically located in the terminal exon and therefore does not have any downstream bound EJC. However, if an mRNA contains a PTC within ~50 nucleotides or greater upstream of the last exon-exon junction, the ribosome stalls at the PTC, giving it the chance to interact with the downstream bound EJC. This triggers the NMD process, which leads to degradation of the PTC-containing mRNA.

The shuttling protein SRSF1 has been shown to stimulate NMD of PTC-containing mRNAs (Zhang and Krainer, 2004). Overexpression of SRSF1 greatly enhanced NMD of two well characterized PTC-containing NMD substrates, β-globin and GPX1. This activity of SRSF1 is independent of its shuttling ability, but requires an intact RS domain. Though the exact mechanism for SRSF1-mediated NMD enhancement is still under investigation, it could be a consequence of SRSF1's ability to promote the pioneer-round of translation or to facilitate the assembly of the EJC complex on the processed mRNA.

d. SRSF1 and the mTOR Pathway.

The PI3K-mTOR signaling pathway is an important cell-cycle regulator and is frequently activated in many cancers (Laplante et al., 2012). Multiple components of the pathway, such as AKT and PI3K, are oncogenes, while negative regulators of the pathway, such as PTEN and TSC1, are powerful tumor-suppressors, and are frequently lost in cancer. The mTOR kinase is part of two independent complexes, each with their own substrate specificity. mTORC1, which contains the rapamycin-sensitive component Raptor, acts as a nutrient/redox sensor and regulates protein synthesis, while mTORC2, with the rapamycin-insensitive component Rictor, is a major regulator of the cytoskeleton and Akt.

SRSF1 overexpression leads to specific activation of the mTORC1 pathway, with increased phosphorylation of its downstream substrates, S6K1 and 4EBP1 (Karni et al., 2008; Michlewski et al., 2008; Bushell et al., 2008). SRSF1-mediated mTORC1 activation bypasses activation of the upstream PI3K/Akt pathway. Though the exact mechanism leading to this activation is still unknown, SRSF1 was reported to directly interact with mTOR in an RNA-independent manner; furthermore, SRSF1 interacts with and inhibits the activity of the phosphatase PP2A, an antagonist of the mTORC1 target S6K1 (Michlewski et al., 2008).

e. SRSF1 and Translation.

Cap-dependent translation of eukaryotic mRNA initiates with the association of the eIF4F complex (composed of the cap-binding protein eIF4E, the scaffold protein eIF4G, and the DEAD-box helicase eIF4A) to the 5'end of the mRNA. This in turn recruits the 40*S* ribosomal subunit, which then scans along the mRNA 5' to 3' to initiate translation at the first start codon

(Hinnebusch et al., 2012). The rate of translation initiation therefore depends on the level of eIF4F complex, which in turn is dependent on the availability of free eIF4E. eIF4E is sequestered away from the eIF4F complex through association with the 4EBP 1-3 proteins. Phosphorylation of the 4EBPs releases eIF4E, leading to enhanced translation initiation.

The shuttling of SRSF1 to the cytoplasm suggests a role for it in the cytoplasmic processes of RNA metabolism, such as translation. Consistent with this idea, SRSF1 was found to associate with polyribosomes in cytoplasmic extracts and to enhance translation of ESE-containing luciferase reporters (Sanford et al., 2004). Enhanced cap-dependent translation by SRSF1 has been attributed to multiple splicing-dependent and -independent mechanisms:

1. SRSF1 regulates alternative splicing of the MAP kinase Mnk2 and favors the formation of the Mnk2b isoform, which phosphorylates the translation initiation factor eIF4E, which in turn enhances translation initiation (Karni et al., 2008).
2. SRSF1 was also found to activate the mTORC1 signaling pathway, either by modulating splicing of its components or by physically interacting with it (Karni et al., 2008; Michlewski et al., 2008). mTORC1 phosphorylates S6 Kinase 1, which in turn phosphorylates 4EBP1, resulting in release of eIF4E.
3. SRSF1 also regulates the splicing of S6 kinase 1 to generate short isoforms, which have been shown to enhance 4EBP1 phosphorylation, leading to enhanced translation initiation (Ben-Hur et al., 2013).

f. SRSF1 and miRNA Processing.

miRNAs are small non-coding RNAs that regulate gene expression at the post-transcriptional level (He et al., 2007). miRNAs are processed from longer precursors called pri-miRNAs, transcribed by RNA Pol II. The pri-miRNA is then processed, first in the nucleus by the RNAse Drosha to form a ~70 nt pre-miRNA, and then in the cytoplasm by another RNAse, Dicer, to generate the mature miRNA. The miRNA is then subsequently loaded into an RNA-induced silencing complex (RISC). The miRNA in the RISC complex typically binds to the 3'UTR of its target mRNA by complementary base pairing, and silences expression of the corresponding gene either by triggering cleavage of the mRNA or by blocking its translation. Recently, SRSF1 was reported to play an important role in processing of a pri-miRNA (Wu et al., 2010). SRSF1 binds

to and regulates the Drosha cleavage step of the miR-7 pri-miRNA and this regulation is independent of its splicing function. Furthermore, expression profiling revealed that this role of SRSF1 is more general and extends to processing of other miRNAs, such as miR-221 and miR-222 as well.

g. SRSF1 and Protein-Sumoylation.

Small ubiquitin-related modifier (SUMO) is a post-translational protein modification group, very similar to ubiquitin (Geiss-Friedlander et al., 2007). The SUMO 1-4 proteins are expressed as precursors that undergo C-terminal cleavage to form the mature protein. Protein sumoylation proceeds through the following steps: (1) the mature SUMO group is activated in an ATP-dependent manner by the SUMO activating enzyme E1 (SEA1/SEA2 heterodimer); (2) it is transferred to SUMO-conjugating enzyme E2 (Ubc9); and (3) it is subsequently transferred to the ε-amino group of lysine on the target protein. Sumoylation of proteins was reported to regulate their cellular localization, function or stability. The process is generally facilitated by another class of proteins, the SUMO E3 ligases, such as PIAS1.

Surprisingly, SRSF1 was found to facilitate protein sumoylation through a splicing-independent mechanism (Pelisch et al., 2010). Overexpression of SRSF1 greatly stimulates protein sumoylation both in vitro and in vivo, and its depletion inhibits overall SUMO conjugation. SRSF1 interacts with Ubc9 and regulates the last step of the process: the transfer of the SUMO group from the intermediate to the substrate protein. In doing so, it displays characteristics of an E3 ligase, acting as a cofactor to facilitate the process. In addition, SRSF1 was also found to interact with and regulate the function of the E3 ligase PIAS1.

h. SRSF1 and Chromatin Association.

SRSF1 and SRSF3 were found to associate with chromatin in a cell-cycle dependent manner (Loomis et al., 2009). Both proteins bind to interphase chromatin, dissociate from mitotic chromosomes and reassociate with post-mitotic chromatin. This association-disassociation is

regulated by M-phase specific phosphorylation of Histone H3 on Ser 10 by Aurora Kinase B. Hyperphosphorylation of the two SR proteins by SRPK1 in the M-phase also plays an important role in regulating their chromatin-binding properties. Furthermore, SRSF1 depletion led to decreased chromatin-association of the HP1 proteins, which are the basic units of heterochromatin organization. Though the functional implications of this are still unknown and largely speculative, it was proposed that the cell-cycle specific association of SRSF1 with chromatin may reflect its possible role in chromatin organization and cell-cycle progression, as well as in transcriptional regulation and as discussed below, the maintenance of genomic stability.

i. SRSF1 and Genome Stability.

Chicken DT-40 cells depleted of SRSF1 were observed to be hypermutagenic, with accumulation of DNA double-strand breaks (DSB) and wide-scale genomic rearrangements (Li et al., 2005a). Further investigation revealed a role for SRSF1 in maintaining genomic stability by preventing formation of R-loops, structures that are formed by hybridization between the DNA template strand and the nascent RNA, which can trigger genomic instability. Deposition of SRSF1 co-transcriptionally from the CTD of RNA Pol II to the nascent mRNA transcript enables it to prevent the formation of R-loops, instead triggering the formation of pre-mRNPs. The association of SRSF1 with interphase chromatin may be another way in which SRSF1 participates in the surveillance of genome integrity, though the exact mechanism is still elusive and needs to be thoroughly investigated.

1.6.2 Regulation of SRSF1 Expression.

In light of the many processes that it regulates, it is not surprising that *SRSF1* is an essential gene and SRSF1-null mice are embryonic lethal (Xu et al., 2005). Tissue-specific deletion of SRSF1 in mouse heart led to lethality about 6-8 weeks after birth, due to heart failure (Xu et al., 2005). These mice were found to have defective Ca^{2+} metabolism, seemingly due to missplicing of the Ca^{2+}/calmodulin-dependent kinase IIδ (CAMKIIδ), leading to a defective contractile apparatus and cardiomyopathy. Knockdown of the SRSF1 orthologue in *C. elegans* also leads to

late embryonic lethality (Longman et al., 2000). Furthermore, loss of SRSF1 expression in chicken DT-40 cells triggers cell-cycle arrest in the G2-phase and apoptosis (Li et al., 2005b).

On the other hand, overexpression of SRSF1 in immortal rodent fibroblasts or human mammary epithelial cells leads to oncogenic transformation, with increased cellular proliferation and protection against apoptosis (Anczukow et al., 2012; Karni et al., 2008).

Presumably to prevent the deleterious consequences of its misregulation, the level of SRSF1 is tightly controlled within the cell. SRSF1 negatively autoregulates its expression through multiple post-transcriptional and translational mechanisms (Sun et al., 2009), so as to prevent the deleterious outcomes of its overexpression. Using tetracycline-induced overexpression of SRSF1 in HeLa cells, the autoregulation of SRSF1 has been systematically dissected to identify the following regulatory check-points:

1. The SRSF1 transcript is itself extensively alternatively spliced to form six different splice isoforms (I-VI). However, only one of these isoforms (isoform I) has a complete ORF and is exported out of the nucleus for translation. While isoforms II-IV are retained in the nucleus, isoforms V and VI contain a PTC and are degraded by NMD. SRSF1 regulates its own splicing and keeps a check on its overexpression by promoting the formation of splice isoforms II-VI.
2. SRSF1 also regulates its expression at the translational level, by shifting the association of its mRNA from polysomes to monosomes—indicative of decreased translation efficiency. This was attributed to a decrease in translation initiation, and depends on the 3'UTR of the SRSF1 mRNA.
3. As outlined above, SRSF1 regulates the processing of the miR-7 miRNA. One of the targets of miR-7 is the SRSF1 mRNA itself. Thus once again, SRSF1 forms an auto-regulatory loop wherein it promotes the formation of miR-7, which in turn silences translation of the SRSF1 mRNA.

Surprisingly, in spite of these safeguards against SRSF1 misregulation, it is frequently over-expressed in many tumor types. Elevated SRSF1 expression can be a result of either disruption of its autoregulatory mechanisms or significant increases in *SRSF1* mRNA (due to increased *SRSF1* copy number, or increased transcription) or both. As described later, since *SRSF1*

overexpression promotes tumorigenesis, studying the factors leading to its elevated expression can be potentially exploited to develop anti-tumor therapy targeting *SRSF1* expression specifically in tumor cells.

1.7 Alternative Splicing and Cancer.

In recent years, it has become increasingly evident that aberrant alternative splicing in cancer cells is a major contributor to the process of tumorigenesis (David et al., 2010). This can include missplicing at a single locus due to mutations, leading to production of tumor-promoting isoforms of critical cell-cycle regulators or wide-scale reprogramming of alternative splicing patterns in cancer due to changes in expression and activity of splicing regulators.

Mutations in cis-acting elements like 5'ss, 3'ss and BPS, or the regulatory enhancer and silencer sequences resulting in aberrant splicing have been reported for a number of tumor-regulating genes. One of the best studied examples is the tumor suppressor *BRCA1*, which frequently carries mutations in its consensus intronic 5' ss and 3' ss, and in degenerate splicing consensus sites in sequences near intron-exon boundaries, giving rise to several splice variants in breast and ovarian cancers that are important for tumor development (Liu et al., 2001; Orban et. al, 2003; Yang et. al, 2003). Another example is an intronic single nucleotide polymorphism in the transcription factor and tumor suppressor gene *KLF6*, which creates a binding site for the SR protein SRSF5 and destroys two other overlapping SR protein binding sites, resulting in increased production of three splice variants and conferring increased susceptibility to prostate cancer (Narla et al., 2005).

Additionally, aberrant expression of splicing regulators in many different cancers can alter the cellular splicing profile to promote tumorigenesis by affecting cell proliferation, apoptosis, metabolism, invasion, metastasis and angiogenesis (David et al., 2010). Many SR proteins display tumor-stage-specific changes in expression levels during mammary tumorigenesis, pointing towards possible concentration-dependent effects on splice-site selection (Stickeler et al., 1999). The expression of the RBFOX2 splicing factor has also been correlated with breast and ovarian cancer, with the protein being frequently downregulated in these tumors (Venables et al., 2009).

SRSF1, *SRSF3*, *SRSF6* as well as *HNRNPA2B1* have been described as potent oncogenes (Cohen-Eliav et al., 2013; Golan-Gerstl et al., 2011; Jia et al., 2010; Karni et al., 2008). While *SRSF1* is an important contributor to breast and lung cancer progression, *SRSF6* is frequently amplified in and promotes lung and colon tumors, and *HNRNPA2B1* activity is associated with glioblastomas.

The elevated expression of these and other splicing regulators results in altered splicing of important tumor-associated gene transcripts. One prominent example is the splicing of the Pyruvate Kinase M gene (*PKM*) which contains two mutually exclusive exons (9 &10) and gives rise to two splice isoforms: M1, containing exon 9; and M2, containing exon 10. While PK-M1 is predominantly expressed in adult differentiated cells, PKM2 expression is associated with proliferating cells, including cancer cells. Tumor initiation and progression involve a splicing switch from the M1 to the M2 isoform, which confers upon the cancer cells the ability to undergo aerobic glycolysis (Christofk et al., 2008). Multiple splicing factors have been identified as key regulators of this splicing switch, such as hnRNPA1, PTB and the oncoproteins SRSF3 and hnRNPA2 (David et al., 2010; Clower et al., 2010; Wang et al., 2012).

Aberrant expression of splicing regulators has also been associated with altered splicing of apoptotic regulators, such as Bcl-x, Caspase 2 & 9 and Fas, the angiogenesis factor VEGFA, whose pro-angiogenic splice isoform is promoted by the oncoprotein SRSF6, regulators of metastasis and invasion, such as CD44, Rac1 and Ron, as well as critical oncogenes and tumor-suppressors, such as Cyclin D1, H-Ras, MDM2, p53 etc. (Venables et al., 2009).

Inappropriate activity of the splicing regulatory proteins in cancer can also be a result of their aberrant post-translational modifications. The regulation of the phosphorylation state of many SR proteins, like SRSF1, SRSF5, SRSF6 and Nsrp1, by kinases like Akt and phosphatases PP1 and PP2A, which themselves play important roles in tumor development, affects their function by altering sub-cellular localization and protein-protein interactions (Blaustein et al., 2007).

1.8 SRSF1 and Cancer.

SRSF1 was the first splicing regulator to be characterized as an oncogene (Karni et al., 2008). A protein microarray across a wide range of tumor tissues and their normal tissue counterparts

revealed SRSF1 to be overexpressed in a number of different tumor types, including lung, breast, colon, kidney and pancreas. Immortal rodent fibroblasts modestly overexpressing *SRSF1* form high-grade sarcomas in nude mice, have high proliferative capacity and are resistant to apoptosis. The pro-oncogenic activities of *SRSF1* have since been extensively studied in breast and lung cancers. Overexpression of *SRSF1* in immortalized human mammary epithelial cells MCF10A enables them to form larger acinar structures in 3-D matrigel assays, corresponding to increased proliferation and delayed apoptosis during morphogenesis (Anczukow et al., 2012). Furthermore, *SRSF1*-overexpressing murine mammary epithelial COMMA-1D cells form malignant tumors upon orthotropic transplantation for mammary reconstitution in mice.

Lung cancer is the most common cause of cancer-related mortality and lung tumors have been shown to show differential expression and splicing of a number of oncogenes and tumor suppressors, compared to normal lung. Analysis of 107 human Non small-cell lung carcinoma (NSCLC) as well as 25 associated normal lung parenchymas revealed *SRSF1* to be the most prominent splicing regulator which is significantly overexpressed in 65% of all analyzed tumors (Gout et al., 2012). Furthermore, *SRSF1* overexpression in lung adenocarcinoma cells led to a more aggressive phenotype, with more anchorage-independent growth as well as greater metastatic potential with EMT. *SRSF1* overexpression also made these cancer cell lines resistant to anti-cancer drugs, such as carboplatin and paclitaxel.

The oncogenic activity of SRSF1 is in part a result of its activity as a splicing regulator. *SRSF1* overexpression regulates the splicing of apoptosis regulators, such as *BIN1* and *BCL2L11* (BIM) (Karni et al., 2008; Anczukow et al., 2012). The BIN1 protein interacts with the MYC oncoprotein and promotes its pro-apoptotic activity (Duhadaway et al., 2003; Prendergast 2000a; Prendergast et al., 2003; Sakamuro et al., 1996). SRSF1 promotes the formation of the BIN1+12A isoform, which includes the 12A exon and is non-apoptotic due to its inability to interact with MYC. SRSF1 also promotes alternative splicing of the BIM mRNA to generate the γ1 and γ2 isoforms, which lack a BH3 domain, required for the pro-apoptotic activity of BIM. Both BIN1 and BIM splicing have been shown to be important contributors to the delayed apoptosis phenotype observed in MCF-10A acini upon *SRSF1* overexpression. The Bcl-2 family member Mcl-1 is another apoptotic regulator which is alternatively spliced by SRSF1 (Gautrey et al., 2012; Moore et al., 2010). Mcl1 expression is associated with poor breast cancer prognosis

and resistance to cancer therapies. Mcl1 has two splice isoforms: the anti-apoptotic Mcl-1L and the pro-apoptotic Mcl-1S. SRSF1 promotes the formation of the anti-apoptotic Mcl-1L isoform in breast cancer.

SRSF1 also alters the splicing of two important regulators in the receptor tyrosine kinase signaling pathways: *RPS6KB1*, which is a downstream effector of the PI3K/Akt pathway and *MKNK2*, an effector in the MAPK/ERK pathway. *RPS6KB1* encodes the protein S6 Kinase 1, which phosphorylates the 40*S* ribosomal subunit protein S6 (Erikson and Maller, 1985). The protein has two known isoforms with different translational starts: a 70-kDa cytoplasmic and an 85-kDa nuclear protein (Berven and Crouch, 2000). The 70-kDa isoform, known as p70S6K, is the most studied and has been implicated in regulation of the cell cycle (Feng et al., 2000), cell growth and proliferation (Dufner and Thomas, 1999) and cell migration (Lambert et al., 2002). *SRSF1* overexpression leads to formation of a novel p31 short isoform of S6K1. This p31 S6K1 isoform is an important downstream effector of *SRSF1*'s oncogenic properties in immortal rodent fibroblasts and is itself oncogenic (Ben-Hur et al., 2013).

Another important splicing target of SRSF1 is the proto-oncogene *RON* (Ghigna et al., 2005). SRSF1 induces Epithelial to mesenchymal transition (EMT) and promotes cell motility and invasion by altering splicing of the Macrophage stimulating protein (MSP) tyrosine kinase receptor RON. SRSF1 mediates exon 11 skipping in the RON transcript, and the resulting protein isoform promotes loss of the epithelial phenotype with acquisition of spindle-shaped morphology and increased cell motility in HEK 293 cells as well as breast and stomach cancer cell lines.

In addition to its role as a splicing regulator, the ability of SRSF1 to activate the tumor-promoting mTORC1 signaling pathway and to enhance translation also contributes to its oncogenic activity (Karni et al., 2008b). Inhibiting mTORC1 activation by treatment with rapamycin inhibits *SRSF1*'s oncogenic activity both in vitro and in vivo. In fact, SRSF1 was reported as an important regulator of Non-small Cell Lung Cancer (NSCLC), disabling the apoptotic response in them by promoting mTORC1-mediated translation of the anti-apoptotic protein Survivin (Ezponda et al., 2010).

