CHAPTER 7

SR Proteins and Related Factors in Alternative Splicing

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Abstract

SR proteins are a family of RNA binding proteins that contain a signature RS domain enriched with serine/arginine repeats. The RS domain is also found in many other proteins, which are collectively referred to as SR-related proteins. Several prototypical SR proteins are essential splicing factors, but the majority of RS domain-containing factors are characterized by their ability to alter splice site selection in vitro or in transfected cells. SR proteins and SR-related proteins are generally believed to modulate splice site selection via RNA recognition motif-mediated binding to exonic splicing enhancers and RS domain-mediated protein-protein and protein-RNA interactions during spliceosome assembly. However, the biological function of individual RS domain-containing splicing regulators is complex because of redundant as well as competitive functions, context-dependent effects and regulation by cotranscriptional and post-translational events. This chapter will focus on our current mechanistic understanding of alternative splicing regulation by SR proteins and SR-related proteins and will discuss some of the questions that remain to be addressed in future research.

Introduction

SR proteins were discovered in the early 1990s by the identification of factors associated with purified spliceosomes,[1,2] by the purification of critical non-snRNP splicing activities in constitutive and alternative splicing,[3-6] and by the analysis of components of a nuclear body that could be selectively precipitated with Mg^{++}.[7] By virtue of its ability to complement splicing-deficient S100 cytoplasmic extracts from HeLa cells and to stimulate splice site switching in HeLa nuclear extracts, SF2/ASF was the first SR protein shown to have dual roles in constitutive and alternative splicing.[3,4,6,8] This observation was quickly extended to other SR proteins.[9-11] The S100 complementation and splice site switch assays have since become standard functional tests for SR proteins isolated from higher eukaryotic organisms.

Sequence analysis has revealed that SR protein family members consist of one or two RNA recognition motifs and a signature RS domain enriched with serine/arginine repeats.[12,13] These structural features have been commonly used to classify SR proteins. Clearly, not all SR proteins behave like prototypical SR proteins. For example, a subset have different fractionation properties and/or are not sufficient to complement S100 extracts. In addition, several new SR protein family members exhibit activities in both constitutive and alternative splicing that are opposite to those possessed by prototypical SR proteins. Because of the functional diversity among SR proteins, we propose to define SR proteins based on their common structural features including at least one RNA recognition motif and an RS domain. Using this classification, several RS

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domain-containing RNA binding proteins, including human TRA2β and RNPS1, can now be classified as SR proteins (Table 1).

In addition to SR proteins, many other splicing factors contain an RS domain. These proteins are collectively referred to as SR-related proteins.[14] In mammalian cells, SR-related proteins include other RNA binding proteins, such as both subunits of the U2AF heterodimer, the U1 snRNP specific protein U1-70K and various enzymes, including several ATPases involved in RNA rearrangement within the spliceosome[15-18] (Table 1). It is generally thought that the RS domains in SR proteins and SR-related splicing factors facilitate spliceosome assembly by mediating protein-protein interactions.[19] However, recent studies have revealed direct binding of the RS domain to critical splicing signals in pre-mRNA transcripts.[20,21]

Interestingly, budding yeast express a few RNA binding proteins that structurally resemble SR proteins.[22] However, there is no direct evidence that these proteins are essential pre-mRNA processing factors in this organism and it is interesting to note in this context that ~5% of the genes in budding yeast contain a single intron and alternative splicing is rare. Therefore, splicing can take place in the absence of SR proteins, which begs the question as to why SR proteins are essential splicing factors in higher eukaryotic cells. The differential requirement for SR proteins in yeast and higher eukaryotic cells probably reflects the fact that the splicing signals in yeast pre-mRNAs are essentially invariant, whereas those in mammals are diverse. Thus, the RS domain in SR proteins may function to strengthen the recognition of weak splicing signals, as has been

