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Functional Study of SR Splicing Factors in a Cellular Genetic System

A dissertation submitted in partial satisfaction of the  
requirements for the degree Doctor of Philosophy

in

Molecular Pathology

by

Shengrong Lin

Committee in charge:

Professor Xiang-Dong Fu, Chair

Professor Steven F. Dowdy

Professor Mark P. Kamps

Professor Michael G. Rosenfeld

Professor Nicholas J. Webster

2007

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Chair

University of California, San Diego

2007

## DEDICATION

I dedicate this dissertation,

to my mother, Yinghua Pu,  
for bringing me the dream of becoming a scientist;

to my father, Baowei Lin,  
for instilling me his skills as a good engineer;

to my wife, Shuo,  
for her love, the most wonderful thing I have.

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The text of Chapter III, in full, is a reprint of the material as it appears in *Molecular Cell*, Volume 20, pp 413-425. I was the primary researcher and the first author in this publication. The co-authors listed in this publication assisted and collaborated with the research which forms the basis for this chapter. I thank my co-authors Ran Xiao, Peiqing Sun, Xiangdong Xu, and Xiang-Dong Fu for permission to use this publication in my dissertation.

The text of Chapter IV, in part or in full, is a reprint of a manuscript in preparation. I was the primary researcher and the first author in this manuscript. The co-authors listed in this manuscript assisted and collaborated with the research which forms the basis for this chapter. I thank my co-authors Gabriela Coutinho-Mansfield, Dong Wang and Xiang-Dong Fu for permission to use this publication in my dissertation.

The text of Chapter V, in part or in full, is a reprint of the manuscript that has been submitted to *Molecular and Cellular Biology*. I was one of the primary researchers and co-authors in this manuscript. My contributions included derivation of inducible cell lines, characterization of cell growth defect and analysis of gene expression profile. I thank my co-authors Ran Xiao, Ye Sun, Jian-Hua Ding, Dave W. Rose, Michael G. Rosenfeld, Xiang-Dong Fu and Xue Li for permission to use this publication in my dissertation.

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### PUBLICATIONS

**Lin S**, Coutinho-Mansfield G, Wang D, and Fu XD. (2007). The SR Family of Splicing Commitment Factors is Required for Transcriptional Elongation in Mammalian Cells. In preparation.

Xiao R, Sun Y, Ding JH, **Lin S**, Rose D, Rosenfeld MG, Fu XD, and Li X. (2007). Splicing regulator SC35 is essential for genomic stability and cell proliferation during mammalian organogenesis. Submitted.

**Lin S** & Fu XD. (2007). SR Proteins and related factors in alternative splicing.

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Sun Z, Cheng X, **Lin S**, Ying K, Xie Y and Mao Y. (2000). The Alternative Splicing of FE65L2 gene generates 4 isoforms. *Journal of Fudan University* 39, 231-234.

## **ABSTRACTS AND TALKS**

**Lin S**, Xiao R, Li X and Fu XD. SC35 Plays a Critical Role in Cell Cycle Progression by Regulating Alternative Splicing of the p53 Deacetylase Sir2 (Oral Presentation). The 11th Annual Meeting of the RNA Society, Seattle, WA. Jun 2006.

Andersen A, **Lin S**, Fu XD, Panning B. Identification of Xist RNA-binding Proteins (Poster). The 11th Annual Meeting of the RNA Society, Seattle, WA. Jun 2006.

**Lin S**, Xiao R, Sun P, Xu X and Fu XD. Delinearizing SR Protein Function in RNA Metabolism by Somatic Complementation in Mouse Embryo Fibroblasts (Oral Presentation). 2005 Meeting on Eukaryotic mRNA Processing, CSHL, Aug 2005.

**Lin S**, Xu X, Xiao R and Fu XD. A Somatic Genetic System to Study SR Protein Function *in vivo* (Oral Presentation). The 9th Annual Meeting of the RNA Society, Madison, WI. Jun 2004.

Huang Y, Ying K, **Lin S**, Gu S, Xie Y, Mao Y. Cloning and Primary Study of OB-RGRP2 Gene. The 18th International Congress of Genetics, Beijing, China. Aug 1998.