1.9 Concluding Remarks

RNA splicing in higher eukaryotes is a critical regulatory step in gene expression. Additionally, alternative splicing is a major contributor to proteomic diversity and organismal complexity in higher eukaryotes. Though significant advances have been made in deciphering the exact mechanisms and identifying the key regulators of the process, there remain numerous questions about the dynamics of how all the different spliceosomal components and splicing regulators work together to ensure efficient and accurate splicing. In addition to ensuring that the correct splice sites are chosen for constitutive splicing of all multi-exon genes—in spite of the low degree of splice site conservation prevalent in the genome—the cell also needs to control the expression of appropriate alternatively spliced isoforms spatially and temporally. Since about 15% of human genetic disease-causing mutations are attributed to splicing defects (Krawczak et al., 1992), a comprehensive understanding of the splicing process globally as well as at a gene-specific level is imperative. Our current knowledge of splice-site selection, based on the interplay between ESE/ISE and ESS/ISS elements, has already enabled us to develop therapeutic strategies using antisense oligonucleotides to correct aberrant splicing of culpable genes in diseases like Spinal Muscular Atrophy (SMA) and Duchenne Muscular Dystrophy (DMD) (Nlend Nlend et al., 2010; Southwell et al., 2012).

The emerging role of splicing regulators in cancer opens up a whole new therapeutic avenue for the disease. In addition to identifying the splicing factors that regulate the cellular alternative splicing profiles, it is also important to characterize the alternative splicing profile itself, in hopes of identifying new critical therapeutic targets. We are well on our way to characterizing the oncogenic activity of the SR protein SRSF1, and its role in human cancers is becoming increasingly clear. In addition, the roles of other members of the SR protein family as well as other splicing regulators in cancer are being explored. The multifunctional character of SRSF1and other SR proteins places further emphasis on the role of splicing as a central regulator of gene expression and cellular homeostasis. Given these facts, it is also important to understand the regulatory mechanisms controlling these critical regulators, which may further enable us to control their disease-associated aberrant expression or activity.

1.10 Figures & Figure Legends

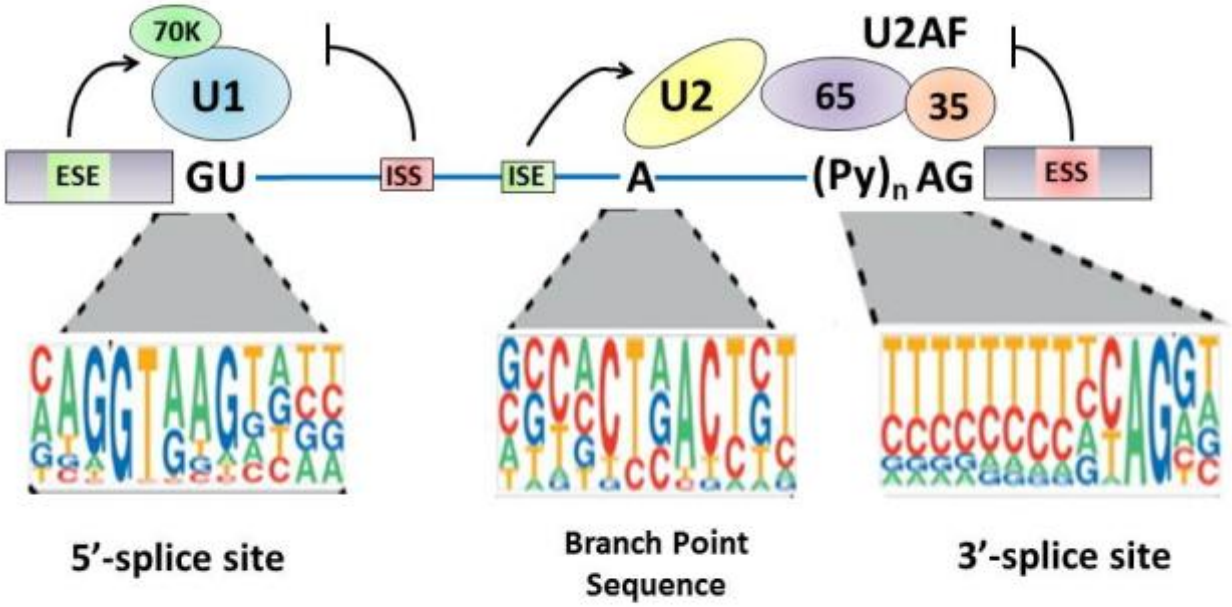

Figure 1.1 cis-regulatory elements of pre-mRNA splicing.

The exon-intron boundaries are defined by consensus sequence elements, namely the 5'-splice (5'-ss) and the 3'-splice site (3'-ss). Spliceosome assembly initiates with the recruitment of the U1 snRNP to the 5'-ss and U2AF to the 3'-ss. The Branch Point sequence, with a conserved adenosine residue required for the first trans-esterification step of the splicing reaction is bound by the U2 snRNP. The assembly of the core spliceosomal components is regulated by trans-acting regulatory proteins binding to the ESE/ISE and ESS/ISS elements. ESE-Exonic Splicing Enhancer; ISE-Intronic Splicing Silencer; ESS-Exonic Splicing Silencer; ISS-Intronic Splicing Silencer.

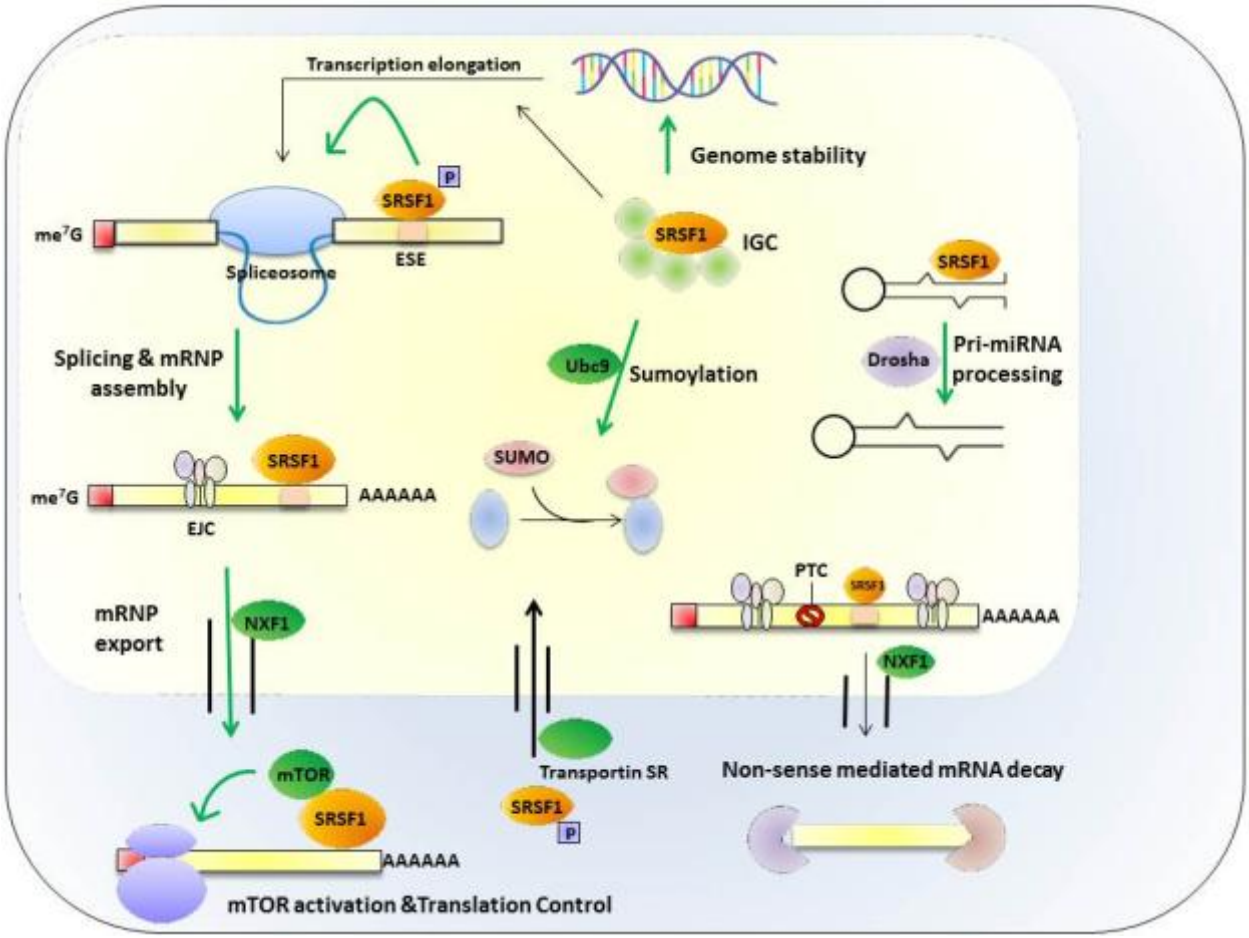

Figure 1.2 The multi-functional SR protein SRSF1.

Splicing-dependent and independent function of SRSF1 in the nucleus and cytoplasm and its differential phosphorylation states. SRSF1-interacting proteins are in green; EJC-Exon Junction Complex; ESE-Exonic Splicing Enhancer; IGC-Inter-chromatin Granule Cluster; PTC-Premature Termination codon; NXF1-Nuclear Export Factor 1; Ubc9-SUMO conjugating enzyme E2;

Chapter 2. Transcriptional Regulation of Oncogenic Splicing Factor SRSF1

Abstract

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. The two oncogenes are significantly co-expressed in lung carcinomas, and MYC knockdown downregulates *SRSF1* expression in multiple lung-cancer cell lines. MYC directly activates transcription of *SRSF1* through two non-canonical 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 a subset of tumors with elevated MYC levels, 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.

2.1 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 et al., 1990; Krainer et al., 1990; Mayeda et al., 1992), SRSF1 regulates other aspects of RNA metabolism, including mRNA stability (Lemaire et al., 2002), nuclear export (Huang et al., 2003), nonsense-mediated mRNA decay (Zhang et al., 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 post-transcriptional and translational mechanisms (Sun et al., 2010; Wu et al., 2010), yet despite this stringent homeostatic control, it is frequently upregulated 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). However, gene amplification can only account for SRSF1 over-expression in some tumors, and there are likely to be other mechanisms that control SRSF1 expression, the loss of which results in its overexpression in cancers. An investigation of how the SRSF1 gene is regulated in normal cells can thus help shed light on how this regulation is altered during tumorigenesis.

Loss of transcriptional regulation might also cause SRSF1 overexpression in tumors. Several studies have aimed at identifying factors that regulate the transcription of genes coding for various splicing factors. E2F1, a member of the E2F family of transcription factors has been shown to up-regulate transcription of the SR protein SRSF2. E2F1 requires SRSF2 to switch the splicing of various apoptotic genes like c-flip, caspases-8 & 9 and Bcl-x to their pro-apoptotic forms, thus mediating E2F1's apoptotic function (Merdzhanov et al., 2008). Another transcription factor complex, β-catenin/TCF-4 upregulates the transcription of SRSF3 and alters cellular alternative splicing patterns (Gonçalves et al., 2008). MYC, another transcription factor, which is also a potent oncogene, has recently been reported to regulate the expression of the splicing repressors hnRNPA1/A2 and PTB1 (David et al., 2010).

The MYC (c-myc) protein is a member of a b/HLH/LZ family of transcription factors that regulate cell growth, differentiation and apoptosis. MYC controls the expression of its target genes by heterodimerizing with its binding partner, Max, through its C-terminal basic-region/helix-loop-helix/leucine-zipper domain. This heterodimerization is required for the binding of MYC to DNA, and through mutational analysis it has been shown to be absolutely essential for MYC's biological functions (Amati, B. et al., 1993; Nilsson & Cleveland, 2003). While MYC is a highly unstable protein whose expression in cells is carefully regulated by both transcriptional and post-transcriptional mechanisms, Max is more stably expressed and is present in stoichiometric excess to MYC. Besides MYC, Max is also known to form homodimers, and to heterodimerize with the Mad-family proteins Mxi1 and Mnt.

MYC can either activate or repress the expression of its target genes. It preferentially binds to sites in genomic DNA with a high CpG dinucleotide content, called CpG islands (Fernandez et al., 2003). The MYC-MAX heterodimer when bound directly to DNA sequence elements called E-box sequence (CANNTG), predominantly CACGTG, activates transcription. In contrast, it represses transcription when tethered to gene promoters and Inr sequence elements through interaction with intermediary proteins such as Miz1. The N-terminal domain of the MYC protein is the transcriptional regulatory domain and contains three highly conserved elements called MYC boxes. These MYC boxes interact with specific binding proteins resulting in transcriptional activation or repression. For instance, MYC Box II, which is required for all biological functions of MYC is essential for transcriptional activation or repression of most, but not all target genes. This transcriptional regulation is brought about through the interaction of MBII with co-activators like TRRAP and TIP 60 which are subunits of Histone acetyl-transferase complexes and co-repressors like TIP48/49, hexameric ATPases that are part of chromatin remodeling complexes.

Multiple high-throughput microarrays and genome wide ChIP-on-ChIP screens have shown that MYC regulates the expression of ~10-15% of all cellular genes (Knoepfler et al., 2007). These include genes participating in cell cycle, survival, protein synthesis, cell adhesion and metabolism, as well as micro RNAs (Dang et al., 2006). The expression of c-MYC protein is frequently misregulated in a wide variety of cancers and by a number of different mechanisms

such as chromosomal translocation and amplifications, increased mRNA and protein stability and growth stimulatory signaling cascades (Spencer et al., 1991).

The MYC protein contributes to many aspects of cellular physiology. Mitogenic stimulation of quiescent cells leads to rapid induction of MYC and is sufficient to drive the entry of the cells into the cell cycle. Furthermore, its sustained expression is required for continuing cell growth and proliferation. Deregulated MYC expression sensitizes cells to cell death and triggers arrest in the Go/G1 cell-cycle phase, and apoptosis in response to growth factor withdrawal. MYC is a potent oncogene; its frequent over-expression in tumors drives unrestricted cell proliferation, suppresses cellular differentiation, promotes cell growth and vasculogenesis, alters cellular metabolism towards aerobic glycolysis, reduces cell adhesion and promotes metastasis, causes epithelial to mesenchymal transition, and results in genomic instability (Evan et al. 1992; Felsher et al., 1999; Dang, 1999; Adhikary et al., 2005; Cho etal., 2010). With few exceptions, the role of many MYC target genes in these cellular functions remains elusive.

Several pieces of evidence point towards a possible role of MYC in transcriptional regulation of the splicing factor SRSF1. Bioinformatic analysis of the SRSF1 promoter reveals the presence of multiple non-canonical, putative MYC binding E-box sequences with a high degree of phylogenetic conservation. In a genome-wide screen aimed at identifying gene promoters that are bound by MYC and are therefore possibly regulated by it, MYC was found to bind to the CpG islands of the SRSF1 promoter (Mao et al., 2003). Furthermore, microarray analysis reveals SRSF1 as one of many genes that is upregulated in response to induction of MYC activity or expression in multiple cell lines, such as human primary fibroblasts, MYC-driven murine pancreatic tumors, neuroblastomas, and cells of the lymphoid lineage (Coller et al., 2000, Schlosser et al., 2005, Wu et al., 2008)

2.2 Results

2.2.1 SRSF1 Expression Correlates with MYC Levels in Human Lung Carcinomas

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 2.1a**). 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 2.1**). 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 2.1b**), 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 2.1b**). 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 (**Figure 2.1c,d**). However, another bronchoalveolar adenocarcinoma cell line, A549, did not show this effect, indicating additional context-dependent levels of control (**Figure 2.2**); this may be due to threshold effects, as A549 cells have relatively low levels of both MYC and SRSF1 (**Figure 2.1b**). The imperfect correlation between SRSF1 and MYC expression in the lung cancer cell lines (**Figure 2.1b**) 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, post-transcriptional, translational, or post-translational levels.

2.2.2 MYC Regulates SRSF1 Expression and Activity

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**

2.3a) and SRSF1 protein (**Figure 2.3b**). As a control for ER.MYC induction, we verified the upregulation of a known MYC target gene, *NCL* (**Figure 2.4a**). Moreover, IMR90 cells transduced with empty vector did not induce *SRSF1* upon 4-OHT treatment (**Figure 2.4b**). 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 (**Figure 2.3a,b**), 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 2.4c**). 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 2.4d**). 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 MNK2 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 2.3c**, **Lanes 1-2**). Another splicing factor, hnRNPA1, which is also positively regulated by MYC (Biamonti et al., 1993; David et al., 2010) and frequently antagonizes SRSF1 (Mayeda et al., 1992), did not alter *MKNK2* or *TEAD1* splicing (**Figure 2.3c**, **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 2.3c**, **lanes 4-5**). Furthermore, induction of ER.MYC in cells transfected with siRNA against *SRSF1* did not trigger a change in *MKNK2* or *TEAD1* splicing (**Figure 2.3c**, **lane 6**), indicating that MYC alters *MKNK2* and *TEAD1* splicing through upregulation of *SRSF1* expression. We also observed that both *SRSF1* over-expression and MYC induction led to a significant increase in the overall *MKNK2* transcript level (**Figure 2.4e**), 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 2.4f**), perhaps due to other splicing factors also being modulated by MYC and counteracting the SRSF1-mediated inclusion of exon 12A.

2.2.3 MYC Binds to and Activates the Human SRSF1 Promoter

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 2.5a**), 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 non-canonical 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 (**Figure 2.1c,d**). Our ChIP analysis revealed significant enrichment of MYC at the *SRSF1* proximal promoter region comprising two E-boxes mapping at −412 and −39 (**Figure 2.5b**). We also detected MYC enrichment at a third E-box at position +146, 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 2.5b**). 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 hetero-dimerization partner MAX (Amati et al., 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 1500-bp genomic fragment of the *SRSF1* promoter, comprising these putative E-boxes (from −1200 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 NIH3T3 cells. We also generated constructs with mutations in the three E-boxes, either individually or together, to an inactive CACTCA sequence (**Figure 2.5c**). MYC overexpression resulted in ~3-fold induction of luciferase activity for the wild-type construct (**Figure 2.5d**), relative to the vector control. The double, but not the individual, mutations of E-boxes 1 and 2 abrogated this MYC-induced activation, suggesting functional redundancy between the two elements (**Figure 2.5d**). Mutation of the third putative non-canonical 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 non-functional (**Figure 2.4d**). We conclude that *SRSF1* is a direct transcriptional target of MYC, with two functional non-canonical E-boxes in its promoter.

2.2.4 SRSF1 is an Important Regulator of MYC-mediated Transformation

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 2.6a)**. As expected, Rat1a-MYC cells showed elevated *SRSF1*expression and increased proliferation, compared to Rat1a-pBabe-Luc control cells (**Figure 2.6b**). *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 phases, compared to the vector control (**Figure 2.7**). 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 2.7**). Rat1a-MYC-SRSF1sh cells showed significantly decreased anchorage-independent growth, compared to Rat1a-MYC cells (**Figure 2.6c**). We conclude that *SRSF1* is a critical MYC target gene, required for MYC's full activity in tumorigenesis.

2.3 Discussion and Future Perspective

The oncogenic transcription factor MYC which is a key regulator of cell growth, proliferation, apoptosis and differentiation is predicted to regulate expression of ~15% of genes in the human genome (Zeller et al., 2003). 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 expression positively correlates with that of SRSF1 in a panel of human lung tumors and lung tumor-derived cell lines. Furthermore siRNA-mediated knockdown of MYC revealed that SRSF1 expression is regulated by MYC in at least some of these cell lines.

Induction of MYC activity in human primary lung fibroblasts led to 2-3 fold increase in SRSF1 mRNA and protein levels. Prompted by preliminary ChIP-Microarray and ENCODE data, we investigated MYC binding and activity at the SRSF1 promoter. Our studies led to the discovery of two functional non-canonical E-boxes in the SRSF1 proximal promoter through which MYC activates SRSF1 transcription.