Table 1. SR proteins and SR-related splicing regulators

Classification	Factors	Key Domains	Functions
Classic SR Proteins	SRp20,[1] SF2/ASF,[2] SC35,[3] 9G8,[4] SRp40,[5] SRp55/B52,[6] SRp75[7]	One or two RRMs plus an RS domain	Constitutive and alternative splicing
Additional SR proteins	hTRA2α,[8] hTRA2β,[9] RNPS1,[10] SRp38,[11] SRp30c,[12] p54,[13] SRrp35,[14] SRrp53,[15] SRp86[16]	One or two RRMs plus an RS domain	Positive and negative regulation of alternative splicing
RNA binding SR related factors	U2AF65,[17] U2AF35,[18] Urp,[19] HCC1/CAPER,[20] U1-70K,[21] hSWAP,[22] Pinin,[23] Sip1,[24] SR-A1,[25] ZNF265,[26] SRm160,[27] SRm300,[28]	RRM, PWI domain, Zn finger plus an RS domain	Splicing factors or co-activators
Enzymes and regulators carrying an RS domain	hPRP5,[29] hPRP16,[30] Prp22/HRH1,[31] U5-100K/ hPRP28,[32] ClkSty-1,[33] 2,[34] 3,[35] CLASP,[36] Prp4K,[37] CrkRS/CRK7/CDK12,[38] CDC2L5,39 CCNL1,[40] CCNL2,[41] SR-cyp,[42]	DEAH box, kinase domains, peptidyl-prolyl isomerase domain	Spliceosome rearrangement and modification of splicing factors

Key literature information and protein sequence for each gene can be found by individual NCBI accession number: [1]NP_003008 [2]NP_008855 [3]NP_003007 [4]NP_001026854 [5]NP_008856 [6]NP_006266 [7]NP_005617 [8]NP_037425 [9]NP_004584 [10]NP_542161 [11]NP_473357 [12]NP_003760 [13]NP_004759 [14]NP_542781 [15]NP_057709 [16]NP_631907 [17]NP_009210 [18]NP_006749 [19]NP_005080 [20]NP_909122 [21]NP_003080 [22]NP_008987 [23]NP_002678 [24]NP_004710 [25]NP_067051 [26]NP_976225 [27]NP_005830 [28]NP_057417 [29]NP_055644 [30]NP_054722 [31]NP_004932 [32]NP_004809 [33]NP_004062 [34]NP_003984 [35]NP_003983 [36]NP_008987 [37]NP_003904 [38]NP_057591 [39]NP_003709 [40]NP_064703 [41]NP_112199 [42]NP_004783

recently documented.[23] In addition, SR proteins are critical for pairing complexes assembled on the 5' and 3' splice sites. This functional requirement may not be critical for splicing in yeast, where introns are relatively short and the communication between splice sites may not require RS domain-mediated interactions during splicing assembly.

The Role of SR Proteins in Splice Site Selection

Prototypical SR proteins, such as SC35, SF2/ASF and 9G8, are required to initiate spliceosome assembly in nuclear extracts. This early function of SR proteins is mediated by their sequence-specific binding to cis-acting elements, which are mostly located in exons and functionally characterized as exonic splicing enhancers (ESEs). The binding specificity of individual SR proteins has been experimentally defined using a technique called SELEX, based either on in vitro binding[14,25] or on the functional consequence of in vitro splicing.[26-28] The ESEs characterized to date have been used to develop an ESE-finder program[29] to assist with the identification of potential cis-acting regulatory elements in pre-mRNAs. While the program is a useful guide for searching for cis-acting regulatory elements in various pre-mRNAs, the information derived is preliminary for several reasons. First, similar analyses have not been extended to other SR proteins. Second, many ESEs may be recognized by non-SR proteins. Third, some complex ESEs may require the action of more than one RS domain-containing splicing factor, as observed in the *Drosophila doublesex* pre-mRNA.[30,31] Consequently, the vast majority of computationally deduced and/or experimentally verified ESEs remain to be characterized with regards to the specific trans-acting factors involved.[32-36] Furthermore, it is unclear as to why SR proteins generally do not bind to intronic sequences that resemble ESEs. An interesting possibility is that SR proteins may bind to all potential sites in an initial scanning mode before stabilization at specific functional ESEs via their interactions with other splicing factors that promote spliceosome assembly.

Two non-exclusive models have been proposed to explain the functional consequence of initial SR protein binding to an ESE (Fig. 1). One model emphasizes the effect of ESE-bound SR proteins on the recruitment and stabilization of additional splicing factors, such as U1 at the 5' splice site[37-39] and the U2AF complex at the 3' splice site.[40-43] Both SR proteins and RS domain-containing splicing co-activators have been implicated in promoting communication between the 5' and 3' splice sites.[44-49] The second model stresses the role of ESE-bound SR proteins in preventing or displacing other RNA binding proteins, such as hnRNP A1, from binding at exonic splicing silencers (ESSs).[50,51] These two mechanisms are likely operating in a synergistic fashion to favor spliceosome assembly on functional splice sites.