#### **SCHOLARSHIPS AND AWARDS**

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# ABSTRACT OF THE DISSERTATION

Functional Study of SR Splicing Factors in a Cellular Genetic System

by

Shengrong Lin

Doctor of Philosophy in Molecular Pathology

University of California, San Diego, 2007

Professor Xiang-Dong Fu, Chair

Pre-mRNA splicing is a critical and highly regulated process in eukaryotic gene expression. Splicing reaction is conducted by hundreds of protein factors, which form a structure called spliceosome. The SR protein family is a group of regulatory splicing factors whose functions have been well-studied in biochemical systems. Typical SR proteins contain one or two RNA recognition motifs at the N-terminus, and serine-arginine repeats at the C-terminus. SR proteins also play important roles in other steps of mRNA metabolism in the cell. Despite extensive biochemical characterization,

the mechanism of SR protein action in the cellular context remains largely elusive. Inactivation of SR proteins by gene targeting indicates that the SR proteins are required for animal development and cell viability. However, the mechanism behind is far from clear. To study how SR proteins act in mammalian cells, we constructed two mouse embryo fibroblast (MEF) cell lines in which the endogenous genes for the SR protein ASF/SF2 and SC35 were each deleted and complemented by prospective exogenous genes expressed from a tetracycline-controlled promoter. Addition of tetracycline/doxycycline in cell culture efficiently turns off the expression of these SR proteins. The inducible cell lines have been used as a genetic model in functional studies.

Several interesting findings observed in these genetic systems form the basis of this dissertation: 1) In mammals, both SR proteins are uniquely required for survival of proliferating cells but dispensable in non-dividing cells. 2) RNA binding motif is required, while RS domain is dispensable, for ASF/SF2 function *in vivo*. 3) Recycling SR proteins in cells is controlled by a sorting mechanism regulated by the phosphorylation state of RS domain. 4) SR proteins are critical for transcriptional elongation in mammalian cells. 5) The elongation defects trigger extensive double-stranded DNA breaks, leading to ATM-mediated activation of p53, inducing p21 expression, and cell cycle arrest.

Our research has shed light on a number of fundamental questions regarding the function of SR proteins in regulated mRNA processing, maintenance of genomic stability and cell proliferation in vertebrates. Moreover, our work has established an experimental platform for future exploration of the cellular functions of SR proteins.



**CHAPTER I**  
**General Introduction**

## **Regulated splicing in Eukaryotes**

Eukaryotic gene expression involves a complex network of multi-step pathways. Pre-mRNA splicing is a necessary step in RNA processing and has been shown to collaborate with other events, such as transcription, mRNA capping and polyadenylation, nuclear export, translation and mRNA surveillance <sup>1</sup>. Like other gene expression events, RNA splicing is known to be under extensive regulation during development and in different cell types. Initial analysis of a draft of human genome showed that about 40-60% of human genes were alternatively spliced <sup>2</sup>. Splicing reactions take place in a complex cellular machine called the spliceosome, which consists of hundreds of different proteins. Among them, a group of splicing factors called SR proteins have been shown to carry out critical functions in constitutive splicing, alternative splicing, and connecting splicing to other steps of RNA metabolism. Chapter II of this dissertation will focus on our current mechanistic understanding of alternative splicing regulated by SR proteins and SR-related proteins, and will discuss some of the questions that remain to be addressed in future research.

## **Essential functions of SR proteins *in vivo***

SR proteins belong to a family of essential splicing factors first identified by a monoclonal antibody mAb104, which recognizes phosphorylated RS domains. 11 typical SR proteins have now been reported, all consisting of one or two N-terminal RNA recognition motifs (RRMs) and a RS domain containing arginine/serine repeats at the C-terminus. All of the typical SR proteins are able to complement the splicing-deficient cytoplasmic S100 extract <sup>3</sup>. Although SR proteins exhibit redundant activities in S100

complementation assays, individual SR proteins examined to date are all required for development and cell viability in vertebrates, indicating each SR protein has unique functions *in vivo*. B52 (homolog to human SRp55) deletion caused lethality during development in *D. melanogaster*<sup>4</sup>. ASF/SF2 has been shown required for cell viability in chicken DT40 cells<sup>5</sup>. Two conditional knockout mouse models generated in our lab showed that both ASF/SF2 and SC35 were required in animal development and cardiomyocyte function<sup>6,7</sup>. However, the reason why cells depleted of SR proteins die remains unknown. It is not clear if the cells die due to a general splicing defect, or changes in splicing of specific targets. The death pathway also remains unclear. In Chapter V of this dissertation, I will further discuss this issue and our findings in detail.