The resulting increase in SRSF1 leads to altered splicing of some but not all of its target gene transcripts. We looked at the splicing of two previously known SRSF1-target genes, the MAPK/ERK signaling pathway kinase *MKNK2* and the transcription factor *TEAD1*. MYC expression altered splicing of both gene transcripts in an SRSF1-dependent manner, and the splicing changes observed were consistent with those induced by SRSF1 activity. MYC activity however fails to alter the mRNA splicing of another SRSF1 target gene-*BIN1*, possibly by regulating expression and activity of additional splicing factors which have a stronger effect than SRSF1 at the BIN1 exon 12A locus.

Since both SRSF1 and MYC are potent oncogenes, we investigated the importance of SRSF1 in the oncogenic activity of MYC. Downregulation of SRSF1 induction upon MYC activation

resulted in decreased proliferation, G1 cell-cycle arrest and decreased anchorage-independent growth. Based on these results we can conclude that *SRSF1* is 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 (Anczukow et al., 2011). 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.

SRSF1 is found to be frequently overexpressed in a wide range of human tumors and is a significant contributor to the process of tumorigenesis. Mechanisms leading to SRSF1 deregulation can help provide valuable insights into the process of tumor initiation and can help us devise ways to restore normal SRSF1 expression, thereby perhaps triggering cancer regression. Here we describe the transcriptional control of SRSF1 by the MYC transcription factor, which can go some ways in explaining cancer-associated SRSF1 overexpression, since MYC activity is itself frequently deregulated in tumors.

While this study provides one possible mechanism of cancer-associated SRSF1 overexpression, there are several additional unexplored possibilities. SRSF1 auto-regulates its expression through multiple post-transcriptional and translational mechanisms and defects in this pathway can either alone or in concert with increased SRSF1 copy number or transcription lead to elevated SRSF1 level in cancer. A good example is the identification of splicing factor Sam68 as a regulator of SRSF1 mRNA splicing (Valacca et al., 2010). Sam68 activity switches alternative splicing of SRSF1 transcript from the NMD targeted isoform to the full-length translatable isoform, leading to increased SRSF1 protein levels. Sam68 mediated increase in SRSF1 expression is associated with EMT and increased cell motility and invasion. We think that continued studies on SRSF1 regulation will keep on revealing other novel and important regulators of SRSF1 expression as well as function, and perhaps provide some therapeutic potential in cancer.

Our study also has significant implications on our current understanding of MYC activity and functions. 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. It is estimated that de-regulated MYC activity is associated with nearly 20% of all human tumors (Dang et al., 2006). Thus, identifying MYC-induced splicing changes, as a whole as well as those mediated through the SRSF1 oncoprotein using high-throughput techniques like RNA Sequencing can provide further insights into the cellular functions of MYC and provide us with potential anti-cancer drug targets.

2.4 Materials and Methods

2.4.1 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 -1200 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.

2.4.2 Cell Culture and Stable Cell Line Generation

IMR90, NIH3T3, 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 h. For MYC induction studies, ER.MYC-expressing cells were grown to confluence and treated with 2 μM 4-OHT for 8 h for RT-PCR, and 48 h for immunoblotting and splicing analysis.

2.4.3 RNA Interference

For inhibition of *MYC* or *SRSF1* expression, cells were seeded (2×10^5 cells per well) in six-well plates in antibiotic-free medium. After 24 h, cells were transfected with 200 pmol short interfering RNA against MYC (Cell Signaling, Catalog No. 6553) or SRSF1 (target sequence 5'-ACGAUUGCCGCAUCUACGU-3') using Lipofectamine RNAiMAX (Invitrogen). After a further 48 h, 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.

2.4.4 Immunoblotting

Cells were lysed in RIPA buffer and protein was quantitated using a Bradford Protein Assay kit (Bio-Rad). 25 μg of total protein 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).

2.4.5 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’-ATGTCGGGAGGTGGTGTGATTC-3’

h*SRSF1*R: 5’-TGTTCCACGGCCGCTTCGAG-3’

r*SRSF1*F: 5’-CGCGACATCGACCTGAAGAAC-3’

r*SRSF1*R: 5’-CCACGACACCAGTGCCATCTCG-3’

*hnRPA1*F: 5’-AAAGACCAGGTGCCCACTTA-3’

*hnRPA1*R: 5’-AATCTTATCCACGGAGTCATGG-3’

*MYC*F: 5’-GGTACAAGCTGGAGGTGGAG-3’

*MYC*R: 5’-AATCTTATCCACGGAGTCATGG-3’

*NCL*F: 5’-TTTCTTTCCTTTGGCTGGTG-3’

*NCL*R: 5’-ATGGCAAGAATGCCAAGAAG-3’

*MKNK2*Ex11F: 5’-CCAAGTCCTGCAGCACCCCTG-3’

*MKNK2*Ex13aR: 5’-GATGGGAGGGTCAGGCGTGGTC-3’

*MKNK2*Ex13bR: 5’-GAGGAGGAAGTGACTGTCCCAC-3’

*BIN1*Ex11F: 5’-CCTCCAGATGGCTCCCCTGC-3’

*BIN1*Ex15R: 5’-CCCGGGGGCAGGTCCAAGCG-3’

β-actinF: 5’-GTGCCCATTTATGAGGGCTA-3’

β-actinR: 5’-CTGGCAGCTCGTAGCTCTTT-3’

2.4.6 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 for the amplicons were as follows:

Amplicon A:

F: 5’-CCCAGCCTGATTTGAATTTT-3’

R: 5’-GAAAATACCGGTCCTCTCAGG-3’

Amplicon B:

F: 5’-GGATTAGACGCACCCTACGA-3’

R: 5’-CGATTTCTCCAGGAATGAGG-3’

Amplicon C:

F: 5’-ACGTAGCCCTCGCAGCAC-3’

R: 5’-GGACTCGAGAACAGGCCTTC-3’

Amplicon D:

F: 5’-CTTTTCGTCACCGCCATGT-3’

R: 5’-GTCCTCGAACTCAACGAAGG-3’

Amplicon E:

F: 5’-GGATTGATGTGAAGGGACGA-3’

R: 5’-TGGAATCCAGAGTCCAAAAT-3’

2.4.7 Luciferase Reporter Assay

500 ng of MYC expression vector, 100 ng of pGL3-Luciferase reporter comprising nucleotides −1200 to +300 of the *SRSF1* promoter—with or without E-box mutations—and 100 ng of pEGFP vector were co-transfected into NIH3T3 cells using Fugene 6 (Roche). 36 h 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.

2.4.8 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.

2.4.9 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) CO_2 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.

2.4.10 Flow Cytometric Cell Cycle Analysis

Rat1a cells transduced with pBABE-hygro, pBABE-MYC, LMP-Puro, LMP-SRSF1sh1, or LMP-SRSF1sh2 were seeded at 1×10^6 cells per 10-cm dish in media supplemented with 10% FBS. Two days later, cell cycle distribution was determined by Flow Cytometry. DNA content was quantitated by staining with propidium iodide (PI) and used to determine cell-cycle distribution by analysis on LSR-II (BD Biosciences) using FACSDiva software (BD Biosciences).

2.4.11 Microarray Analysis

The GEO GSE2109 dataset 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 s.d. 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).

2.4.12 Statistical Analysis

All histograms were plotted using mean ± s.d. 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.

2.5 Acknowledgement

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. We thank Martin Akerman and Olga Anczukow for Analysis of Microarray data from human lung tumors for MYC-SRSF1 correlation analysis.

2.6 Figures and Figure Legends

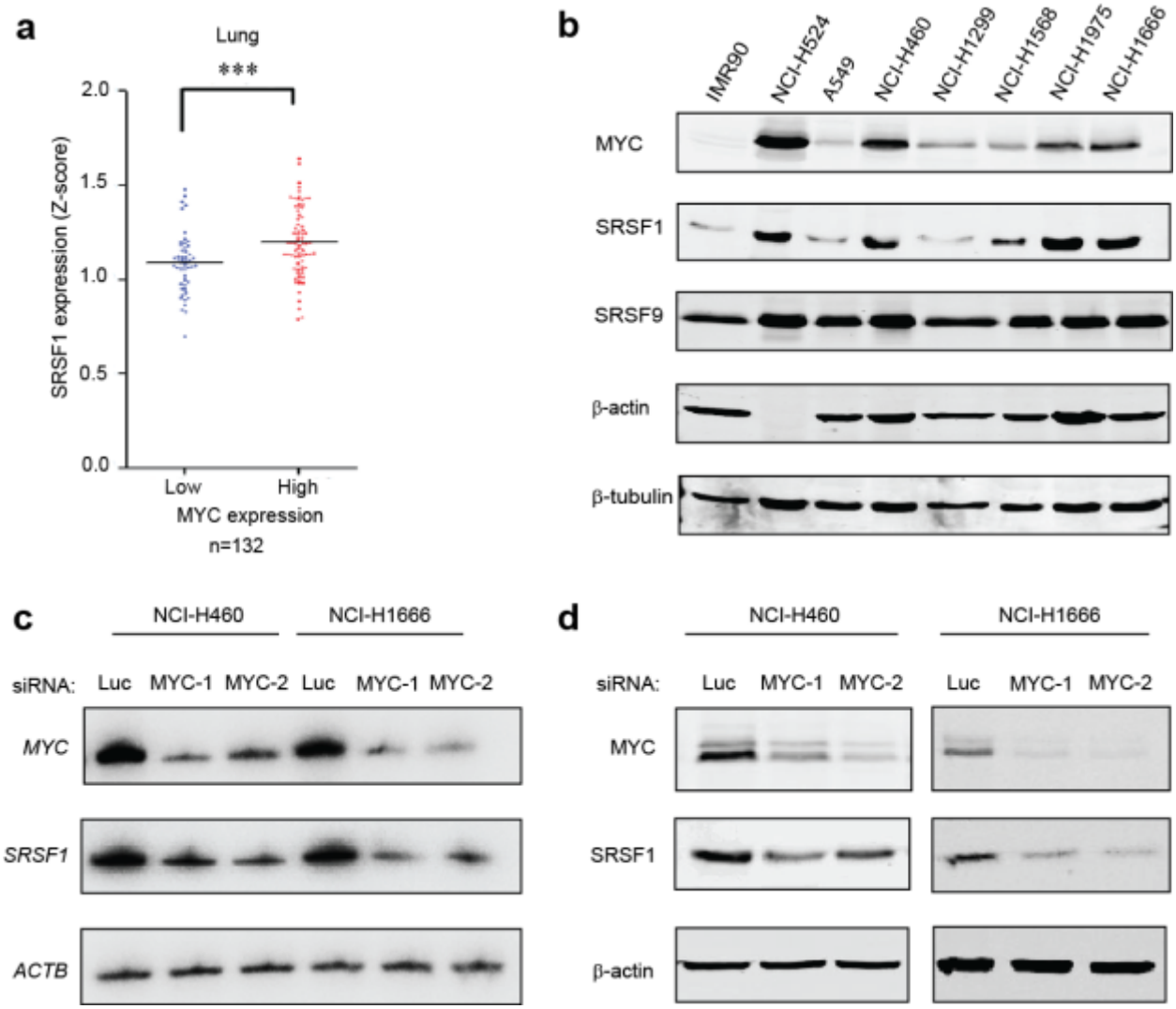

Figure 2.1 *SRSF1* expression correlates with MYC levels in human lung tumors and cell lines.
(**a**) *SRSF1* expression profile from microarray analysis of 132 lung tumors (expO). The data were normalized to Z-score and divided into two categories: tumors expressing high or low MYC levels. The dot plot 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.

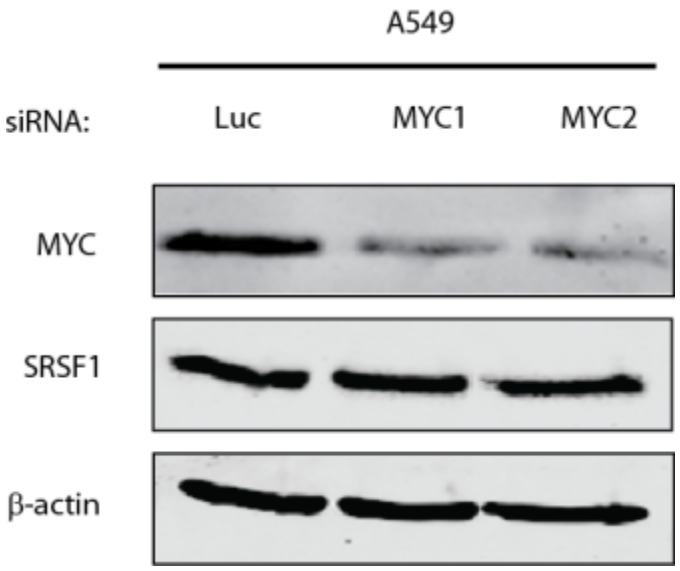

Figure 2.2 Effect of MYC knockdown on SRSF1 expression in A549 cells.

Immunoblotting of *SRSF1* and *MYC* in the lung cancer cell line A549 transfected with a control luciferase siRNA or one of two siRNAs against *MYC*. β-actin was used as loading control.

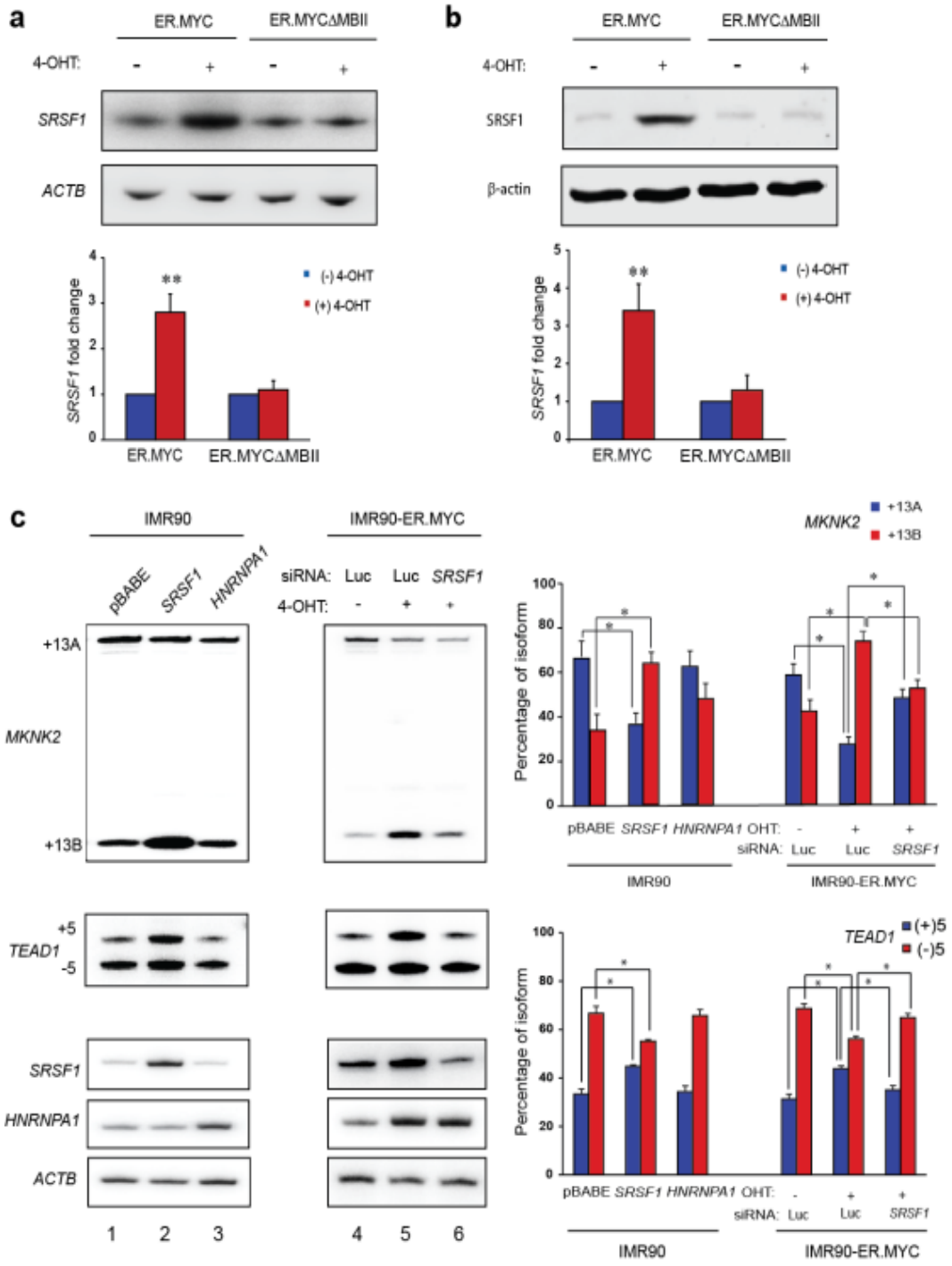

Figure 2.3 MYC regulates *SRSF1* expression and alternative splicing of an SRSF1 target gene.

(**a**) RT-PCR and (**b**) Immunoblotting of SRSF1 from IMR90-ER.MYC or IMR90-ER.MYCΔMBII cells induced with 4-OHT. Error bars, s.d.; n=3; t-test ***P*<0.01. (**c**) RT-PCR of *MKNK2* 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, s.d.; n=3, **P*<0.05.

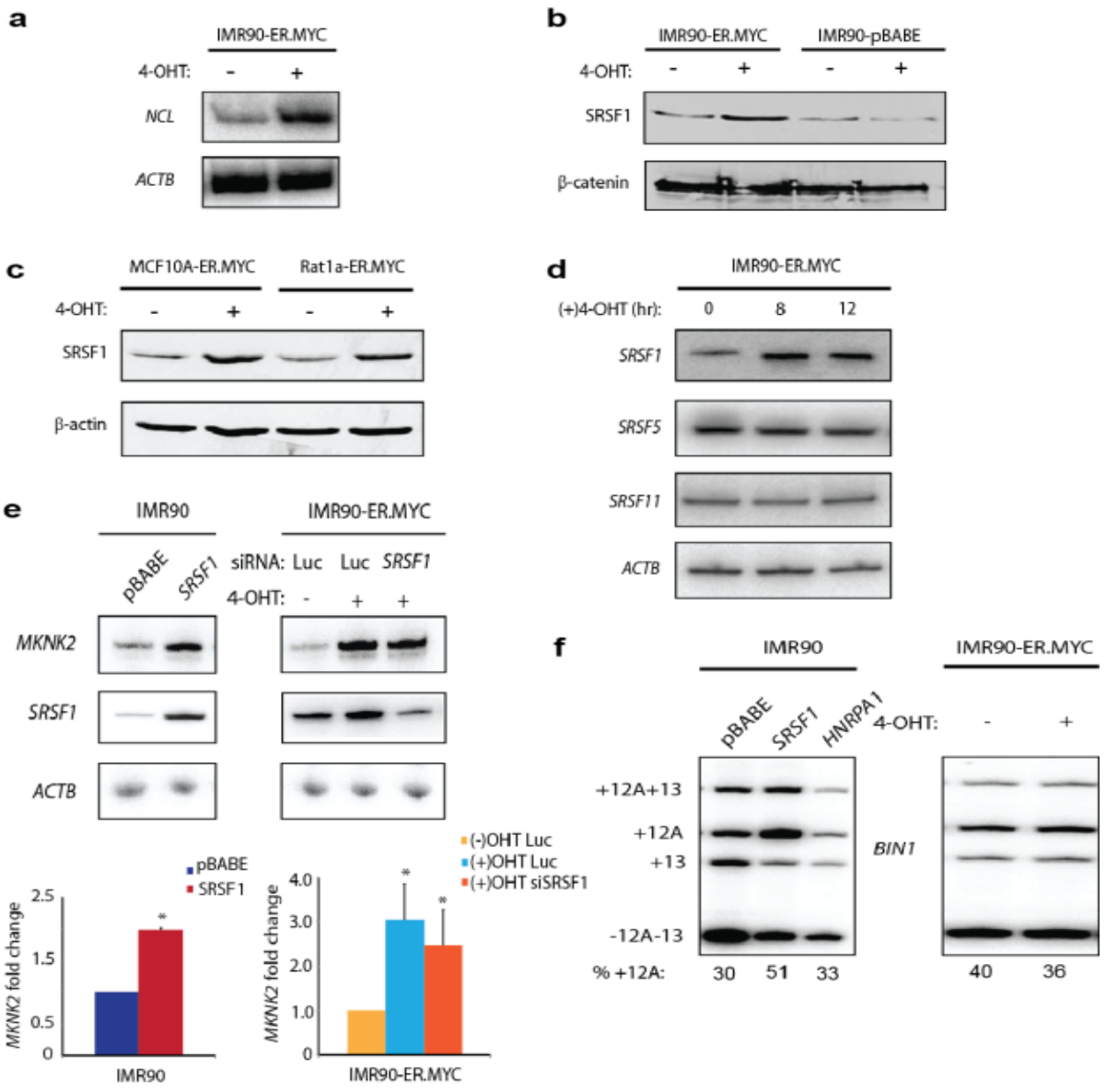

Figure 2.4 MYC induction regulates SRSF1 expression and activity.