The early function of SR proteins in splice site recognition is probably similar in both constitutive and alternative splicing. Based on in vitro analysis of several prototypical SR proteins in alternative splicing, binding of SR proteins promotes the selection of proximal sites over distal ones in alternative 5' or 3' splice site choices.[8,9,52,53] In such processes, splice site selection may be dictated by the intrinsic strength of the competing splice sites and/or the frequency of competing exonic splicing silencer (ESS) sequences.[54] SR protein binding may enhance complex assembly on both strong and weak splice sites to make them equally competitive.[55] The proximal site is then selected because of the insulating function of SR proteins, allowing the closest pair of splice sites to be linked in later spliceosome assembly events[56] (Fig. 1). This insulating function may play a critical role in preventing exon skipping during the removal of multiple introns in a pre-mRNA transcript.

The ability of SR proteins to bind RNA is essential for the activity of SR proteins in both constitutive and alternative splicing.[57-59] In contrast, the RS domain seems to be important for constitutive splicing, but dispensable in alternative splicing, at least for the small number of pre-mRNA substrates analyzed.[58,60] The reason why the RS domain is not required for alternative splicing is not completely understood. It is possible that SR proteins lacking the RS domain may be sufficient to compete with the binding of negative splicing factors to adjacent splicing silencer sequences.[50,51] Given the fact that the dispensability of the RS domain in alternative splicing has only been tested with a limited number of alternative splicing substrates, it remains possible that certain alternative splicing events may require the domain to promote the selection of weak splice sites.

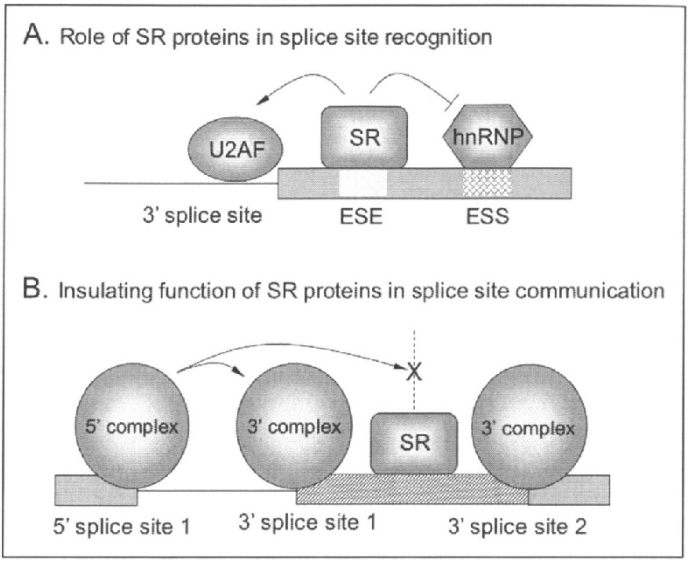

Figure 1. Role of SR proteins in splice site selection. A) An ESE-bound SR protein may stimulate complex assembly at a nearby functional splice site and/or antagonize the negative effect of an hnRNP protein on spliceosome assembly. B) An insulating function of SR proteins may promote the selection of the proximal splice site and prevent the use of the distal splice site.

SR Proteins Modulate Alternative Splicing in Both Ways

As described above, SR proteins seem to promote exon inclusion and the selection of intron-proximal splice sites over distal ones. However, further studies indicate that different SR proteins may influence splice site selection in both positive and negative fashions. Three distinct mechanisms by which SR proteins negatively modulate splice site selection have been reported in the literature (Fig. 2). SR proteins may recognize some intronic sequences that resemble ESEs, therefore resulting in the activation of an intronic cryptic splice site at the expense of a native splice site[61] (Fig. 2A). Mechanistically, this mode of negative regulation is similar to the activity of SR proteins in promoting the selection of a proximal, weak splice site in competition with a strong, distal one.

SR proteins may be actively involved in suppressing splice sites in a substrate-dependent manner. This was observed in SR knockout cardiomyocytes, where loss of SF2/ASF induced exon inclusion in the alternatively spliced CaMKIIδ gene.[62] While the direct effect of SR proteins in CaMKIIδ exon skipping event remains to be confirmed by in vitro analysis, a more recent study demonstrated that SF2/ASF acted on an ESE to promote exon skipping in the *Ron* proto-oncogene.[63] Similarly, SRp30c was found to suppress splice site selection of an alternative exon in the hnRNP A1 gene.[64] While the mechanism for these SR protein-dependent exon skipping events remains elusive, the phenomenon may be related to a number of earlier observations that different SR proteins appear to have opposite effects on regulated splicing.[65-69] In these cases, different SR proteins may act on their respective cis-acting elements to antagonize each other, thereby influencing the final choice of alternative splice sites. The opposite effects observed with different SR proteins may be due to the possibility that some SR proteins are more productive in promoting splice site selection than others, such that less productive SR proteins may interfere with productive ones in a competitive manner (Fig. 2B). Furthermore, it was recently shown that the positive and negative effects may be also related to the location of SR protein binding site with respect to splice sites.[70]