### **Multiple functions of SR proteins**

SR proteins were first discovered as splicing activators and regulators. They act as bridges in splicing reactions by recruiting and stabilizing small nuclear RNPs (snRNP) to pre-mRNA (exon-intron junction and branch point), and connecting different snRNPs during spliceosome assembly. Their functions in splicing can be divided into three categories: constitutive exon-dependent functions (for weak splicing sites), exon-independent functions (for basal splicing) and regulated exon-dependent functions (for alternative splicing)<sup>3</sup>. According to previous *in vitro* and *in vivo* results, some of the above functions are redundant. Furthermore, the function required for splicing of different exons may vary. Increasing evidence suggests that the splicing reaction is temporally and mechanistically coupled with other processes in RNA metabolism including transcription, translation and RNA degradation<sup>1</sup>. For example, ASF/SF2 was

reported to directly affect the stability of PKCI-r mRNA independent of transcription or splicing <sup>8</sup>, and to stimulate protein syntheses in the cytoplasm <sup>9</sup>. Recently, nucleocytoplasmic shuttling SR protein 9G8, SRp20 and ASF/SF2 have been implicated as adaptors for TAP-dependent mRNA export. This function requires dephosphorylation of the RS domain <sup>10, 11</sup>. Although these functions beyond splicing are intriguing, no evidence before our study showed whether they are essentially required *in vivo* or not.

### **Regulation of SR protein function by phosphorylation**

The RS domain of SR proteins undergoes extensive phosphorylation on serine residues *in vivo*, which can be detected by a specific antibody (mAb104) against phosphorylated arginine/serine repeats. *In vitro* binding assays suggested phosphorylation may enhance the specificity of protein-protein binding and prevent nonspecific sticking of the charged RS domain to RNA <sup>12</sup>. In splicing assays, phosphorylation of SR proteins is required for initiating spliceosome assembly. Nuclear extracts treated with the phosphatase PP1 block splicing and spliceosome assembly at the earliest detectable stage <sup>13</sup>. Once the spliceosome is fully formed, dephosphorylation appears to be necessary for re-arranging the spliceosome on its substrates. Thio-phosphorylated ASF/SF2, which can not be dephosphorylated, can support spliceosome assembly but blocks the following steps of the splicing reactions <sup>14</sup>. Dephosphorylation of the RS domain is also required in post-splicing steps. Only hypo-phosphorylated shuttling SR proteins are able to recruit TAP proteins for nuclear export of mature mRNA <sup>10, 11</sup>.

### **Shuttling property of SR proteins and RNA export**

An important property of the RS domain is its ability to direct intracellular trafficking of SR proteins. SR proteins can be separated into nucleocytoplasmic shuttling and non-shuttling classes<sup>15</sup>. Shuttling SR proteins play a role in mRNA nuclear export due to their ability to interact with the crucial RNA export factor TAP<sup>16</sup>. Because SR proteins bind to exons in both pre-mRNA and spliced mRNA, it was unclear why only spliced mRNA is selected for export. The puzzle was solved recently by the observation that the RS domain in shuttling SR proteins associated with post-splicing RNP is hypo-phosphorylated. The dephosphorylated state is required for interaction with TAP<sup>10, 11</sup>. So, what happens to non-shuttling SR proteins during the transition from splicing to export? Non-shuttling SR proteins do not have the capability to interact with TAP, and do not leave the nucleus. Therefore, how would they avoid potential interference with mRNA export, if both shuttling and non-shuttling SR proteins provide similar functions in the splicing reaction? Mammalian cells must have a strategy to deal with this problem to allow a smooth transition from splicing to export, especially when shuttling and non-shuttling SR proteins are both involved in processing of a precursor mRNA. In Chapter III of this dissertation, I will describe a genetic complementation system derived from conditional knockout mice used to address the function and regulation of SR proteins *in vivo*. Using this system, we demonstrate that ASF/SF2 and SC35 are each required for cell viability, but surprisingly, the effector RS domain of ASF/SF2 is dispensable for cell survival in MEFs. Although shuttling SR proteins have been implicated in mRNA export, prevention of ASF/SF2 from shuttling had little impact on mRNA export. We found that shuttling and non-shuttling SR proteins are segregated in an orderly fashion during mRNP maturation, indicating distinct recycling pathways for

different SR proteins. We have further shown that this process is regulated by differential dephosphorylation of the RS domain, thus revealing a sorting mechanism for mRNP transition from splicing to export.

### **Transcription and splicing coupling**

In eukaryotes, processing a mature mRNA is a highly complex and tightly regulated process. Coupling between individual steps in this dynamic process is believed to ensure efficiency and accuracy of gene expression. Therefore it is not surprising that more and more reports have emerged suggesting sophisticated interactions among the various steps of gene expression. In this introduction, I will only focus on links between two steps of mRNA processing: transcription and splicing.

It is a well-accepted concept that transcription and splicing are spatially and temporally coupled *in vivo*. Previous investigations of gene transcription products revealed a considerable percentage of co-transcriptional splicing events in eukaryotic cells<sup>17</sup>. This phenomena occurs more frequently in proximal exons than distal exons of long genes<sup>18,19</sup>. Apparently, due to the constrained time of polymerase processing from any specific exon to the end of the transcript, short genes and proximal exons are more inclined to be co-transcriptional spliced<sup>20</sup>.