(a) RT-PCR of *NCL* in IMR90-ER.MYC cells. Induction of *MYC* by 4-OHT treatment for 8 h led to upregulation of *NCL* mRNA. **(b)** Immunoblotting of SRSF1 in IMR90 cells transduced with empty vector or ER.MYC and treated with 4-OHT for 48 h. β-catenin was used as a loadingcontrol. **(c)** Immunoblotting of *SRSF1* in MCF10-ER.MYC and Rat1a-ER.MYC cells induced with 4-OHT for 48 h. β-actin was used as a loading control. **(d)** RT-PCR of *SRSF1*, *SRSF5* and *SRSF11* in IMR90-ER.MYC cells, induced with 4-OHT for 8 h and 12 h. *ACTB* was used as a loading control. **(e)** RT-PCR of MKNK2 total mRNA using preimers in constitutive exons 3 and 5 in IMR90 cells overexpressing SRSF1 and IMR90-ER.MYC cells treated with 4-OHT for 48 h. Error bars, sd; n=3; *P<0.05 **(f)** RT-PCR of *BIN1* mRNA isoforms in IMR90-ER.Myc cells treated with 4-OHT. IMR90 cells overexpressing *SRSF1* or *HNRNPA1* are shown as controls. As previously reported, SRSF1 overexpression resulted in increased exon 12A inclusion (Karni et al., 2007). Neither hnRNPA1 overexpression nor MYC induction affected the +12A isoform, relative to the control.

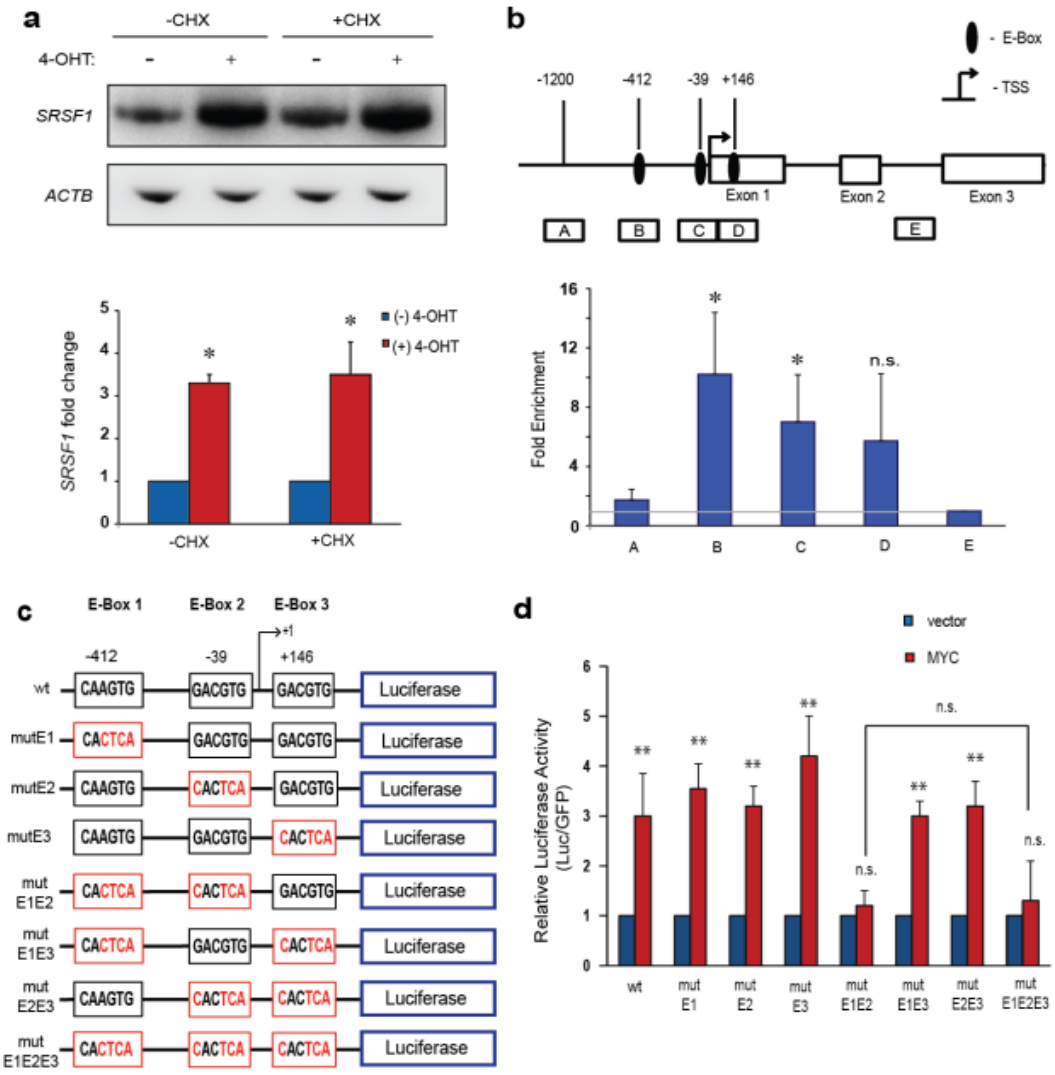

Figure 2.5 MYC binds to and activates the human *SRSF1* promoter.

(**a**) RT-PCR of IMR90-ER.MYC cells treated with 4-OHT, with or without cycloheximide. Error bars, s.d.; 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, s.d.; n=3; t-test *P<0.05; n.s., not significant. (**c**) Diagram of the wild-type *SRSF1* promoter, comprising three non-canonical 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**) co-transfected with *MYC* cDNA or vector control into NIH3T3 cells. Luciferase activity was normalized to co-transfected GFP, and the relative activity is plotted. Error bars, s.d.; n=3; t-test ***P*<0.01; n.s., not significant.

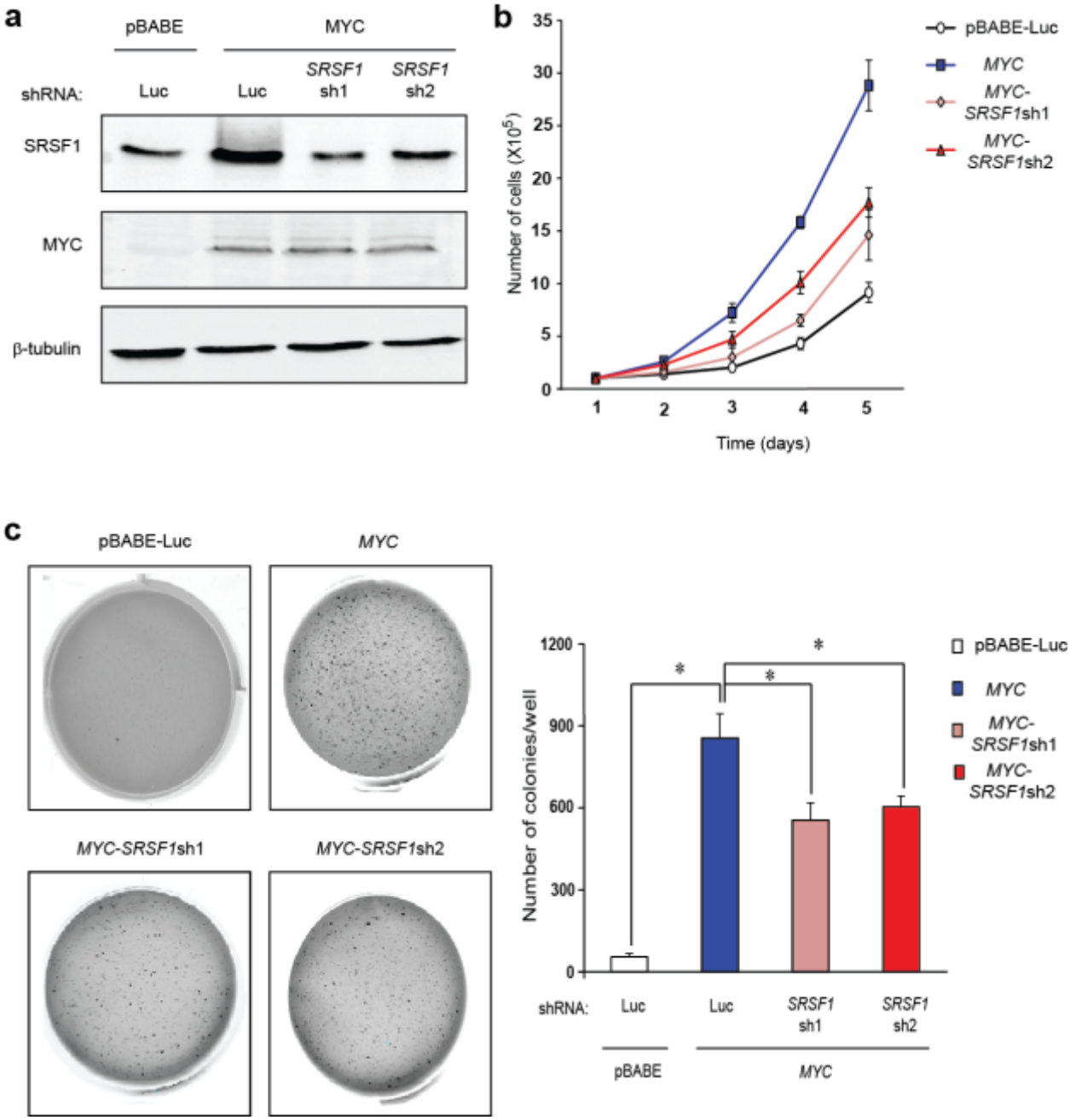

Figure 2.6 SRSF1 knockdown impairs anchorage-independent growth of MYC-transformed cells.

(**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, s.d.; n=3. (**c**) Anchorage-independent growth of cell lines from (**a**) in soft-agar colony-formation assays. Error bars, s.d.; n=3; t-test *P<0.05.

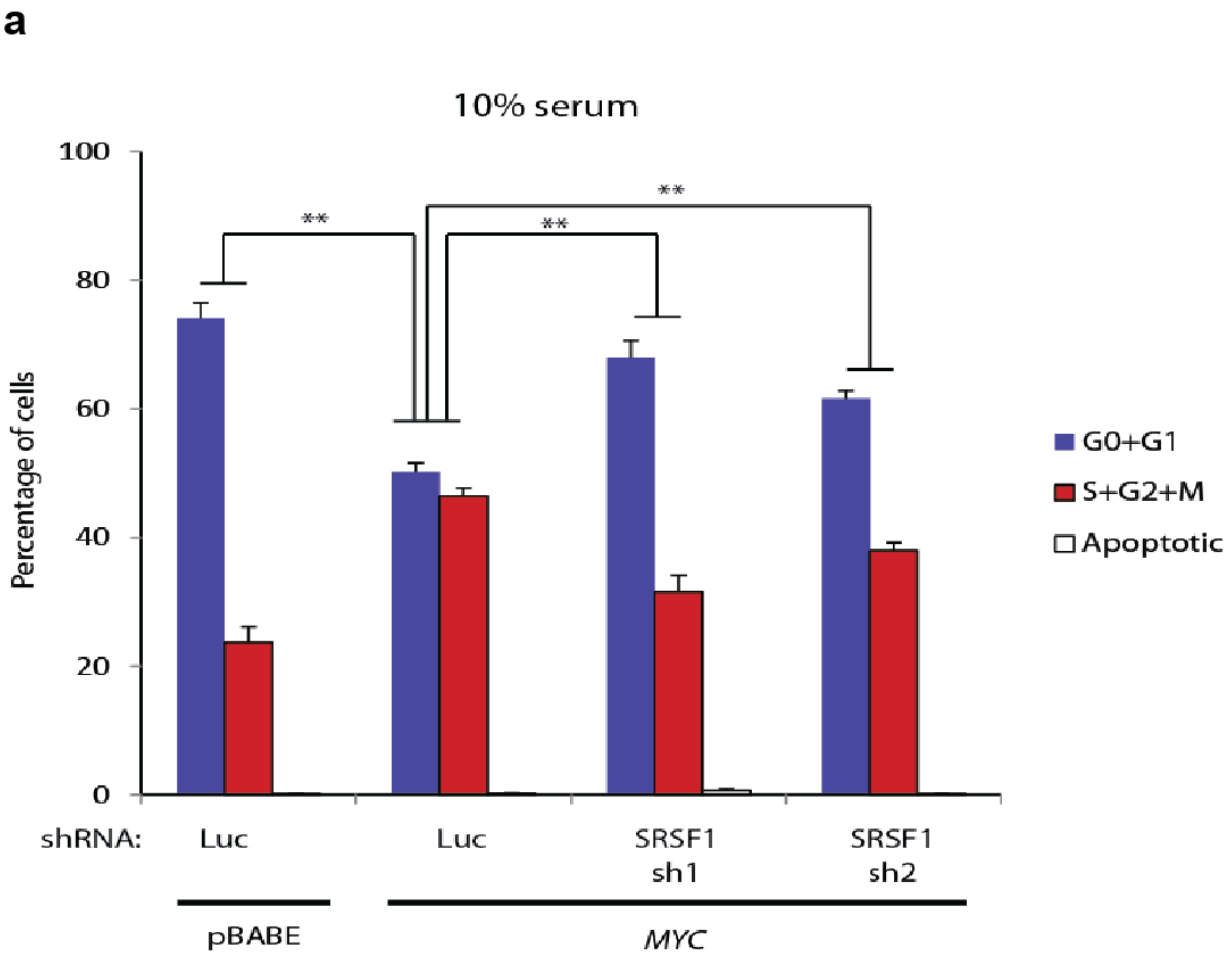

Figure 2.7 SRSF1 induction is important for MYC-mediated cell proliferation.

(**a**) Cell-cycle profile of indicated cell lines by PI staining of DNA and Flow Cytometry analysis. Error bars, sd; n=3; *P<0.05

2.7 Table and Table Legend

Co-expression with MYC in Lung tumors

Gene Name	Aliases	p-value
HNRPH1	hnRNPH1	0.004583
PTBP1	hnRNPI	0.020213
SRSF1	SF2/ASF	0.014976
SRSF2	SC35	0.657895
SRSF5	Srp40	0.657895
SRSF7	9G8	0.824561
SRSF11	Srp54	0.931556
HNRPA2B1	hnRNPA2B1	0.490873
HNRPA1	hnRNPA1	0.663001

Table 2.1 Splicing factors co-overexpressed with MYC in Lung tumors

Expression of various splicing factors (SF) in lung tumors profiled by microarray (data from Expression Project for Oncology, Gene Expression Omnibus GSE2109) was normalized to Z-score and divided into four categories: tumors expressing high levels of MYC and high/low levels of the SF; low levels of MYC and high/low levels of SF. This categories were represented as a contingency table and analyzed with a Fisher's exact tests. The resulting p-vlaues are shown in this table.

Chapter 3. Role of Oncogenic Splicing Factor SRSF1 in Regulation of p53 and Cellular Senescence

Abstract

Splicing and translation are highly regulated steps of gene expression. Altered expression of proteins involved in these processes can be deleterious. Therefore, the cell has many safeguards against such misregulation. We report that the oncogenic splicing factor SRSF1, which is overexpressed in many cancers, stabilizes the tumor-suppressor protein p53 by abrogating its MDM2-dependent proteasomal degradation. We show that SRSF1 is a necessary component of an MDM2/ribosomal-protein complex—separate from the ribosome—that functions in a p53-dependent ribosomal-stress checkpoint pathway. Consistent with the stabilization of p53, increased SRSF1 expression in primary human fibroblasts decreases cellular proliferation and ultimately triggers oncogene-induced senescence (OIS). These findings underscore the deleterious outcome of SRSF1 overexpression and identify a cellular defense mechanism against its aberrant function. Furthermore, they implicate the RPL5-MDM2 complex in OIS, and demonstrate a link between spliceosomal and ribosomal components—functioning independently of their canonical roles—to monitor cellular physiology and cell-cycle progression.

3.1 Introduction

The SR proteins are a phylogenetically conserved protein family involved in constitutive and alternative splicing (Long et al., 2009). SR proteins play additional key roles in the interplay between various steps in gene expression (Zhong et al., 2009). The shuttling SR protein SRSF1 is an essential, prototypical family member that functions in multiple steps of gene expression besides splicing, including chromatin remodeling, transcription, nonsense-mediated mRNA decay (NMD), mRNA export and stability, and translation (Das et al., 2007; Loomis et al.,2009; Michlewski et al., 2008; Sanford et al., 2004; Zhang et al., 2004). Furthermore, alterations in SRSF1 expression affect cell-cycle progression and cell viability (Li et al., 2005). For example, SRSF1 knockdown promotes apoptosis by altering the splicing of pro-apoptotic genes (Moore et al., 2010). *SRSF1* is a proto-oncogene that is overexpressed in many different cancers, e.g., because of an increase in its gene copy number or through transcriptional activation by MYC (Anczuków et al., 2011; Das et al., 2011; Karni et al., 2007). The oncogenic properties of SRSF1 are mediated in part through altering splicing of various oncogenes and tumor suppressors (Anczuków et al., 2011; Ghigna et al., 2005; Karni et al., 2007), as well as through activation of the mTOR pathway (Karni et al., 2008; Michlewski et al., 2008).

Normal cells resist oncogenic transformation by activating an intricate anti-tumorigenic pathway, mediated by multiple cell-cycle regulators and tumor suppressors. The tumor-suppressor protein p53 (TP53) is one such critical regulator of cellular homeostasis. In response to cellular stress, multiple mitogenic and genotoxic stresses converge to induce a p53-dependent response, resulting in cell-cycle arrest, apoptosis, DNA repair, or replicative senescence (Ko et al., 1996). Furthermore, aberrant activation of oncogenes in primary cells likewise activates p53-mediated tumor-suppressive barriers, leading to cell-cycle arrest and the onset of premature cellular senescence. This phenomenon, referred to as oncogene-induced senescence (OIS), is one of the ways cells guard themselves against oncogenic transformation.

Considering its central role in controlling the cell-cycle, the regulation of p53 is critically important for cell viability. The major regulator of p53 is the ubiquitin ligase MDM2. Upon binding to p53, MDM2 promotes p53 nuclear export and ubiquitylation, leading to its

degradation. Additionally, MDM2 binding conceals the N-terminal activation domain of p53, inhibiting its transcriptional activity (Boyd et al., 2000). Many factors regulate p53 by altering its interaction with MDM2, including a subset of ribosomal proteins (RPs) that function independently of the ribosome (Dai and Lu, 2004; Dai et al., 2004; Horn et al., 2008; Zhang et al., 2003). One such ternary complex, consisting of L5, L11, and L23, binds and sequesters MDM2, blocking its ability to ubiquitylate p53, and consequently increasing p53 protein stability and activity (Dai et al., 2004). The RP-MDM2-p53 pathway acts as a stress sensor for aberrant ribosome biogenesis and function, which can be triggered in response to various cues, such as nutrient changes (Bhat et al., 2004) and oncogenic activation, that impose a large burden on the translational machinery. For example, the RP-MDM2-p53 pathway forms an effective barrier against MYC-induced lymphomagenesis (Macias et al., 2010). Furthermore, mutations in the RP-interacting regions of MDM2 have been found in human tumors (Schlott et al., 1997).