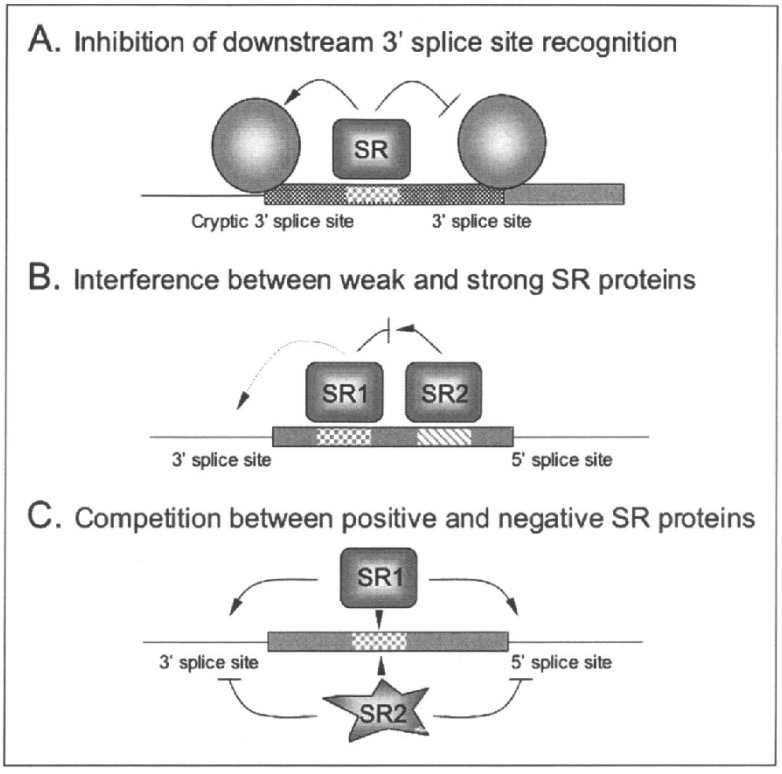

Figure 2. Positive and negative effects of SR proteins on splice site selection. A) An SR protein may bind to an intronic sequence resembling an ESE, thereby activating an upstream cryptic 3' splice site and inhibiting the use of the normal, downstream 3' splice site. B) The function of an ESE-bound SR protein (SR2) may be blocked by another ESE-bound SR protein with a weaker activity in splicing activation. C) The same cis-acting ESE may be recognized by both positive and negative SR proteins.

Aside from the substrate-dependent effects of typical SR proteins, some SR proteins appear to only function in splicing in a negative fashion (Fig. 2C). The best characterized example is SRp86, which appears to antagonize typical SR proteins in splice site selection.[71-73] Likewise, the SR protein p54, which was initially identified as a U2AF65-interacting protein, promotes the selection of an intron-distal splice site in the E1A pre-mRNA.[74] In a recent functional screen using a tau-based alternative splicing reporter, p54 was found to compete with hTRA2β for binding to an ESE and to promote exon skipping.[75] Joining this list of "negative" SR proteins are two new SR-related RNA binding proteins, SRrp35 and SRrp40 (also known as NSSR, TASR or SRp38), which should be classified as SR proteins.[76] SRp38 was isolated as an alternative splicing regulator in several independent studies.[76-79] Interestingly, SRp38 normally seems to have little activity in splicing. However, following heat shock and during cell mitosis, dephosphorylation of the RS domain of SRp38 results in a strong inhibitory effect on splicing.[80,81] However, when the RS domain of SRp38 was linked to an MS2 binding site or to the RNA recognition motif (RRM) of a typical SR protein, the hybrid protein appeared to act as a typical splicing activator, like other SR proteins.[79,82] Thus, both the RNA binding activity and the phosphorylation state of its RS domain contribute to the inhibitory effect of SRp38 on splicing.

How Do SR-Related Splicing Factors Regulate Alternative Splicing?