Additional evidence of transcription/splicing coupling is that these two mRNA processing machineries share some factors. Inclusion of transcription factors in splicing complexes has been reported in proteomic analyses<sup>21, 22</sup>. Among the identified transcription/splicing factors are some considered to play dual-functions in both processes, which coordinate two huge protein complexes in the bigger event of gene

expression<sup>23</sup>. For example, thermogenic coactivator PGC-1, when recruited to transcriptional complexes on promoters, can affect alternative splicing<sup>24</sup>. More generally, it has been shown that transcriptions initiated from different promoters might affect the recognition of downstream splicing signals through differential recruitment of transcriptional factors and splicing machineries<sup>25,26</sup>. However, detailed mechanisms and specific factors that regulate these events still remain unclear.

RNA polymerase II C-terminal domain (CTD) plays critical roles in regulating transcription and coupling transcription to other steps of mRNA processing like 5' capping, 3' cleavage/polyadenylation and splicing<sup>27</sup>. In human Pol II, the CTD comprises 52 heptad consensus repeats as YSPTSPS. Truncation of those repeats causes defects in mRNA processing<sup>28</sup>. Cellular localization and immunoprecipitation assays showed that the CTD recruits and loads SR splicing factors to nascent transcripts in a phosphorylation-dependent manner<sup>29</sup>. Moreover, the CTD has also been shown to facilitate spliceosome assembly on exons that are separated by a long intron<sup>23,30</sup>.

### **Pol II elongation/pausing and splicing**

A series of studies done by Kornblihtt and colleagues using minigene models suggested that transcription might control alternative splicing patterns by regulating pol II elongation rate and processivity<sup>31</sup>. They hypothesized that this might be due to the fact that splice sites in alternative exons are relatively weak, and final splicing outcomes are the result of competition between two alternative sites. Therefore, the splicing choice may be influenced by the rate of transcription. For example, the EDI exon of the fibronectin gene has a weaker 3' splicing site compared to the downstream competing

exon. If the polymerase processes fast enough, both splicing sites will be transcribed in a short time and the spliceosome will assemble on the strong splicing site to promote splicing, resulting in the exclusion of the alternative EDI exon. On the contrary, if Pol II elongation is slow or transcription complex pauses between the two exons, the spliceosome would have sufficient time to assemble on the weak EDI splicing site resulting in inclusion of the weak exon in the final product <sup>31</sup>. This hypothesis was supported by several reports in which the polymerase elongation rate was manipulated by DRB, a drug that inhibits CTD phosphorylation and impairs elongation <sup>32</sup>, or by a mutation affecting elongation <sup>33</sup>. In the cellular context, specific internal pausing sites and/or chromatin structures may affect polymerase processivity, and indeed, modification of the chromatin structure has been shown to affect alternative splicing <sup>32,34</sup>. In addition, the use of alternative promoters may recruit different transcription factors/co-factors and elongation factors, thereby altering the transcription rate from different promoters. This elongation-control-splicing model could explain the observed phenomena that transcriptions from different promoters may affect downstream splicing events.

### **Converse coupling between transcription and splicing**

It has been known for decades that the presence of an intron in a cDNA construct enhances expression of the cDNA in transfected cells. However, the underlying mechanism for this remains unclear. Recently, Fong and Zhou demonstrated that the human transcription elongation factor TAT-SF1 recruits spliceosomal U small nuclear ribonucleoproteins (UsnRNPs) to the common elongation factor P-TEFb to significantly stimulate elongation. Because purified TAT-SF1/snRNPs complex also has splicing



activities *in vitro*, this finding suggests that splicing may reciprocally regulate transcription<sup>35</sup>. In addition, U1 snRNP is found in association with the general transcription factor TFIID at gene promoter regions and stimulates transcription initiation<sup>36</sup>. However, the specific component in the splicing complex that is involved in regulating transcription initiation and elongation remains undefined. In Chapter IV of this dissertation, I will demonstrate that SR proteins are critical for transcription elongation in mammalian cells. Analysis of nascent transcripts revealed gene-specific blockage of transcription elongation in SR protein-depleted cells. Remarkably, the defect could be functionally rescued with purified SR protein, suggesting a direct role of SR proteins in this process. The elongation defects triggered extensive double-stranded DNA breaks, leading to ATM-mediated activation of p53, induced p21 expression, and cell cycle arrest. These findings suggest that timely recognition of splicing signals by SR proteins in nascent transcripts is a driving force for transcription elongation in vertebrates.