To identify and study how SRSF1 protein-protein interactions regulate the multiple cellular processes in which SRSF1 is involved, as well as to uncover other potential functions, our laboratory has built an SRSF1 interactome through quantitative Isotopic Differentiation of Interactions as Random or Targeted (I-DIRT) IP-MS experiment using inducible T7 tagged SRSF1 Hela cells. Briefly, I-DIRT is a quantitative form of mass spectrometry in which, by mixing differentially labeled control and experimental lysates at equal concentrations prior to the IP, one can determine which peptides found to interact with the target (T7-SRSF1) come from the control cells, and which come from the experimental cells. A Light/Heavy ratio of around 1 indicates that, though the proteins were identified to interact with overexpressed T7-SRSF1, this interaction was not formed in the cell, but instead formed post-lysis; whereas a Light/Heavy ratio greater than 3 indicates that the majority of the peptides stem from the experimental T7-SRSF1-overexpressing cells, and thus the interaction is indeed physiological.

While we identified many SRSF1 interacting partners regulating the already known functions of SRSF1, we were intrigued to detect in vivo interaction between SRSF1 and the ribosomal protein L5 (RPL5). This observation was made more interesting by the fact that RPL5 was the only ribosomal protein to be identified as a true SRSF1-interacting partner. Furthermore this interaction persisted even in the present of nuclease.

Though SRSF1 has been shown to regulate translation and reportedly co-sediments with monosomes and polysomes in cytoplasmic extracts (Sanford et al., 2004), these results indicate that the SRSF1-RPL interaction is independent of RNA and the ribosome. As mentioned previously, RPL5 does form an extra-ribosomal complex with the E3 ubiquitin ligase MDM2 and mediates the ribosomal stress response. Hence we hypothesized that SRSF1 could be an additional, as yet undetected component of this complex. Accordingly, we investigated the potential involvement of SRSF1 in the ribosomal stress pathway as well as the consequences of the overexpressed SRSF1-RPL5 interaction on cellular physiology.

3.2 Results

3.2.1 SRSF1 is a Component of the RPL5-MDM2 Complex

As RPL5 has functions separate from its roles in ribosome function and biogenesis, we used co-IP of endogenous SRSF1 to examine whether SRSF1 is also a component of the RPL5/11/23-MDM2 complex. Consistent with the I-DIRT data, we detected an interaction between endogenous SRSF1 and RPL5; however, we did not detect interactions between endogenous SRSF1 and RPL11 or RPL23 (though overexpressed T7-SRSF1 did co-IP with RPL11 in HeLa cells, data not shown). In addition, we found that SRSF1 interacts with the E3 ubiquitin ligase MDM2 (**Figure 3.1a**). As variations in the canonical RPL5/11/23-MDM2 complex have been previously reported (Horn and Vousden, 2008), our data suggest that SRSF1 is part of an SRSF1-RPL5-MDM2 complex distinct from the canonical complex.

To verify that the ribosome is not involved in the interaction of SRSF1 and RPL5, as well as to identify where in the cell this interaction takes place, we immunoprecipitated endogenous SRSF1 from nuclear and cytoplasmic fractions of HeLa cells. We detected no interaction between endogenous SRSF1 and RPL5 in the cytoplasmic fraction, but endogenous SRSF1 efficiently co-IPd with RPL5 in the nuclear fraction (**Figure 3.1b**). Taken together, these data indicate that SRSF1 does not interact directly with the intact ribosome, and that the SRSF1-RPL5 interaction occurs in the nucleus, independently of the ribosome or the large subunit.

3.2.2 SRSF1 Stabilizes p53 through RPL5 and is Necessary for Ribosomal Stress-Induced p53 Activation

Induction of ribosomal stress, such as by treatment with 5 nM actinomycin D, a concentration that specifically inhibits RNA polymerase I, thereby perturbing ribosome biogenesis, has been shown to trigger the formation of the RP-MDM2 complex (Dai and Lu, 2004; Dai et al., 2004; Horn et al., 2008; Zhang et al., 2003). Interestingly, we observed that the interaction of endogenous SRSF1 with RPL5 and MDM2 was strengthened upon actinomycin D treatment (**Figure 3.1a**), highlighting a physiological role for this complex in response to ribosomal stress.

As the major role of the RPL-MDM2 complex is to sequester MDM2 and stabilize p53 (Dai and Lu, 2004; Dai et al., 2004; Horn et al., 2008; Zhang et al., 2003), we investigated whether overexpression of SRSF1 affects p53 expression in non-transformed human BJ fibroblasts. We generated doxycycline-inducible BJ cells overexpressing SRSF1, or with empty vector as a control. Induction of SRSF1, but not the control, led to increased levels of p53 protein, similar to those seen by treatment with 5 nM actinomycin D (**Figure 3.2a**). Overexpression of two other SR proteins, SRSF2 and SRSF9 did not have any effect on p53 expression (**Figure 3.2e**).

This increase in p53 was not due to a direct interaction between SRSF1 and p53 (**Figure 3.1a,** and **3.2b**), nor to changes in transcription, splicing, or mRNA stability of the major p53 isoform, as p53 mRNA levels did not change upon overexpression of SRSF1 (**Figure 3.2c**). Furthermore, overexpression of SRSF1 and/or actinomycin D treatment did not lead to activation of p14/ARF (**Figure 3.2c**), an upstream activator of p53, whereas both mRNA and protein levels of the p53 target gene p21 increased when p53 was upregulated by either means (**Figure 3.2c** and **3.2d**).

Though the interaction studies suggest that SRSF1 does not interact with the ribosome (Fregoso et al., 2013), considering the involvement of SRSF1 in translation, we analyzed the effects on p53 expression in BJ cells of nuclear-retained SRSF1 (SRSF1-NRS) (Cazalla et al., 2002), a chimeric protein unable to regulate translation (Sanford et al. 2004). Stable overexpression of wild-type SRSF1 or SRSF1-NRS increased p53 expression to comparable

levels (**Figure 3.3a**). Furthermore, SRSF1 has been shown to activate the mTOR pathway (Karni et al., 2008; Michlewski et al., 2008), which in turn regulates p53 mRNA translation (Astle et al., 2012). However, we saw no reduction in the ability of SRSF1 to induce p53 when cells were pretreated with the mTOR inhibitor rapamycin (**Figure 3.3b**). We conclude that SRSF1 increases p53 protein expression and activity, independently of direct or indirect effects on p53 transcription, splicing, mRNA stability, or translation.

To measure the effect of SRSF1 on p53 protein stability, we performed a cycloheximide-chase experiment in BJ fibroblasts, and monitored p53 levels with and without SRSF1 induction (**Figure 3.3c**). Wild-type p53 had a half-life of ~80 min in the control cells. Overexpression of SRSF1 greatly increased the stability of p53, to the extent that no appreciable decline in p53 levels was seen over a 150-min time course, suggesting that SRSF1, like additional components of the RPL-MDM2 complex, inhibits degradation of p53.

To directly determine whether SRSF1 influences p53 ubiquitylation, we measured the ubiquitin status of p53, with and without SRSF1 overexpression in H1299 cells, which lack endogenous p53 (Dai et al., 2004). Transient overexpression of T7-SRSF1 with Flag-p53 and His-Ub decreased the levels of ubiquitylated p53, while increasing the steady-state levels of p53 (**Figure 3.3d**). Thus, the effect of SRSF1 on p53 expression occurs at the level of protein stability, with SRSF1 blocking the ubiquitylation and proteasome-mediated degradation of p53.

We next determined whether this increased stability of p53 upon SRSF1 overexpression is dependent on the interaction of SRSF1 with RPL5. siRNA-mediated knockdown of RPL5 in BJ cells severely abrogated the effect of SRSF1 overexpression on p53, whereas a luciferase siRNA had no effect (**Figure 3.4a**), consistent with the inability of SRSF1 to interact with MDM2 upon RPL5 knockdown (**Figure 3.4b**). Furthermore, upon knockdown of SRSF1, actinomycin D (Sun et al., 2007) treatment no longer induced p53 protein accumulation to the same levels as in the control, and inhibited the RPL5-MDM2 interaction (**Figures 3.4c and 3.4d)** suggesting that SRSF1 is necessary for upregulation of p53 by ribosomal stress. Knockdown of SRSF1 alone did not affect p53 protein levels, as compared to the luciferase control (**Figure 3.4c**). Importantly, DNA-damage-induced p53 activation was not affected by knockdown of

SRSF1 (**Figure 3.5**), indicating that SRSF1 functions specifically through the ribosomal-stress pathway. Taken together, our data indicate that SRSF1 is dependent on its interaction with RPL5 to increase the stability of p53, and that SRSF1 is a necessary component of the RPL-MDM2 complex that stabilizes p53 in response to ribosomal perturbation.

3.2.3 SRSF1 Overexpression Induces Senescence in Primary Fibroblasts

As *SRSF1* is a proto-oncogene whose overexpression is sufficient to transform immortal rodent fibroblasts (Karni et al., 2007), this role of SRSF1 in stabilizing p53 may seem counterintuitive. However, overexpression of other potent oncogenes, such as H-Ras^{V12}, in primary cells likewise induces p53 activity, leading to a state of premature cellular senescence termed oncogene-induced senescence, or OIS (Chicas et al., 2010; Serrano et al., 1997). In this way, p53 forms an essential barrier against cellular transformation upon oncogenic stress (Courtois-Cox et al., 2008). We therefore characterized cell morphology and senescence-associated β-galactosidase (SA-β-gal) accumulation to determine whether modest overexpression of SRSF1 in non-transformed BJ fibroblasts induces senescence. Prolonged overexpression of SRSF1 resulted in enlarged and flattened cells with an increased number of intracellular vesicles—phenotypes typical of senescence (Chicas et al., 2010; Serrano et al., 1997) (**Figures 3.6a,** right panel and **3.6b**). In addition, SRSF1 overexpression resulted in a 5-fold increase in the number of SA-β-gal-stained cells, compared to uninduced control cells (**Figure 3.6a**, right panel).

As senescence occurs with a gradual decline in cell proliferation (Chicas et al., 2010; Serrano et al., 1997), we used EdU (a BrdU analog) incorporation to measure cell proliferation in the presence of overexpressed SRSF1 over a seven-day period. Within four days of SRSF1 overexpression, cell proliferation was drastically reduced to only 20% of control cells (**Figure 3.6c**). Senescence-associated heterochromatic foci (SAHF), an additional hallmark of senescent cells (Narita et al., 2006; Chicas et al., 2010), were also observed in SRSF1-overexpressing but not in control cells (**Figure 3.6b**). Two additional SR proteins-SRSF3 and SRSF9, which did not induce p53 expression (**Figure 3.2e**) also did not induce cellular senescence (**Figure 3.7a**).However, unlike other senescence-inducing oncogenes, such as Ras, Mos, and Cdc 6 (Di

Mocco et al., 2006), SRSF1 overexpression did not trigger an early phase of hyper-proliferation (**Figure 3.6c**), nor did it cause detectable DNA damage, as Western blotting and immunofluorescence showed no increase in γ-H2AX levels or CHK1 phosphorylation (**Figure 3.7b,** left and right panels, respectively). Furthermore, modest overexpression of SRSF1 in p53-null MEFs (**Figure 3.7c**) failed to trigger premature cellular senescence (**Figure 3.7d**), indicating that the ability of SRSF1 to activate the tumor-protective senescence response is dependent on an intact p53 pathway. This is once again in contrast to Ras^{V12}-induced senescence, for which p53 has been reported to be dispensable for OIS in primary human fibroblasts (Serrano et al., 1997).

Based on these results, we predict that tumors driven by SRSF1 overexpression would be compromised in their p53 tumor-suppressive pathway, with mutations, loss or silencing of p53 itself, or any of its regulators or critical downstream target genes. To test this hypothesis, we analyzed public microarray data from different cancer types to ascertain the *TP53* expression status of *SRSF1*-overexpressing tumors (**Figure 3.8**). We found significant anti-correlation between *SRSF1* and *TP53* expression in cancers of the kidney (282 tumor samples), colon (293 tumor samples), and breast (352 tumor samples), with *SRSF1*-overexpressing tumors having a tendency to downregulate their *TP53* expression.

3.2.4 RRM1 is Required for Interaction with the RPL5-MDM2 Complex, p53 Induction, and OIS

To further dissect the role of the SRSF1-RP-MDM2 complex in regulating p53 and inducing premature senescence, we examined the interaction of several SRSF1 domain mutants (**Figure 3.9**) with the other components of the complex. Whereas the ΔRRM2 mutant was indistinguishable from wild-type SRSF1, the ΔRRM1 mutant was almost completely defective in interacting with RPL5 or MDM2 (**Figure 3.10a**). Interestingly, the ΔRS mutant, which is defective in nuclear-cytoplasmic shuttling, and primarily accumulates in the cytoplasm (Cáceres et al., 1998), still co-immunoprecipitated with MDM2—though the interaction was much weaker than that for wild-type SRSF1 or ΔRRM2—but it did not interact with RPL5 (**Figure 3.10a)**. Similarly, whereas SRSF1-NRS was able to interact with RPL5 and MDM2, SRSF1-AAA, another mutant of SRSF1 that accumulates in the cytoplasm (Sinha et al., 2010), failed to interact

with RPL5, yet weakly interacted with MDM2 and very marginally induced p53 protein (**Figures 3.10b** and **3.10c**). We were unable to observe induction of p53 protein in HeLa cells, in which p53 protein is rapidly degraded due to the expression of HPV16-E6 (May et al., 1991), unless we massively overexpressed SRSF1. Therefore, we examined the effects of these various mutants on induction of p53 and premature cellular senescence in BJ cells. Whereas ΔRRM1 was unable to induce p53, both ΔRRM2 and ΔRS overexpression led to a partial increase in p53 protein (**Figure 3.11a**), though in both cases the degree of p53 induction was lower than observed with wild-type SRSF1. Furthermore, only ΔRRM1 overexpression failed to slow down cell proliferation, as assayed by EdU labeling (**Figure 3.11b**). Unlike the wild-type protein, none of the deletion mutants was able to induce premature cellular senescence (**Figure 3.11c**), despite the ability of ΔRS and ΔRRM2 to both induce p53 expression and growth arrest to some extent; we attribute this to a threshold effect for p53 induction to elicit senescence. These data suggest that SRSF1 interacts with the RPL-MDM2 complex through RRM1, and through this interaction SRSF1 leads to senescence in primary fibroblasts.

3.3 Discussion and Future Perspective

We have identified a new role of SRSF1 in regulating p53 protein stability and cell viability, summarized in our proposed model (**Figure 3.12**). We found that endogenous SRSF1 is an essential component of an RPL5-MDM2 complex; through these interactions, it increases the cellular pool of active p53. Furthermore, p53 induction resulting from overexpression of SRSF1 in primary human fibroblasts leads to OIS through a pathway not previously implicated in this oncogenic-stress response.

Using quantitative MS, we found that SRSF1 interacts specifically with RPL5, whereas the remaining RP interactions are non-specific. This interaction occurs independently of the ribosome and of rRNA or mRNA, as nuclease treatment did not disrupt SRSF1-RPL5 binding. Although SRSF1 co-sediments with actively translating ribosomes, and influences translation when bound to exonic splicing enhancers in mature mRNA (Sanford et al., 2004; Michlewski et

al., 2008), the main cellular pool of SRSF1 does not appear to interact directly or stably with intact ribosomes.

In addition to RPL5, we identified the E3 ligase MDM2 as an SRSF1-interacting protein. Previous reports described a complex of RPL5/11/23-MDM2 (Dai and Lou, 2004; Dai et al., 2004; Horn et al., 2008; Zhang et al., 2003); however this interaction was not observed by I-DIRT or by co-IP with endogenous SRSF1 (though RPL11 was detected by co-IP when T7-tagged SRSF1 was overexpressed). The apparent lack of bona fide interactions with L11 and L23 suggests that SRSF1 functions in a separate complex from other, previously described RPL-MDM2 complexes. It is possible that SRSF1 competes with L11 or L23 for binding to RPL5 and/or MDM2, therefore displacing components of the complex. Whether particular complexes are formed in response to distinct stress signals, and whether these complexes activate distinct cellular responses in response to induction of ribosomal stress will be important questions to pursue.

SRSF1 expression within cells is tightly controlled (Sun et al., 2010). It is auto-regulated, reflecting the fact that changes in SRSF1 expression can be deleterious to cells. Indeed, even a modest 2-fold overexpression of SRSF1 in immortal murine NIH3T3 fibroblasts, which have a compromised p53 pathway, has been shown to transform them, by promoting proliferation and inhibiting apoptosis (Karni et al., 2007). Here we show that a powerful tumor-suppressive barrier has apparently evolved to guard against overexpression of SRSF1, further emphasizing SRSF1's potential as a potent oncoprotein. Although it remains unclear how oncogenic pathways can overcome the autoregulation of SRSF1 expression, we show here that enforced overexpression of SRSF1 in normal human primary fibroblasts with intact p53 activates the p53 tumor-suppressive pathway. Consequently, the cells are forced into a state of premature senescence, which protects the host against transformation.

Interestingly, premature senescence induced by SRSF1 overexpression in normal cells is distinct from the classical Ras-induced senescence (Serrano et al., 1997), which is primarily a DNA-damage response induced by hyper-proliferation and is dependent on p16 activation (Bartkova et al., 2006; Chicas et al., 2010; Di Mocco et al., 2006). In contrast, SRSF1-induced

senescence does not result in an early hyper-proliferative phase, DNA damage, or activation of p14/ARF, and it is likely due to aberrant activation of the ribosomal-stress pathway, involving distinct regulators and mediators. Thus, the differences in the senescence phenotypes may stem from the distinct underlying mechanisms of induction. Furthermore, we show that SRSF1 is directly involved in mounting the anti-tumorigenic response to its own overexpression, through initiating the formation of an SRSF1-RPL5-MDM2 complex, and thereby stabilizing p53. Hence, it appears that SRSF1 autoregulates not only its expression but also its function, so as to allow the organism to resist oncogenic transformation.

Aberrant activation of the PI3K/AKT signaling pathway likewise induces premature cellular senescence in the absence of hyper-proliferation or DNA damage (Alimonti et al., 2010; Astle et al., 2011). However, AKT-induced senescence is dependent on mTOR, a protein kinase that controls cell growth (Astle et al., 2011). SRSF1 is also an activator of the mTOR pathway, but it bypasses upstream PI3K/AKT signaling (Karni et al. 2008). Considering the critical role of mTOR in regulation of ribosome biogenesis (Mayer et al., 2006), these studies further emphasize the emerging concept of the central role that the ribosome plays in regulating cellular homeostasis and oncogenesis. Additionally, this is the first time that ribosomal stress has been implicated in OIS; however, whether this is a common mechanism of tumor protection caused by ribosomal perturbation, or is unique to the *SRSF1* proto-oncogene will need further investigation.

Considering p53's role in preventing SRSF1-induced oncogenesis, it is apparent that SRSF1 overexpressing tumors are in all probabilities compromised in their p53 tumor suppressive pathway. This could be achieved by silencing mutations in the *TP53* gene, or mutations in regulators of p53 expression or activity. >50% of human tumors are found to carry point mutations in *TP53*-especially in its DNA-binding domain (Hollestein et al., 1991; Petitjean et al., 2007)), while many tumors with wild-type *TP53* have no active p53 protein due to factors such as overexpression of MDM2 or deletion of the p53 positive regulator ARF, etc. Since p53 is a key barrier to SRSF1-mediated tumorigenesis, it provides us with a potential therapeutic target. Though still in nascent stages, there are strategies under development aimed at reactivating mutant p53, or activating wild-type p53 in tumors (Wang & Sun, 2010). For instance, for tumor-associated mutant p53 with an altered conformation that prevents DNA binding, synthetic

peptides like CDB3 (derived from p53-binding protein 2) (Friedler et al., 2002) or small molecules like PRIMA-1 and MIRA-1 (Bykov et al., 2002; Bykov et al., 2005) can bind and stabilize mutant p53 in its active conformation and restore DNA-binding, leading to transactivation of p53 target genes. Additionally, small molecule such as Nutlins can disrupt p53 binding to MDM2, thus preventing its ubiquitylation and subsequent degradation.