In the past, SR-related alternative splicing regulators were often referred to as mammalian homologues of splicing regulators identified in *Drosophila*, such as hTRA2α and hTRA2β[83]. Because these splicing factors can be classified as SR proteins, we will focus our discussion on the other RS domain-containing splicing factors listed in Table 1. One example is the U2AF heterodimer, which is comprised of U2AF65 and U2AF35. These proteins are structurally related to SR proteins, but have distinct features: U2AF65 contains an N-terminal RS and three RRMs, whereas U2AF35 carries a C-terminal RS domain, but no RRM. The U2AF heterodimer is believed to play a critical role in the definition of 3' splice site selection in both constitutive and alternative splicing. Indeed, recent RNAi knockdown studies showed that the U2AF heterodimer is directly involved in regulating splicing in both *Drosophila* and human cells.[84,85] Unlike SR proteins, however, U2AF does not seem to affect 3' splice site choice in a dosage dependent manner. Instead, the U2AF heterodimer appears to be the target for replacement by other polypyrimidine tract binding proteins, such as Sxl in *Drosophila*[86] or PTB in vertebrates.[87-89]

Besides U2AF, a growing number of RS domain-containing proteins have been implicated in alternative splicing, including the mammalian homologue of suppressor-of-white-apricot[90] and a large Zn-finger protein ZNF265[91] (Table 1). Interestingly, several kinases, such as Clk/Sty,[92,93] CrkRS,[94] Prp4K,[95] and CDC2L5,[96] and the regulator subunits cyclin L1[97,98] and L2,[99,100] also contain an RS domain. While these kinases have exhibited effects on alternative splicing in transfected cells, only Clk/Sty is known to target and directly phosphorylate SR proteins. These kinase systems have the potential to link signal transduction pathways to regulated splicing in mammalian cells.

A recent large-scale RNAi screen found, surprisingly, that constitutive splicing factors are also capable of altering the splice site choice. Among these unexpected alternative splicing regulators are the ATPase Prp5 and Prp22,[85] the mammalian homologues of which carry an extra RS domain.[15,18] This finding is surprising because regulation of alternative splicing has been generally thought to take place in early stages of spliceosome assembly and these ATPases are known to act during the splicing reaction after the spliceosome is fully assembled. However, a more recent kinetic study demonstrated that, despite the fact that splice sites are paired in the absence of ATP, they are flexible and exchangeable within the E complex until they are locked in the A complex in the presence of ATP.[101] Thus, many factors that act after spliceosome assembly may still be capable of functioning as regulators in alternative splicing. This finding is consistent with the role of Prp5, Prp22 and other "late" splice factors in regulated splicing. The recent recognition of the dynamic nature of the spliceosome provides a conceptual framework for understanding how many known factors for constitutive splicing show an ability to modulate alternative splicing.[102]

Functional Requirement of SR Proteins In Vivo

While regulated splicing was initially recognized and extensively studied by genetics in the *Drosophila* system, most concepts and mechanistic insights into the regulation of alternative splicing by SR proteins and SR-related proteins have been based on biochemical analysis in vitro or in transfected cells. It is therefore important to test and extend the biochemical studies to in vivo systems. To this end, the RNAi approach has been used to determine the role of SR proteins in *C. elegans*.[103] Strikingly, most SR protein knockdowns resulted in no detectable phenotype, except for a late embryonic lethal phenotype induced by RNAi against SF2/ASF. These findings suggest an extensive functional overlap among the SR family of splicing factors in this model organism. A more extensive RNAi screen performed in *Drosophila* S2 cells revealed the role of several SR proteins and SR-related splicing factors in alternative splicing.[85] Although the RNAi approach has been applied to mammalian cells to demonstrate specific requirements of SR proteins in alternative splicing,[104,105] a similar systematic undertaking remains to be extended to the mammalian system where regulated splicing may be more dynamic and thus more complex.

Complementary to the RNAi approach, gene targeting in chicken DT40 cells and in mice has permitted the analysis of SR proteins in vivo. A study performed on SF2/ASF knockout DT40 cells revealed that SF2/ASF is required for cell viability,[106] has an unexpected role in maintaining genomic

stability,[107] and has a regulatory function in DNA fragmentation during apoptosis.[108] At least one of these in vivo functions (DNA fragmentation) was linked to SF2/ASF-regulated alternative splicing.[108] These studies have significantly extended our understanding of SR proteins in vivo.

So far, all SR protein knockout mice studied to date have shown an early embryonic lethal phenotype, thus demonstrating the fundamental function of SR proteins in vivo.[62,109,110] Surprisingly however, SC35 seems to be dispensable in nondividing mature cardiomyocytes, indicating that SR proteins are not universally required for cell viability in vivo.[111] This observation is in agreement with an RNAi result in *C. elegans*.[103] Importantly, specific alternative splicing events have been directly linked to some defined phenotypes in SC35 and SF2/ASF knockout mice, showing that SR proteins are indeed regulators of alternative splicing in mammalian cells.