### **SR proteins and cell viability**

The SR family of splicing factors has been extensively studied at the biochemical level for their diverse roles in RNA metabolism and gene expression<sup>37</sup>. However, because of the lethal phenotype observed at cellular and animal levels, few studies have linked their activities to specific pathways to elucidate their biological functions *in vivo*. Because all SR protein knockouts in mammals have caused embryonic lethality and cell death, loss of a specific SR protein may be sufficient to induce global effects that ultimately cause cell mortality. These effects might not be limited to splicing, as catastrophic defects in constitutive/alternative splicing were not extensively detected in

SR depleted cells/animals. Manley and colleagues recently demonstrated a remarkable genomic instability phenotype induced by DNA damage that was linked to R-loop formation in ASF/SF2-deficient chicken DT40 cells<sup>38,39</sup>. However, genomic instability may not be the only cause of cellular lethality, because preventing R-loop formation and DNA damage by overexpressing RNaseH still cause cell death in DT40 cells. In Chapter V of this dissertation, I will discuss the characterization of SR protein depletion induced cell lethality. By using inducible somatic genetic complementation systems, we discovered that the SR protein SC35 plays a critical role in cell proliferation, but surprisingly, it is dispensable in terminally differentiated mature cardiomyocytes in the heart. In conditional knockout MEFs, we found that depletion of SC35 induced cell cycle arrest at the G2/M phase. Remarkably, this defect in cell cycle progression could be partially relieved by inactivation of the *p53* tumor suppressor gene, indicating a key role of *p53* in the SC35-mediated cell proliferation pathway. Analysis of the *p53* activation mechanism revealed hyperphosphorylation of *p53* at the site known to be modified by activated ATM<sup>40</sup>, consistent with double strand DNA breaks observed in SC35-deficient MEFs. We also detected *p53* hyperacetylation, which was linked to the increased expression of the *p53* acetyltransferase *p300*<sup>41-45</sup>, and the aberrant splicing of the *p53* deacetylase *Sirt1*<sup>46-49</sup>. These observations provide evidence for a general role of SR proteins in maintaining genomic stability and cell proliferation in vertebrates.

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## **CHAPTER II**

### **SR Proteins and Related Factors in Alternative Splicing**

**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 co-transcriptional 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 1990's by the identification of factors associated with purified spliceosomes,<sup>1,2</sup> by the purification of critical non-snRNP splicing activities in constitutive and alternative splicing,<sup>3-6</sup> and by the analysis of components of a nuclear body that could be selectively precipitated with Mg<sup>++</sup>.<sup>7</sup> 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.<sup>3,4,6,8</sup> This observation was quickly extended to other SR proteins.<sup>9-11</sup> 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.<sup>12,13</sup> 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 domain-containing RNA binding proteins, including human TRA2 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.<sup>14</sup> In mammalian cells, SR-related proteins include other RNA binding proteins, such as both subunits of the U2AF heterodimer and the U1 snRNP specific protein U1-70K, and various enzymes, including several ATPases involved in RNA rearrangement within the spliceosome<sup>15-18</sup> (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.<sup>19</sup> However, recent studies have revealed direct binding of the RS domain to critical splicing signals in pre-mRNA transcripts.<sup>20,21</sup>

Interestingly, budding yeast express a few RNA binding proteins that structurally resemble SR proteins.<sup>22</sup> 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 recently documented.<sup>23</sup> 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<sup>24,25</sup> or on the functional consequence of in vitro splicing.<sup>26-28</sup> The ESEs characterized to date have been used to develop an ESE-finder program<sup>29</sup> 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.<sup>30,31</sup> 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.<sup>32-36</sup> 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 (Figure II-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<sup>37-39</sup> and the U2AF complex at the 3' splice site.<sup>40-43</sup> Both SR proteins and RS domain-containing splicing co-activators have been implicated in promoting communication between the 5' and 3' splice sites.<sup>44-49</sup> 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).<sup>50,51</sup> 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<sup>8,9,52,53</sup> 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.<sup>54</sup> SR protein binding may enhance complex assembly on both strong and weak splice sites to make them equally competitive.<sup>55</sup> 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<sup>56</sup> (Figure II-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.<sup>57-59</sup> 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.<sup>58,60</sup> 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.<sup>50,51</sup> 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.

### **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 (Figure II-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<sup>61</sup> (Figure II-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 CaMKII gene.<sup>62</sup> While the direct effect of SR proteins in CaMKII 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.<sup>63</sup> Similarly, SRp30c was found to suppress splice site selection of an alternative exon in the hnRNP A1 gene.<sup>64</sup> 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.<sup>65-69</sup> 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 (Figure II-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.<sup>70</sup>