As mentioned previously, TP53 mutations in tumors are mostly missense mutations in the central DNA-binding domain of the protein, with six 'hot spots' where the mutations occur mostly commonly. Interestingly, at least a third of these missense mutations in the DBD of p53 have been found to be gain-of-function mutations, even conferring upon them oncogenic activities (Freed-Pastor & Prives et al., 2012). These mutant p53 proteins do not recognize the wild-type p53 DNA consensus sequence, are more stable and have decreased pro-apoptotic , growth-arrest and senescence promoting activity and confer chemoresistance (Blandino et al., 1999) to tumors. Furthermore, they can promote tumorigenesis by binding and sequestering other anti-tumor proteins such as p63 and p73, and regulate transcription of distinct sets of target genes responsible for the gain of functions such as chemoresistance (Scian et al., 2005), genomic instability (Wang et al., 1998; Song et al., 2007) and promotion of metastasis (Lang et al., 2004; Oliver et al., 2004; Noll et al., 2011). Whether mutant p53 have their own distinct transcriptional activity is still under investigation, but many of these mutant p3 proteins have been found to interact with other transcription factors, thereby possibly regulating their functions (Di Aagostino et al., 2006; Liu et al., 2011; Hwang et al., 2011; Freed-Pastor et al., 2012).

It would be interesting to analyse *SRSF1*-overexpressing tumors for mutations in the *TP53* gene. While inactivating mutations will remove the barrier of SRSF1-induced senescence, gain-of-function mutations in *TP53* can actually promote the oncogenic potential of SRSF1 overexpressing cells. Furthermore, *SRSF1* overexpression can potentially enhance the stability of these oncogenic p53 proteins, resulting in oncogenic cooperation and a more aggressive phenotype. Wide-scale analysis of human tumors can shed more light on the status of *SRSF1* co-expression with wild-type and mutant p53 protein, thus helping us design more effective strategies for cancer treatment.

3.4 Materials and Methods

3.4.1 Plasmids

pCG-T7-SRSF1, pCG-T7-SRSF1-ΔRRM1, pCG-T7-SRSF1-ΔRRM2, pCG-T7-SRSF1-ΔRS, pCG-T7-SRSF1-NRS, and pCG-T7-SRSF1-AAA were described previously (Cáceres et al., 1998; Cazalla et al., 2002; Sinha et al., 2010). pMSCV-TT-IRES-Puro was modified from the TMP vector (Dickins et al., 2005) and was a gift from Scott Lowe (MSKCC). pMSCV-TT-T7SRSF1 constructs were generated by subcloning the *SRSF1* cDNAs from the pCG-T7-SRSF1 plasmids into pMSCV-TT-IRES-Puro after amplification with Xho1-forward and EcoRl-reverse primers. pCDNA3.1-p53 and pCDNA3.1-HA-Ub were purchased from Addgene. For primer sequences, see Supplemental Methods.

3.4.2 Cell Culture, Virus and Stable Cell Line Generation

BJ, HeLa, U2OS, and NCI-H1299 cell culture conditions were as recommended by ATCC. Viruses were produced as described (Karni et al., 2007). To generate stable doxycycline-inducible cell lines, Tet-on Advanced HeLa, BJ, and U2OS cells (Clontech) were infected with pMSCV-TT-T7SRSF1 constructs or pMSCV-TTIRES-Puro, and selected with puromycin (Sigma). For inducible expression of SRSF1, doxycycline was added at 0.01 to 1.0 μg/mL for 36 h. For I-DIRT (Tackett et al., 2005), HeLa cells were passaged for six doublings in DMEM (Thermo) without L-lysine and L-arginine, supplemented with either L-lysine-2HCl and L-arginine-HCl for light media, or $^{13}C_6$ L-lysine-2HCl and $^{13}C_6$ $^{15}N_4$ L-arginine-HCl (Thermo) for heavy media, to a final concentration of 0.1 mg/mL each.

3.4.3 Cell Lysis and Protein Analysis

For protein analysis of whole-cell lysates, cells were lysed in RIPA buffer plus Protease Inhibitor Cocktail EDTA-free (Roche). For whole-cell lysate preparation followed by IP, cells were first lysed in NP-40 Lysis Buffer (0.05-0.5 % (v/v) NP-40, 100-500 mM NaCl, 50 mM Tris, pH 7.4, 1 mM DTT) plus protease inhibitor cocktail. Lysates were sequentially passed through a syringe

with a 20G, 22G, and 26G needle. Nuclear/cytoplasmic fractionation was adapted from (Allemand et al., 2005). Where indicated, nuclease was added (1 U/mL of RNase A (Ambion), 40 U/mL RNase T1 (Ambion), 500 U/mL Benzonase Nuclease (EMD) plus 2 mM $MgCl_2$) and incubated on ice for 30 min. Lysates were cleared by high-speed centrifugation at 13,000 g for 15 min at 4 ^{o}C.

3.4.4 Immunoblotting

Lysates were separated by SDS-PAGE, probed with the indicated antibodies, and when appropriate, quantified using an Odyssey infrared-imaging system (LI-COR Biosciences). Primary antibodies against the following proteins/epitopes were used: T7 (Novagen), SRSF1 (AK-96, CSHL), RPL5, RPL11, and RPL23 (GeneTex), MDM2 (2A10, Abcam), p53 (DO-1, EMD Bioscience), β-actin (BD Biosciences), p21 (Abcam), γ-H2AX (Upstate Cell Signaling Solutions), phospho-CHK1 (Cell Signaling), p53 polyclonal (Abcam). Secondary antibodies were HRP-conjugated goat anti-mouse or anti-rabbit (Biorad) for chemiluminescent detection, and IR dye 800 or 680 anti-rabbit or anti-mouse (LI-COR Biosciences) for infrared detection.

3.4.5 Immunoprecipitations

Antibody capture and crosslinking to Dynabeads Protein G (Invitrogen) were performed according to the manufacturer's specifications. For I-DIRT IPs, heavy and light lysates were combined at equal total-protein concentrations, determined by the Bradford assay, before incubation with beads. All IPs were incubated while rotating for 45 min at 4 ºC and then washed five times with Lysis Buffer.

3.4.6 Cycloheximide Chase Assay

2×10^5 cells plated in 6-well plates were treated with 10 μg/mL cycloheximide for the indicated times, followed by lysis and immunoblotting.

3.4.7 RNA Interference

The RPL5 siRNA pool was purchased from Dharmacon. BJ and U2OS cells were reverse-transfected with RNAiMax (Invitrogen) at a final concentration of 20 nM. Thirty-six h after transfection of the siRNA, doxycycline was added for an additional 36 h, and where indicated, 5 nM actinomycin D was added for 8 h.
Additional siRNA target sequences:
SRSF1: 5'-ACGAUUGCCGCAUCUACGU-3'
Luciferase: 5'-'CGUACGCGGAAUACUUCGA-3'

3.4.8 RT-PCR Analysis

RT-PCR was performed as described (Karni et al., 2007). For a complete list of primers, see Supplemental Experimental Procedures.

3.4.9 In Vivo Ubiquitination Assays

The ubiquitination assay was adapted from William P Tansey, Cold Spring Harb Protoc; 2006; doi:10.1101/pdb.prot4616.

3.4.10 Senescence-associated β-galactosidase Assay

The SA-β-gal assay was adapted from (Chicas et al., 2010). SRSF1 expression was induced by Dox addition on day 0 and analyzed on day 7.

3.4.11 EdU Cell Proliferation Assay

EdU incorporation was measured using a Click-It EdU Cell Proliferation Assay Kit (Invitrogen). SRSF1 expression was induced by Dox addition on day 0 and analyzed on days 2, 4, and 7. Cells were imaged using a Zeiss Axiovert 200M fluorescent microscope.

3.4.12 Statistical Analysis

Where appropriate, the data are presented as the means ± s.d. Data points were compared using unpaired two-tailed Student's t-tests, and *P*-values are indicated in the figure legends.

3.5 Acknowledgements

We thank Oliver Fregoso for initiating the study, performing the Mass spectrometry analysis of SRSF1-interacting proteins and collaboration on the design and performance of experiments. We thank S. Lowe and R. Sordella for helpful comments. We thank O. Anczuków-Camarda and Martin Akerman for help with analysis of Microarray data from human tumors to determine *TP53-SRSF1*expression correlation

3.6 Figures and Figure Legends

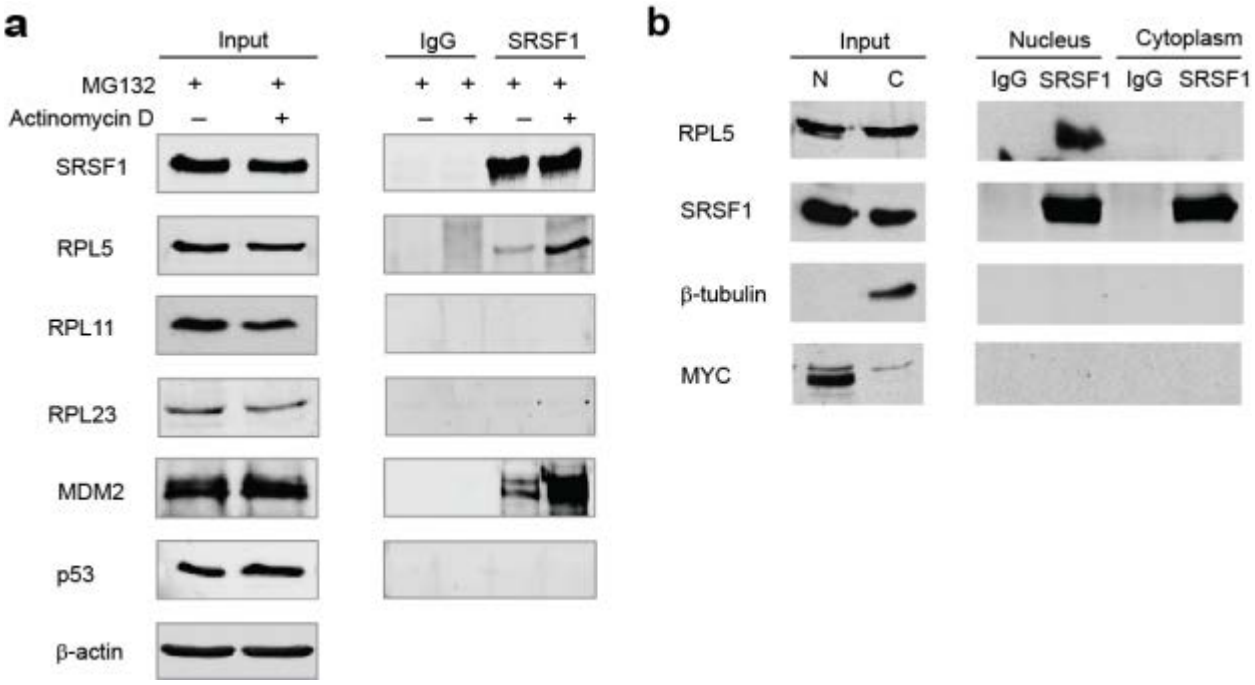

Figure 3.1 SRSF1 is a component of a nuclear RP-MDM2 complex.

(a) BJ fibroblasts were either left untreated or treated with 5 nM actinomycin D for 8 h, followed by 50 μM of proteasomal inhibitor MG132 for 8 h. Lysates were immunoprecipitated with either control IgG or AK96 monoclonal antibody against SRSF1. Whole-cell lysates (Input) and IPs were analyzed with the indicated antibodies. **(b**) Nuclear and Cytoplasmic fractions obtained from sub-cellular fractionation of Hela cell lysate were subjected to immunoprecipitation with the AK96 antibody against SRSF1. Whole cell lysates and Immunoprecipitates were analysed by immunoblitting using the indicated antibodies. MYC protein was used as a nuclear marker while β-tubulin was used as a cytoplasmic marker to assess the quality of fractionation.

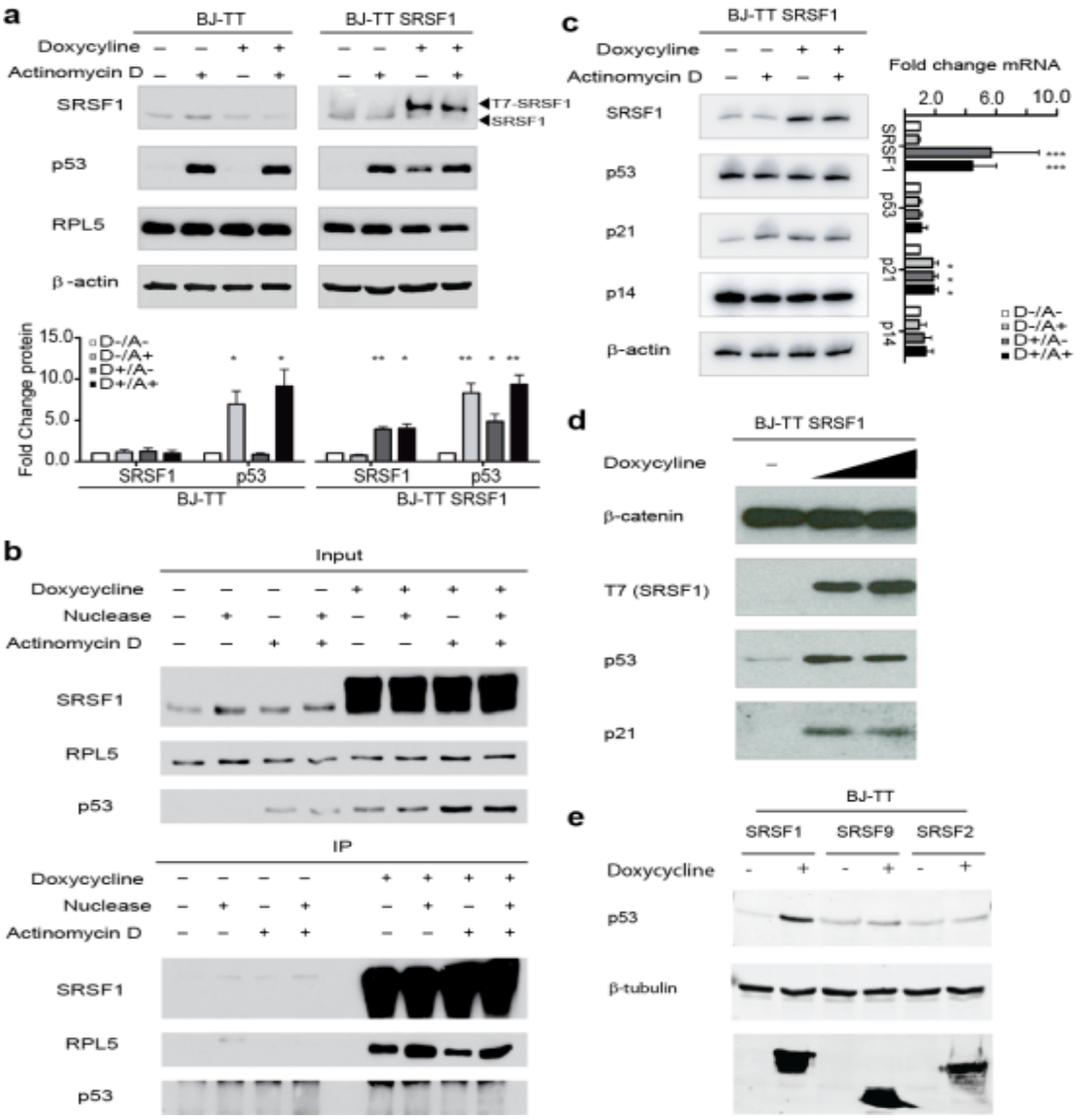

Figure 3.2 SRSF1 overexpression induces p53 protein expression and activity.

(a) BJ TT7-SRSF1 fibroblasts or empty-vector control (BJ TT7) were treated with doxycycline for 36 h and 5 nM actinomycin D for 8 h and analyzed by immunoblotting, as indicated. Values represent fold change in protein levels, relative to a loading control. Representative western blots are show. Data are means +/-s.d. (n=3), *P<0.05, **P<0.01, *** P<0.001. **(b)** BJ TT7-SRSF1 cells were treated with doxycycline (0.1 µg/mL) for 36 h and actinomycin D (5 nM) for 8 h as indicated, lysed, with or without nuclease treatment and immunoprecipitated with T7 monoclonal antibody. Whole cell lysates (Input) and Immunoprecipitates (IP) were analyzed by immunoblotting with the indicated antibodies. The asterisk indicates non-specific binding. **(c)** Total RNA from BJ TT7-SRSF1 cells was amplified by radioactive RT-PCR and analyzed by native PAGE. Values represent fold change in mRNA levels relative to β-actin. Statistics are as in panel **(a)**. **(d)** BJ TT7-SRSF1 cells were induced with increasing concentrations of doxycycline, from 0.1 to 10 µg/mL. Cells were collected in RIPA buffer and analyzed by immunoblotting with the indicated antibodies. **(e)** BJ TT7-SRSF1, BJ TT7-SRSF3 and BJ TT7-SRSF9 were treated with doxycycline for 36 h and 5 nM actinomycin D for 8 h and analyzed by immunoblotting, as indicated.

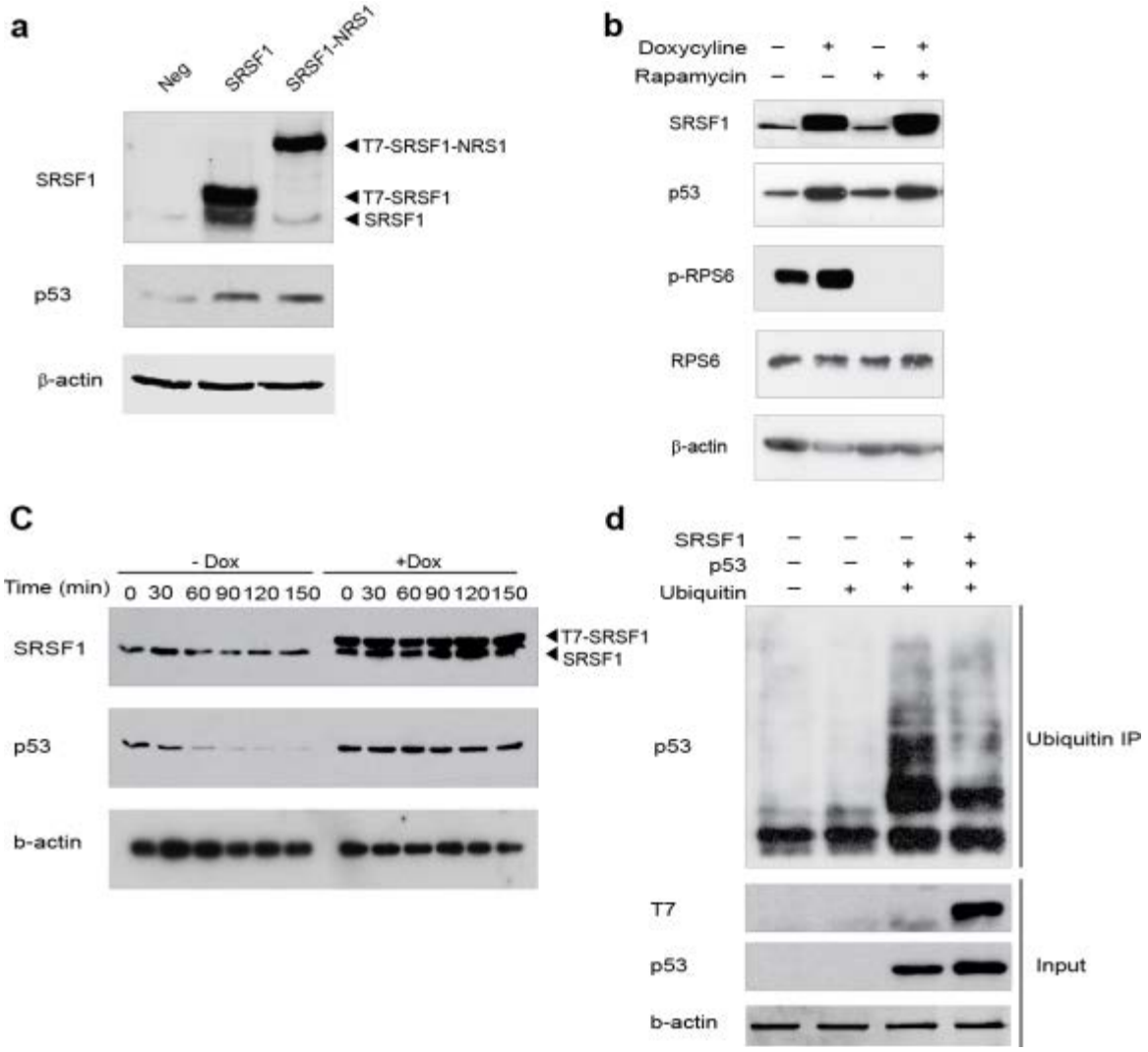

Figure 3.3 SRSF1 blocks ubiquitylation and increasing stability of p53 protein.