Interestingly, an SF2/ASF mutant lacking the RS domain could rescue cell viability in SF2/ASF-depleted mouse embryonic fibroblasts.[112] Because the RS domain in SF2/ASF is required for constitutive splicing but dispensable in alternative splicing in most cases, this observation suggests that most cellular malfunctions might result from defects in alternative splicing. This possibility is consistent with the studies of the SF2/ASF orthologue in *Drosophila*, in which dASF appeared to lack any activity in constitutive splicing, but functioned as a regulator in alternative splicing.[113] Furthermore, the global pattern of gene expression was not dramatically altered in SR protein-depleted cells, indicating that inactivation of individual SR proteins may not cause widespread defects in constitutive splicing.[111,114]

SR Proteins as Splicing Regulators In Vivo: Why So Few Targets?

Members of the SR family of splicing factors are among the bestcharacterized splicing regulators and have been extensively studied by biochemical analysis. One surprising finding from the study of SR protein knockout cells was that most splicing events (both constitutive and alternative) remained unaltered in response to depletion of individual SR proteins in vivo. This result has been assumed to be due to functional redundancy among SR proteins, which may be explained by two potential mechanisms (Fig. 3). First, more than one SR protein may be able to recognize

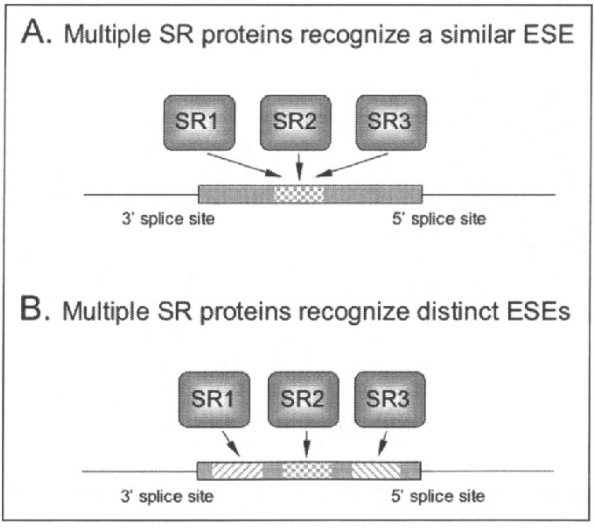

Figure 3. Potential functional redundancy of SR proteins. A) Multiple SR proteins may recognize the same ESE in a pre-mRNA. B) Multiple SR proteins may interact with several distinct ESEs in a pre-mRNA. As a result, deficiency of a single SR protein may have little effect on most constitutive splicing events.

a similar set of ESEs present in most exons; this has been observed in vitro with SF2/ASF and hTRA2β, which are both are capable of recognizing purine-rich ESEs.[25,61,83,115] Second, most exonic sequences appear to harbor multiple ESEs that are responsive to distinct SR proteins,[116] which may act independently or in a synergistic manner.[31,117] As a result, many splicing events may be responsive to SR protein overexpression, but relatively insensitive to down regulation or depletion of a single SR protein. Overexpression of SR proteins may exert a dominant effect on exons containing related ESEs. Therefore, caution must be taken in interpreting overexpression results in transfected cells, in which an affected splicing event may not be the natural substrate for the SR protein under study. This problem can be addressed by comparing results from both overexpression and RNAi knockdown studies.

According to the theory of functional redundancy, one might expect a more prevalent effect of SR protein depletion on alternative splicing versus constitutive splicing in vivo, since alternative splicing is often coupled with weak splice sites in conjunction with specific ESEs.[33] In this regard, alternative splicing would be more dependent on individual ESEs and thus more sensitive to variations in SR protein expression. As a result, SR proteins may be collectively essential, but individually dispensable for constitutive splicing in most cases. On the other hand, individual SR proteins may each control a defined spectrum of substrates via weak splice sites coupled with ESEs and these substrates may be limited in type or in number. Therefore, SR proteins may function as alternative splicing regulators in vivo more extensively than previously appreciated. The challenge is in identifying key alternative splicing events involving specific SR proteins and to link these molecular alterations to defined biological phenotypes.