Aside from the substrate-dependent effects of typical SR proteins, some SR proteins appear to only function in splicing in a negative fashion (Figure II-2C). The best characterized example is SRp86, which appears to antagonize typical SR proteins in splice site selection.<sup>71-73</sup> 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.<sup>74</sup> In a recent functional screen using a tau-based alternative splicing reporter, p54 was found to compete with hTRA2 for binding to an ESE and to promote

exon skipping.<sup>75</sup> 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.<sup>76</sup> SRp38 was isolated as an alternative splicing regulator in several independent studies.<sup>76-79</sup> 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.<sup>80,81</sup> 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.<sup>79,82</sup> 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 hTRA2 and hTRA2.<sup>83</sup> 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 regulated splicing in both *Drosophila* and human cells.<sup>84,85</sup> 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*<sup>85</sup> or PTB in vertebrates.<sup>86-88</sup>

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<sup>88</sup> and a large Zn-finger protein ZNF265<sup>91</sup> (Table 1). Interestingly, several kinases, such as Clk/Sty,<sup>92,93</sup> CrkRS,<sup>94</sup> Prp4K,<sup>95</sup> and CDC2L5,<sup>96</sup> and the regulator subunits cyclin L1<sup>97,98</sup> and L2,<sup>99,100</sup> 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,<sup>85</sup> the mammalian homologues of which carry an extra RS domain.<sup>15,18</sup> 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.<sup>101</sup> 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.<sup>102</sup>

### **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*.<sup>103</sup> 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.<sup>83</sup> Although the RNAi approach has been applied to mammalian cells to demonstrate specific requirements of SR proteins in alternative splicing,<sup>104,105</sup> 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,<sup>104</sup> has an unexpected role in maintaining genomic stability,<sup>107</sup> and has a regulatory function in DNA fragmentation during apoptosis.<sup>108</sup> At least one of these *in vivo* functions (DNA fragmentation) was linked to SF2/ASF-regulated alternative splicing.<sup>108</sup> 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*.<sup>62,109,110</sup> Surprisingly however, SC35 seems to be dispensable in non-dividing mature cardiomyocytes, indicating that SR proteins are not universally required for cell viability *in vivo*.<sup>111</sup> This observation is in agreement with an RNAi result in *C. elegans*.<sup>112</sup> 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 embryo fibroblasts.<sup>112</sup> 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.<sup>113</sup> 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.<sup>111,114</sup>

### **SR proteins as splicing regulators in vivo: Why so few targets?**

Members of the SR family of splicing factors are among the best-characterized 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 (Figure II-3). First, more than one SR protein may be able to recognize a similar set of ESEs present in most exons; this has been observed in vitro with SF2/ASF and hTRA2, which are both capable of recognizing purine-rich ESEs.<sup>25,41,83,115</sup> Second, most exonic sequences appear to harbor multiple ESEs that are responsive to distinct SR proteins,<sup>116</sup> which may act independently or in a synergistic manner.<sup>31,117</sup> 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.<sup>33</sup> 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 Kristen 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.<sup>118-122</sup> 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.<sup>123-126</sup> 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.<sup>127-129</sup> Phosphorylation and dephosphorylation are both required,<sup>130</sup> because it was found that experimental induction of SR protein hyper- and hypo-phosphorylation impaired splicing.<sup>92</sup> 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.<sup>112,131</sup> This is due to a full phosphorylation/dephosphorylation cycle does not have to occur in a single SR protein for each round of the splicing reaction.<sup>129</sup> 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.<sup>90,132-135</sup> 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<sup>136,137</sup> 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<sup>138,139</sup> 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.<sup>140</sup> 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<sup>93,141-143</sup> and their recruitment to the transcriptional machinery has been shown to facilitate co-transcriptional splicing in the nucleus.<sup>139,144,145</sup> 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,<sup>139,141</sup> Clk/Sty,<sup>94,132,134</sup> and Akt.<sup>136,137</sup> 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.<sup>140,146</sup> 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.<sup>147</sup> 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.<sup>141,143,148</sup> A recent RNAi study showed a major impact of SRPK1 depletion on SR protein phosphorylation in vivo.<sup>149</sup> 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.<sup>127,128,150,151</sup> Several PP2A family members have been co-purified with spliceosomal components.<sup>152</sup> 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.<sup>153</sup> In particular, because SRp38 is particularly sensitive to dephosphorylation in response to mitotic transitions and heat shock,<sup>81</sup> 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<sup>154</sup> 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.<sup>155</sup>

### **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.<sup>156,157</sup> 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.<sup>63,158,159</sup> 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.<sup>63</sup> In knockout mice, SF2/ASF was found to play a critical role in the developmental control of CaMKII alternative splicing in the heart, resulting in differential cellular targeting of the kinase and malfunction in Ca<sup>++</sup> signaling in cardiomyocytes.<sup>62</sup> 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.<sup>160-165</sup>

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.<sup>157</sup> 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.<sup>166</sup> 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.<sup>166-169</sup> 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 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.