(a) BJ cells were transduced with empty vector, T7-SRSF1, or T7-NRS-SRSF1. After 48 h, whole-cell lysates were analyzed by immunoblotting with the indicated antibodies. **(b)** BJ TT7-SRSF1 cells were treated with or without doxycycline for 36 h, followed by rapamycin (200 nM) for 8 h. Lysates were analyzed by immunoblotting with the indicated antibodies. **(c)** BJ TT7-SRSF1 cells were treated with or without doxycycline for 36 h, followed by cycloheximide (10 μg/mL) for the indicated times. Lysates were analyzed by immunoblotting.
(d) H1299 cells lacking endogenous p53 were transfected with His-Ub, Flag-p53, and/or T7-SRSF1 plasmids, or the corresponding empty vectors. Cells were lysed under denaturing conditions and incubated with nickel-agarose beads for 3 h. Input and nickel-bound proteins were analyzed by immunoblotting with the indicated antibodies.

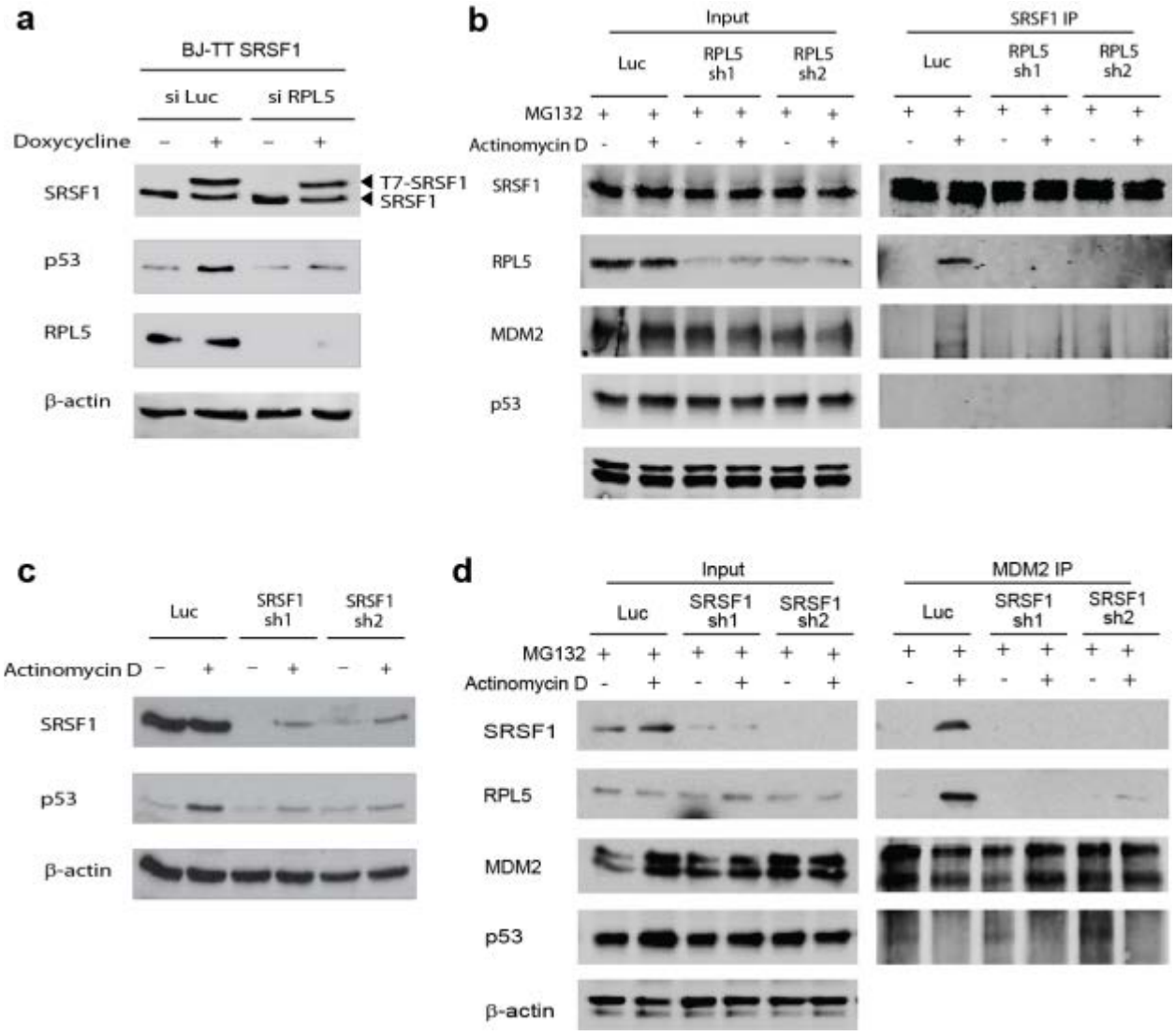

Figure 3.4 SRSF1-RPL5 interaction is important for MDM2 sequestration and p53 induction upon nucleolar stress.

(a) BJ TT7-SRSF1 cells were transfected with luciferase or a pool of RPL5 siRNA for 36 h, followed by doxycycline induction for 36 h, as indicated, and analyzed by immunoblotting. **(b)** U2OS cells transduced with luciferase or RPL5 shRNA were treated with 5 nM actinomycin D for 8 h, followed by 50 μM MG132 for 8h as indicated. Lysates were immunoprecipitated with AK96 monoclonal antibody against SRSF1. Whole cell lysates (Input) and Immunoprecipitates (IP) were analysed by immunoblotting with the indicated antibodies. **(c)** U2OS cells were transduced with luciferase or SRSF1 shRNA, selected with puromycin, followed by actinomycin D (5 nM) treatment for 8 h, as indicated, and analyzed by immunoblotting. **(d)** U2OS cells transduced with luciferase or SRSF1 shRNA were treated with 5 nM actinomycin D for 8 h, followed by 50 μM MG132 for 8h as indicated. Lysates were immunoprecipitated with anti-MDM2 antibody (2A10, Abcam). Whole cell lysates (Input) and Immunoprecipitates (IP) were analysed by immunublotting with the indicated antibodies.

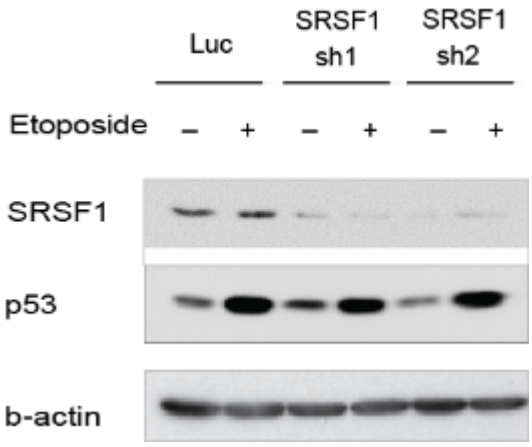

Figure 3.5 SRSF1 mediated p53 induction is specifically through the nucleolar stress pathway.

(a) U2OS cells were transduced with luciferase or SRSF1 shRNA, followed by etoposide treatment for 12 h, as indicated, and analyzed by immunoblotting.

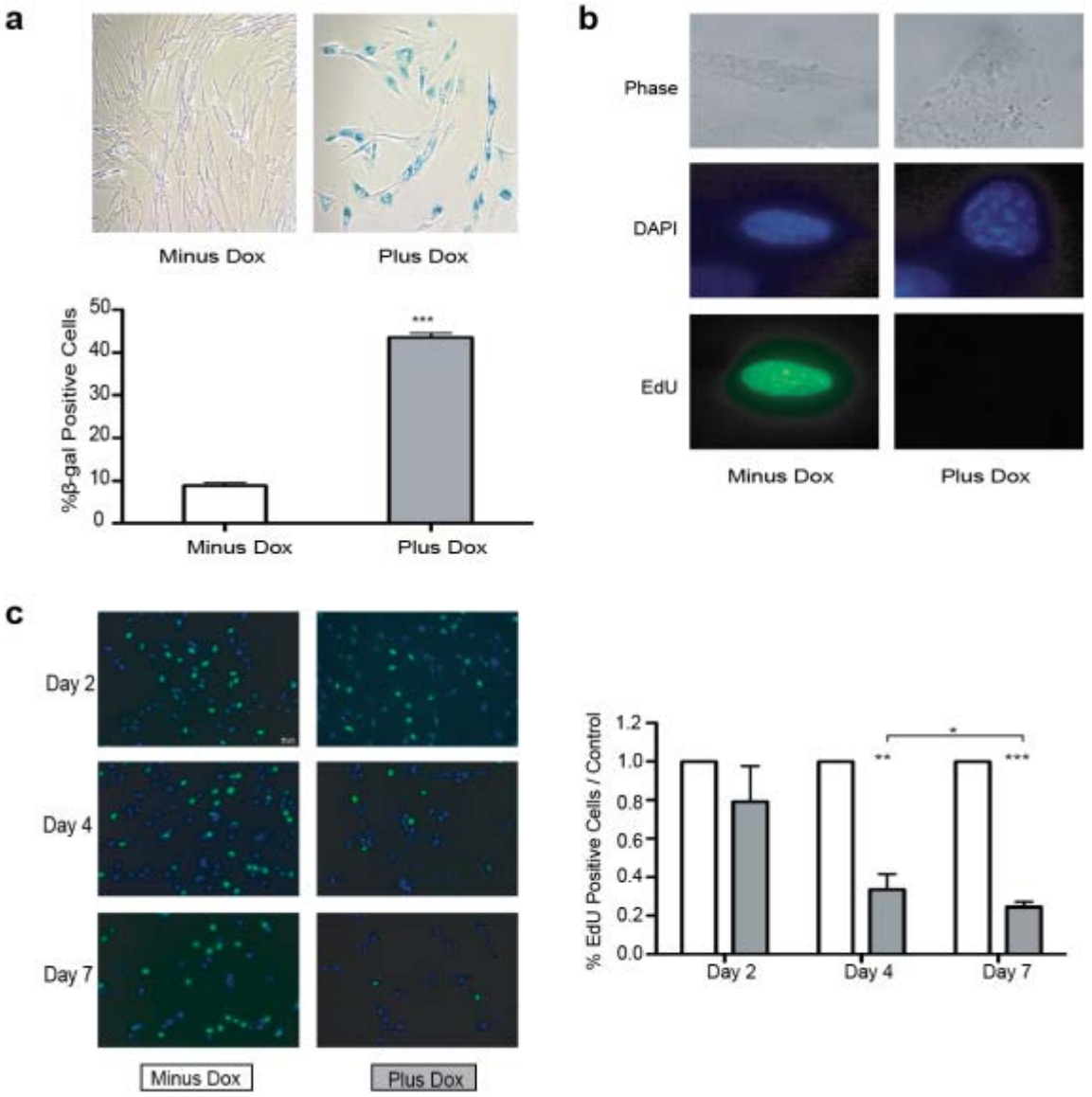

Figure 3.6 Overexpression of SRSF1 Leads to Senescence of Primary Fibroblasts.

(a) BJ TT7-SRSF1 cells were induced with doxycycline for 7 d, fixed, stained with X-gal, and observed at 20× magnification (right panels). 200 cells were counted for each condition (left panel); n=6. Means +/- s.d. are shown; ****P* = 0.0003. **(b)** Representative day-7 induced and control BJ TT7-SRSF1 cells imaged at 63X for morphology (phase, top), DNA (DAPI, middle), and proliferation (EdU, bottom). **(c)** BJ TT7-SRSF1 cells were induced with doxycycline for 2, 4, or 7 d, incubated with 10 μM EdU, and observed at 20× magnification (top panels). 100 cells were counted for each condition (lower panel); n=6. Means +/- s.d. are shown; **P* = 0.05, ***P* = 0.005, ****P* = 0.0008.

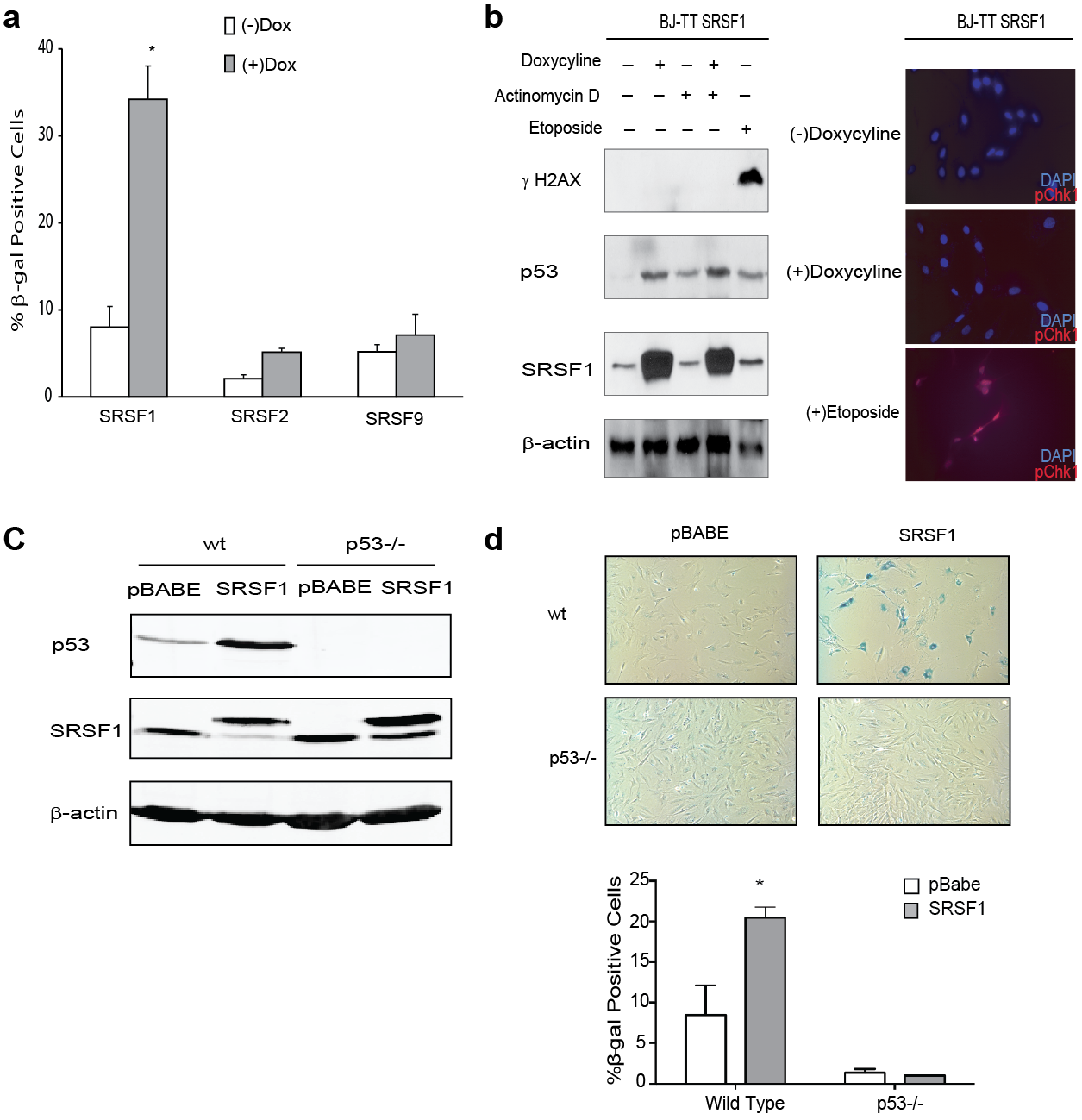

Figure 3.7 SRSF1 induced senescence is p53 dependent and does not cause DNA damage.

(a) BJ TT7-SRSF1, BJ TT7-SRSF3 and BJ TT7-SRSF9 fibroblasts were induced with doxycycline for 7 days, fixed and stained with X-gal. 200 cells were counted for each condition; n=3. Mean +/-s.d. are shown, *P=0.05. **(b)**BJ TT7-SRSF1 cells were treated with or without doxycycline, actinomycin D, or etoposide, and analyzed by immunoblotting (left) or immunofluorescence (right) for the DNA-damage markers γ-H2AX or phospho-CHK1, respectively. 50 nM etoposide was used as a positive control. **(c)** Wild-type and p53-null MEFs were transduced to overexpress SRSF1. Cells were lysed under denaturing conditions and immunoblotted with the indicated antibodies. **(d)** Wild-type or p53-null MEFs transduced with control or T7-SRSF1-expressing retroviruses were fixed and stained with X-gal (top panels). 200 cells were counted for each condition (lower panels); n=3. Means +/-s.d. are shown; *P = 0.05.

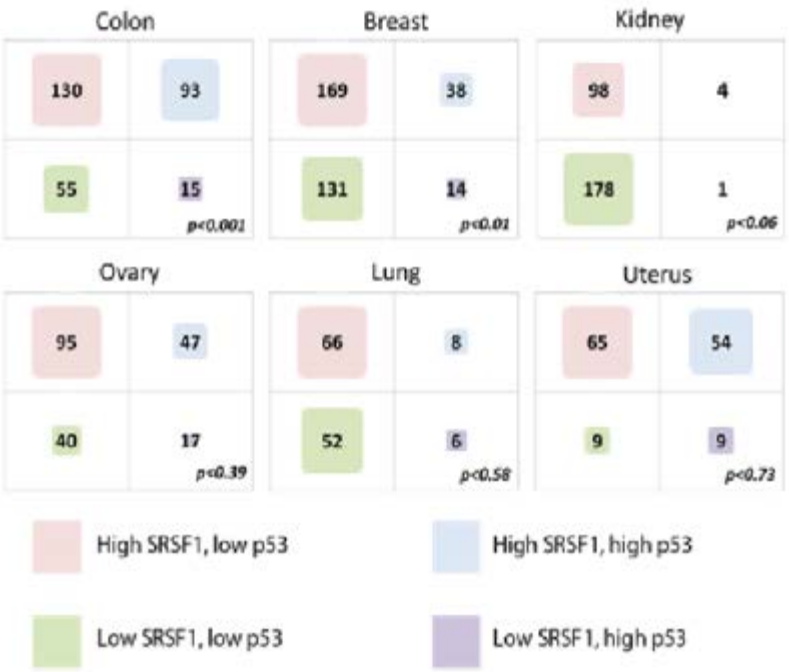

Figure 3.8 SRSF1 expression inversely correlates with p53 levels in human tumors.

Expression of *SRSF1* and *TP53* were profiled from microarray data from a collection of human tumors (GSE2109). The data were normalized to Z-score (see Supplemental Experimental Procedures) and divided into categories corresponding to tumors expressing high or low *SRSF1* or *TP53* levels. The plot shows the distribution of the tumors for each condition. The size of the squares is proportional to the number of observations. Fisher test P-values are shown at the bottom.