Regulation of SR Splicing Regulators

SR proteins and SR-related splicing factors are direct effectors in alternative splicing and are likely subject to regulation at the transcriptional and post-translational levels. Additional regulation likely takes place in response to cell signaling events. Regulation of SR proteins and other splicing regulators by signaling is reviewed in the chapter by Lynch in this book. Accordingly, we will focus our discussion on how alternative splicing may be achieved by regulating the SR family of splicing factors. While SR and SR-related proteins are ubiquitously expressed in most tissues and cell types, differential expression of SR proteins has been reported in certain tissues and cell types in response to signaling.[118-122] In general, however, little is known about how SR proteins are regulated at the transcriptional level and about the functional consequences of such regulation on specific alternative splicing events in specific biological pathways. SR proteins have also been found to be auto-regulated or regulated in trans by other SR proteins at the level of alternative splicing.[123-126] These regulatory mechanisms may help maintain homeostasis of SR protein expression in most cell types.

SR proteins are extensively modified by phosphorylation in their RS domains. Several early studies indicated that phosphorylation was essential for SR proteins to function in spliceosome assembly and that dephosphorylation was critical for RNA catalysis within the spliceosome.[127-129] Phosphorylation and dephosphorylation are both required,[130] because it was found that experimental induction of SR protein hyper- and hypo-phosphorylation impaired splicing.[92] However, mutations that mimic hyper- and hypo-phosphorylation of a single SR protein, such as substitution of RS repeats by RE or RG dipeptides in the RS domain, still supported splicing in vitro and complemented SR protein-depleted cells for viability.[112,131] This is likely because a full phosphorylation/dephosphorylation cycle does not have to occur in a single SR protein for each round of the splicing reaction.[129] For instance, a splicing reaction can be accomplished by using a thio-phosphorylated (phosphatase-resistant) SR protein to stimulate initial spliceosome assembly and using another dephosphorylatable SR protein to complete later steps in the splicing reaction.

Because the activity of SR proteins in constitutive splicing is clearly modulated by phosphorylation, it is conceivable that regulated phosphorylation may have a profound influence

on alternative splicing. Indeed, overexpression or inhibition of an SR protein-specific kinase has been shown to modulate splice site selection.[97,132-135] The activation of various signal transduction pathways has also been shown to affect alternative splicing via, at least in part, differential phosphorylation of SR proteins.[136,137] However, we are far from understanding how SR protein phosphorylation might affect the activity of SR proteins in constitutive and regulated splicing. While phosphorylation of the RS domain is generally believed to prevent SR proteins from non-specific binding to RNA, the impact varies with respect to RS domain-mediated protein-protein interactions that enhance the interaction in certain cases and suppress the interaction in others.[138,139] Importantly, it is essentially unknown as to which proteins are actually engaging in the interaction with the RS domain of an SR protein within the spliceosome and how such interactions might be influenced by phosphorylation. Moreover, SR proteins are phosphorylated at multiple sites in their RS domains.[140] It is currently unclear whether the activity of SR proteins might be affected by phosphorylation in a context or site-specific manner. Finally, phosphorylation has been shown to regulate the localization of SR proteins[95,141-143] and their recruitment to the transcriptional machinery has been shown to facilitate cotranscriptional splicing in the nucleus.[139,144,145] Because SR proteins are known to affect alternative splicing in a dosage-dependent manner, the impact of phosphorylation on the availability (localization) and targeting efficiency (recruitment) of SR proteins may contribute to the complex pattern of alternative splicing in mammalian cells.

One approach to investigate the regulation of splicing by phosphorylation is to identify and characterize specific kinases and phosphatases involved in the process. To date, several protein kinases have been implicated as SR protein kinases, including SRPKs,[141,143] Clk/Sty,[93,132,134] and Akt.[136,137] The family of SRPK and Clk/Sty kinases catalyzed phosphorylation of SR proteins in multiple sites in the RS domain, but with different substrate specificity.[140,146] It is important to emphasize the fact that these kinases were mostly identified by in vitro kinase assays and their effect on splicing, if any, was only tested in transfected cells. Genetic evidence will be required to firmly establish the enzyme-substrate relationship for all of the reported SR protein kinases. In *Drosophila*, a Clk/Sty-related kinase has been shown to phosphorylate endogenous SR proteins and more importantly, mutations in the kinase altered the sex determination pathway.[147] The SRPK family of kinases was initially identified based on their ability to alter the localization of SR proteins in interphase cells as well as during cell mitosis.[141,143,148] A recent RNAi study showed a major impact of SRPK1 depletion on SR protein phosphorylation in vivo.[149] These observations provide genetic evidence for the involvement of these kinases in SR protein phosphorylation in vivo; how these kinases are involved in the regulation of alternative splicing is an important subject for future studies.