## ACKNOWLEDGEMENTS

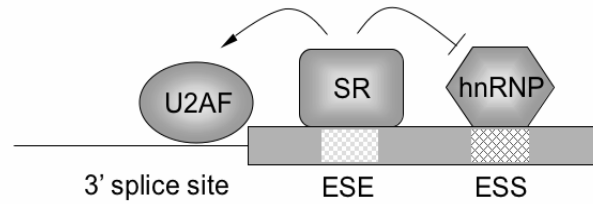
The text of Chapter II, in part or in full, is a reprint of the material as it appears in a chapter of *Alternative Splicing in the Postgenomic Era, Eureka Bioscience Series*, 2007. I was one of the two authors in this publication. I thank my co-author Dr. Xiang-Dong Fu for permission to use this publication in my dissertation.

**Table II-1. SR proteins and SR-related splicing regulators**

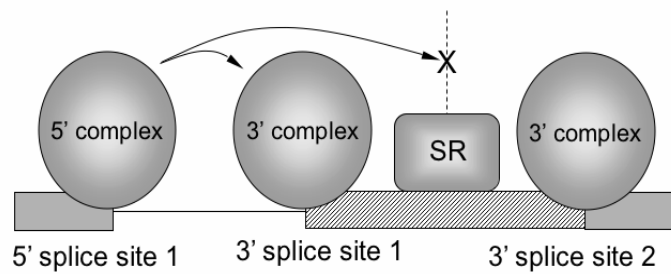
<b>Classification</b>	<b>Factors</b>	<b>Key Domains</b>	<b>Functions</b>
Classic SR Proteins	SRp20, <sup>1</sup> SF2/ASF, <sup>2</sup> SC35, <sup>3</sup> 9G8, <sup>4</sup> SRp40, <sup>5</sup> SRp55/B52, <sup>6</sup> SRp75 <sup>7</sup>	One or two RRMs plus an RS domain	Constitutive and alternative splicing
Additional SR proteins	hTRA2a, <sup>8</sup> hTRA2b, <sup>9</sup> RNPS1, <sup>10</sup> SRp38, <sup>11</sup> SRp30c, <sup>12</sup> p54, <sup>13</sup> SRrp35, <sup>14</sup> SRrp53, <sup>15</sup> SRp86 <sup>16</sup>	One or two RRMs plus an RS domain	Positive and negative regulation of alternative splicing
RNA binding SR related factors	U2AF65, <sup>17</sup> U2AF35, <sup>18</sup> Urp, <sup>19</sup> HCC1/CAPER, <sup>20</sup> U1-70K, <sup>21</sup> hSWAP, <sup>22</sup> Pinin, <sup>23</sup> Sip1, <sup>24</sup> SR-A1, <sup>25</sup> ZNF265, <sup>26</sup> SRm160, <sup>27</sup> SRm300, <sup>28</sup>	RRM, PWI domain, Zn finger plus an RS domain	Splicing factors or co-activators
Enzymes and regulators carrying an RS domain	hPRP5, <sup>29</sup> hPRP16, <sup>30</sup> Prp22/HRH1, <sup>31</sup> U5-100K/hPRP28, <sup>32</sup> ClkSty-1, <sup>33</sup> 2, <sup>34</sup> 3, <sup>35</sup> CLASP, <sup>36</sup> Prp4K, <sup>37</sup> CrkRS/CRK7/CDK12, <sup>38</sup> CDC2L5, <sup>39</sup> CCNL1, <sup>40</sup> CCNL2, <sup>41</sup> SR-cyp, <sup>42</sup>	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

A. Role of SR proteins in splice site recognition



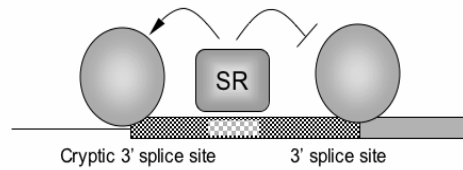
B. Insulating function of SR proteins in splice site communication



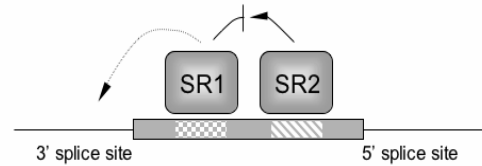
**Figure II-1**

**Figure II-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.

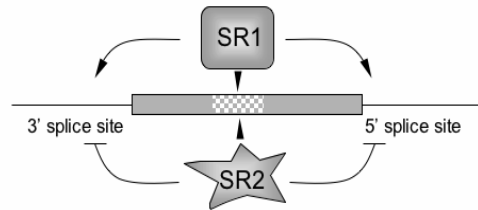
A. Inhibition of downstream 3' splice site recognition