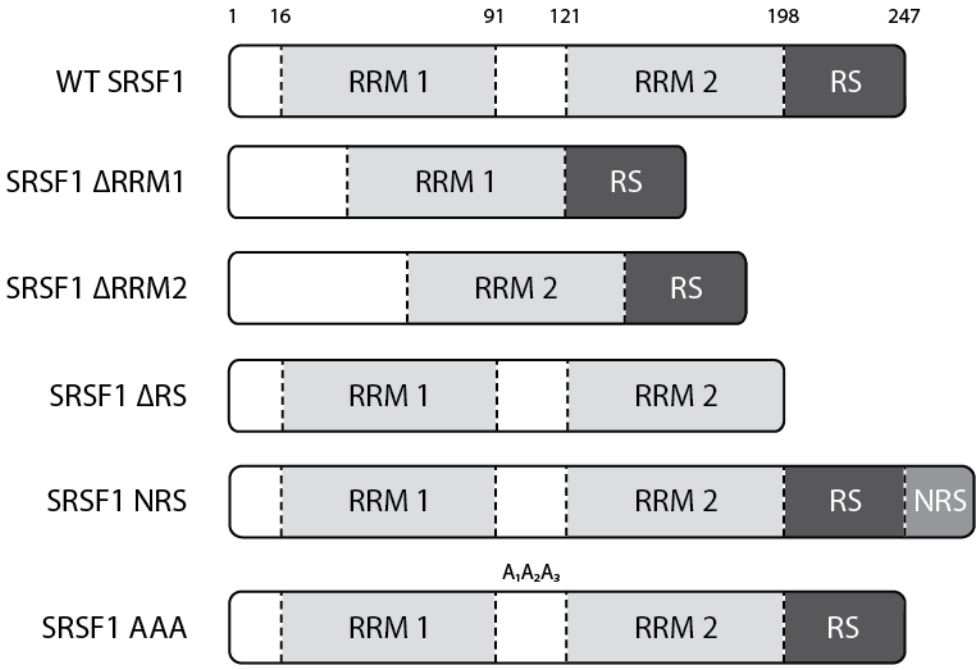

Figure 3.9 Schematic representation of wild-type SRSF1, deletion mutants lacking either RRM1, RRM2, or the RS domain, NRS1 construct consisting of a C-terminal fusion to a nuclear retention signal from SRSF2, and the AAA mutant.

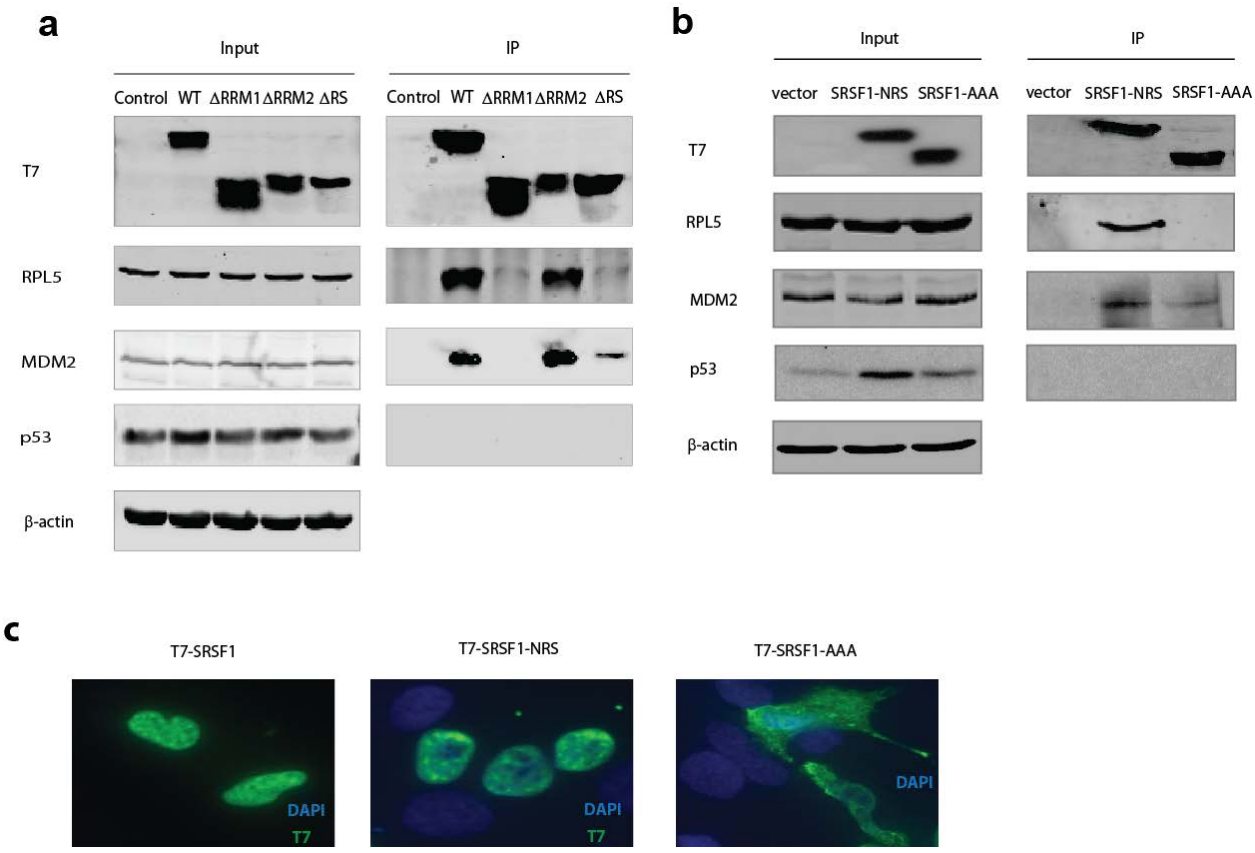

Figure 3.10 Nuclear localization and RRM1 domain of SRSF1 are required for interaction with RP-MDM2.

(a) HeLa cells were transfected with wild-type SRSF1 and domain-deletion mutants. Lysates were immunoprecipitated with T7 monoclonal antibody, with nuclease treatment. Whole-cell lysates (Input) and IPs were analyzed by immunoblotting with the indicated antibodies. **(b)** Lysates from HeLa cells transfected with T7-SRSF1-NRS or T7-SRSF1-AAA mutant construct were immunoprecipitated with T7 monoclonal antibody. Whole-cell lysates (Input) and Immunoprecipitate (IP) were analysed by immunoblotting with the indicated antibodies. **(c)** Immunofluorescence staining using anti-T7 monoclonal antibody to show the localization of T7-tagged SRSF1-NRS or SRSF1-AAA mutant proteins, transfected into HeLa cells. DAPI was used to stain the nucleus.

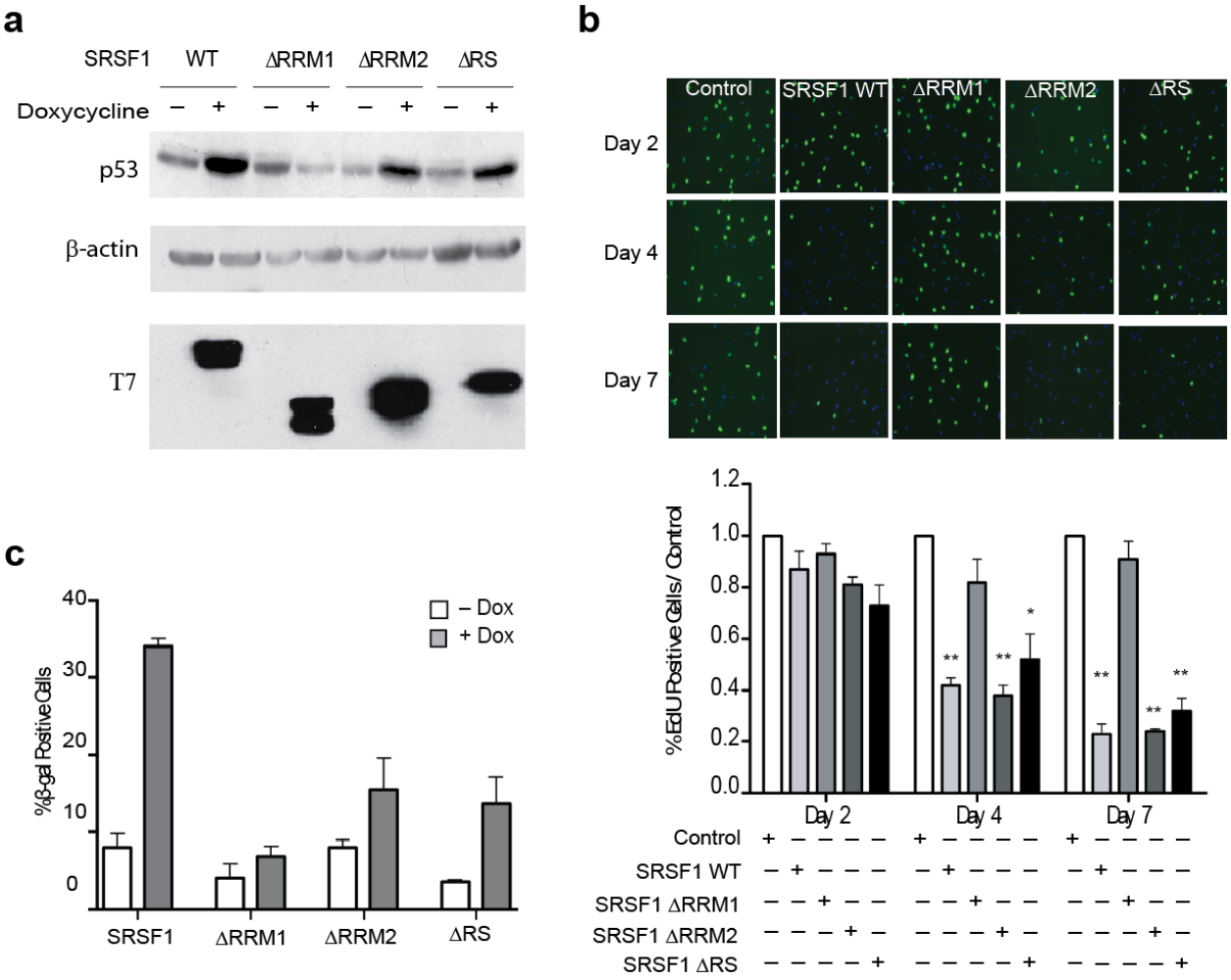

Figure 3.11 RRM1 domain of SRSF1 is required for p53 induction and cell-cycle arrest.

(a) BJ TT7-SRSF1, BJ TT7-SRSF1-ΔRRM1, BJ TT7-SRSF1-ΔRRM2 and BJ TT7-SRSF1-ΔRS cells were treated with doxycycline for 36 h and analyzed by immunoblotting, as indicated. **(b)** BJ TT7-SRSF1, BJ TT7-SRSF1-ΔRRM1, BJ TT7-SRSF1-ΔRRM2 and BJ TT7-SRSF1-ΔRS cells were induced with doxycycline for 2, 4, or 7 d, treated with 10 μM EdU, and observed at 20x magnification.and incubated with 10 μM EdU. One hundred cells were counted for each condition; n=3. Means +/-s.d. are shown, **P* = 0.05, ***P* = 0.005. **(c)** BJ TT7-SRSF1, BJ TT7-SRSF1-ΔRRM1, BJ TT7-SRSF1-ΔRRM2 and BJ TT7-SRSF1-ΔRS cells were treated with doxycycline for 7 d, fixed, and stained with X-gal. 200 cells were counted for each condition; n=2. Ranges are shown.

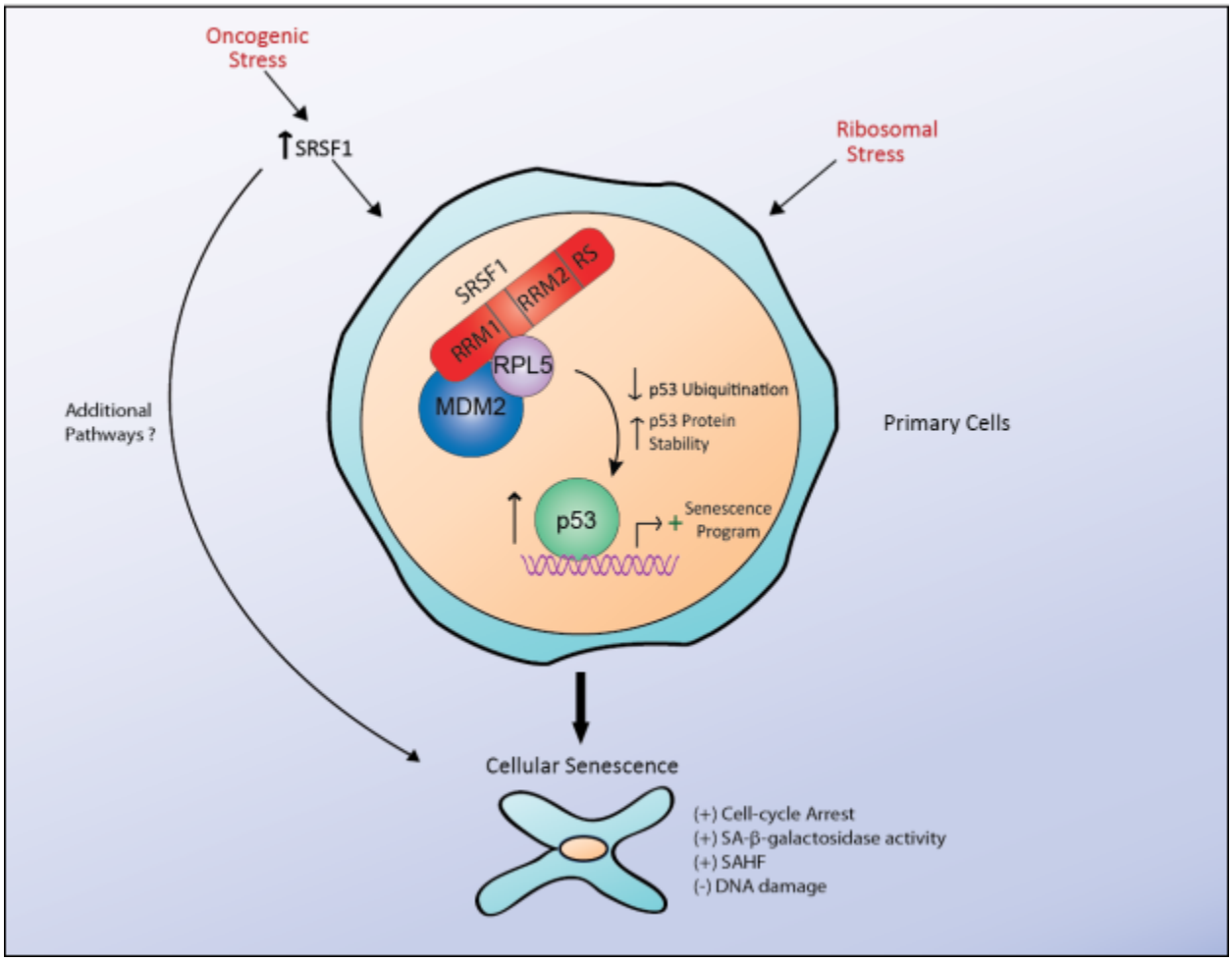

Figure 3.12 A model for SRSF1's Role in Ribosomal-stress Pathway and Oncogene-Induced Senescence.

We have identified SRSF1 as a critical component of the RP-MDM2 complex, which is formed in response to induction of ribosomal stress. Sequestration of the E3 ligase MDM2 in this complex results in decreased ubiquitylation and increased stability of the tumor-suppressor p53 protein. Moreover, we have identified and characterized an anti-tumorigenic response that primary cells mount in response to overexpression of the SRSF1 oncoprotein, which triggers the formation of a nuclear ternary SRSF1-RPL5-MDM2 complex, leading to activation of the p53-mediated tumor-suppressive pathway and OIS.

Chapter 4 : Concluding Remarks

The splicing factor SRSF1 is a proto-typical member of the SR protein family. Since its discovery, SRSF1 has been implicated in the regulation of numerous biological processes. Furthermore, SRSF1 is a potent proto-oncogene and is frequently over-expressed in a variety of tumors. Though SRSF1 amplification is reported in a sub-set of tumors, very little was known about the processes and factors regulating SRSF1 expression and over-expression in cancer.

During the course of my thesis dissertation, I identified the oncogenic transcription factor MYC as a regulator of SRSF1 expression. Expression of MYC positively associated with SRSF1 expression in a panel of human lung, breast and colon tumors. The *SRSF1* promoter contains two non-canonical MYC binding sites that work together to positively regulate SRSF1 expression upon MYC binding. Interestingly, I found SRSF1 upregulation by MYC to be an important contributor to MYCs own oncogenic activity. Neutralizing SRSF1 induction led to a significant decline in the growth rate and anchorage-independent growth of MYC overexpressing rat fibroblasts.

These findings add a new dimension to our current understanding of MYCs activity in tumorigenesis. My study shows that we can now expand the potential mediators of MYCs oncogenic activity to include targets that are alternatively spliced in response to MYC-mediated differential regulation of splicing factors such as SRSF1. This is promising since it provides us with additional MYC targets that can have significant therapeutic potential. Modulating the altered splicing profile of such targets using anti-sense oligonucleotide technology can prove to be an effective anti-tumorigenic approach. Identifying the altered splicing profiles in cells and tumors with aberrant MYC using high-throughput deep sequencing will therefore be very informative in understanding and potentially treating MYC-driven tumors.

In addition to dissecting in part the transcriptional regulation of SRSF1, I also identified a tumor-protective mechanism cells adopt to protect themselves against aberrant SRSF1 overexpression. SRSF1 was found to be a key regulator of the ribosomal stress response pathway. Upon induction of ribosomal stress, SRSF1 associates with the ribosomal protein RPL5 and together they bind and sequester the E3 ubiquitin ligase MDM2. This results in the decreased ubiquitylation and increased stability of the primary MDM2 target, the tumor-suppressor transcription factor p53.

I also identified a novel pathway of Oncogene-induced senescence wherein stabilized p53 upon SRSF1 overexpression in primary fibroblasts results in cell-growth arrest and cellular senescence. We believe this to be a tumor-protective mechanism adopted by primary cells to protect themselves against overexpression of oncogenic *SRSF1*. It is therefore intuitive that tumors driven by SRSF1-overexpression would have a disabled p53-mediated tumor suppressive pathway. Reactivation of p53 expression or activity can thus serve as an effective barrier against tumors with SRSF1 overexpression. This further emphasizes the need to develop effective therapeutic strategies aimed at restoring p53 function to SRSF1 overexpressing tumors.

A significant number of tumors are characterized by mutations in the *TP53* gene. While initially these mutations were thought to deactivate wild-type p53 function, it is increasingly becoming evident that most of these mutations are mis-sense mutations that result in production of mutant p53 protein that actually have pro-oncogenic activity. Contrary to wild-type p53, oncogenic p53 isoforms are very stable; however exact mechanisms that contribute to their higher stability are as yet unknown. Since SRSF1 overexpression stabilizes the p53 protein, an intriguing possibility is that tumors overexpressing SRSF1 carry mutant p53 which protect the cells against SRSF1-induced senescence. In turn, SRSF1 can be an important contributor to increased stability of mutant p53 protein. SRSF1 and oncogenic mutant p53 can therefore show oncogenic cooperation resulting in a more aggressive tumorigenic phenotype. Investigating the status of *TP53* in SRSF1 overexpressing human tumors can shed light on this, and can be used as a prognostic marker.

In conclusion, my thesis research has further expanded our current knowledge of the role and significance of *SRSF1* as an oncogene, in addition to identifying its novel function in the ribosome-stress pathway and as a mediator of OIS. It would be interesting to expand the current study to other SR proteins, and determine whether these functions are a characteristic of the whole family or are more specific to one or a few members.

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Chapter 1

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