The action of kinases is often counteracted by phosphatases. Unfortunately, phosphatases specifically involved in SR protein dephosphorylation are largely unknown. In vitro, both PP1 and PP2A were able to act on SR proteins and activated splicing.[127,128,150,151] Several PP2A family members have been copurified with spliceosomal components.[152] Intriguingly, a recent study demonstrates the essential role of both PP1 and PP2A phosphatases in the second step of splicing, but their main substrates are U2 and U5 snRNP components, instead of SR proteins, indicating that multiple phosphatases are involved in the splicing regulators and those specific for SR proteins remain to be identified and functionally characterized.[153] In particular, because SRp38 is particularly sensitive to dephosphorylation in response to mitotic transitions and heat shock,[81] it will be of great interest to identify the phosphatase(s) responsible and the potential role of these enzymes in regulated splicing. Interestingly, although alternative splicing is not common in budding yeast, a member of the SRPK family of kinases is conserved in the organism and is responsible for phosphorylation of the SR-related RNA binding protein Npl3p.[154] This action is counteracted by the yeast PP1 family phosphatase Glc7p, suggesting that the mammalian counterpart of Glc7c may function as an SR protein-specific phosphatase.[155]

SR Protein-Regulated Splicing in Development and Disease

As splicing is an essential component of gene expression and a key point in expression regulation, splicing defects have been linked to various diseases in humans.[156,157] Given the role of SR proteins and related splicing factors in alternative splicing and cell growth control, they are primary candidates for causing specific disease phenotypes. Available evidence indicates that SR proteins may be involved in development and disease in several ways. First, they may function as critical regulators of disease-causing genes, such as oncogenes or tumor suppressor genes.[62,158,159] Consistent with this possibility, a recent study showed that the alternative splicing of the *Ron* proto-oncogene was subject to regulation by SF2/ASF and the protein product from an alternatively spliced isoform appeared to contribute directly to the invasive behavior of tumor cells.[63] In knockout mice, SF2/ASF was found to play a critical role in the developmental control of CaMKIIδ alternative splicing in the heart, resulting in differential cellular targeting of the kinase and malfunction in Ca^{++} signaling in cardiomyocytes.[62] Because SR proteins affect alternative splicing in a dosage-dependent manner, it is conceivable that altered expression of SR proteins may manifest the effect by changing the alternative splicing pattern of their target genes, thereby causing specific defects in the regulation of cell proliferation and differentiation. Indeed, altered expression of SR proteins and SR protein-specific kinases has been detected in multiple types of cancer.[160-165]

The second way for SR proteins to act in disease pathways lies in their ability to recognize specific point mutations and small deletions directly in disease-causing genes, thereby manifesting the disease phenotype via the mutation-triggered alternative splicing events.[157] One of the best such examples is the disease gene *SMN* in spinal muscular atrophy (SMA). The *SMN* gene is duplicated in the human genome, but the disease phenotype is only associated with molecular defects in the *SMN1* gene.[166] The reason why *SMN2* is insufficient to complement the defective *SMN1* gene in SMA is because of a point mutation in exon 7 in the *SMN2* gene which converts an ESE to an ESS, thereby causing exon skipping to result in a partly defective gene product.[166-169] These findings illustrate how some silent mutations may be linked to specific diseases because of their impact on the regulatory information embedded in the sequence. Therefore, although SR proteins and SR-related splicing factors have not yet been directly mapped as disease genes, they may play a larger role in the expression of specific disease phenotypes than previously anticipated. This may be one of the major tumor selection mechanisms resulting from an unstable genome due to defects, for example, in the DNA repair pathway.

Concluding Remarks

Despite the significant progress that has improved our understanding of alternative splicing mechanisms and the functional consequences of regulated splicing in development and disease, we are still confronted with a large array of challenges, which may be expressed in the following questions: (1) Why do SR proteins generally recognize exonic splicing enhancers, but not similar sequences in introns? (2) Which protein(s) interact with the RS domains of SR proteins during spliceosome assembly? (3) Why is the RS domain differentially required for constitutive and alternative splicing? (4) What is the molecular basis by which some SR proteins act positively and others act negatively on splicing? (5) To what extent do SR proteins share redundant functions in splicing? (6) How do SR proteins cooperate with other splicing RNA binding proteins to regulate alternative splicing? (7) How are SR proteins regulated in vivo and in response to signals? (8) To what extent does the activity of SR proteins in alternative splicing contribute to their functional requirement in development and cell proliferation control? In this chapter, we have speculated on some of these questions based on the available evidence. Additional experiments that address these biological and mechanistic questions are clearly needed to understand the function and regulation of this important class of splicing regulators in development and disease.

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