B. Interference between weak and strong SR proteins



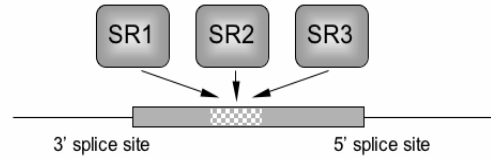
C. Competition between positive and negative SR proteins



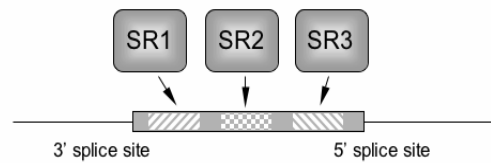
**Figure II-2**

**Figure II-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.

A. Multiple SR proteins recognize a similar ESE



B. Multiple SR proteins recognize distinct ESEs



**Figure II-3**

**Figure II-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.



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## **CHAPTER III**

### **Dephosphorylation-Dependent Sorting of SR Splicing Factors during mRNP Maturation**

## Dephosphorylation-Dependent Sorting of SR Splicing Factors during mRNP Maturation

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### Summary

SR proteins are a family of sequence-specific RNA binding proteins originally discovered as essential factors for pre-mRNA splicing and recently implicated in mRNA transport, stability, and translation. Here, we used a genetic complementation system derived from conditional knockout mice to address the function and regulation of SR proteins *in vivo*. We demonstrate that ASF/SF2 and SC35 are each required for cell viability, but, surprisingly, the effector RS domain of ASF/SF2 is dispensable for cell survival in MEFs. Although shuttling SR proteins have been implicated in mRNA export, prevention of ASF/SF2 from shuttling had little impact on mRNA export. We found that shuttling and nonshuttling SR proteins are segregated in an orderly fashion during mRNP maturation, indicating distinct recycling pathways for different SR proteins. We further showed that this process is regulated by differential dephosphorylation of the RS domain, thus revealing a sorting mechanism for mRNP transition from splicing to export.

### Introduction

SR proteins are a family of conserved splicing factors in metazoans and play diverse roles in RNA metabolism from pre-mRNA splicing to mRNA export to protein translation (Black, 2003; Huang and Steitz, 2005). SR proteins have a bipartite structure containing one or two RNA recognition motifs (RRMs) at the N terminus and a signature RS domain enriched in arginine/serine dipeptide repeats at the C terminus (Fu, 1995; Graveley, 2000). The RRM in SR proteins are responsible for sequence-specific binding to exonic splicing enhancers (ESEs). The RS domain can function as a general activation domain for splicing when linked to a heterogeneous RNA binding motif (Graveley and Maniatis, 1998), and most RS domains seem to be functionally interchangeable (Chandler et al., 1997; Dauwalder and Mattox, 1998; Wang et al., 1998). In addition, RS domains appear to harbor various signals for nuclear and subnuclear localization for SR proteins (Hedley et al., 1995; Caceres et al., 1998).

Biochemical analyses indicate that ESE bound SR proteins promote spliceosome assembly via RS domain-mediated recruitment of splicing factors (Wu and Maniatis, 1993) and/or by sequestering inhibitory RNA binding proteins bound to exonic splicing silencers (ESSs) (Kan and Green, 1999; Zhu et al., 2001). More recently, the RS domain was found to directly contact RNA in a stage-specific manner in assembled spliceosomes, raising the possibility that the RS domain may be a crucial contributor to the recognition of *cis*-acting splicing signals during spliceosome assembly (Shen and Green, 2004; Shen et al., 2004). Interestingly, while essential for constitutive splicing, the RS domain is dispensable for alternative splicing both *in vitro* and in transfected cells (Caceres and Krainer, 1993). How the RS domain is differentially required for constitutive and alternative splicing remains an unsolved question.

In addition to bona fide SR proteins, many other mammalian splicing factors carry an RS domain, including both subunits of the U2AF heterodimer and most ATPases involved in spliceosome rearrangement during splicing (Graveley, 2000). The RS domain in the N terminus of U2AF65 was shown to contact RNA at the branchpoint via electrostatic interactions, and the binding specificity is guided by RRM-mediated binding at the downstream polypyrimidine tract (Valcarcel et al., 1996). U2AF35 carries a C-terminal RS domain, which may be responsible for contacting the conserved AG dinucleotide at the end of an intron (Merendino et al., 1999; Wu et al., 1999; Zorio and Blumenthal, 1999). Thus, the U2AF heterodimer appears to cover all critical splicing signals surrounding the polypyrimidine tract at the 3' splice site. Interestingly, however, the RS domain in either subunit, but not both, could be removed without compromising the essential function of U2AF in *Drosophila* (Rudner et al., 1998). The mechanism for this observation remains to be understood.

Another important property of the RS domain is its ability to direct intracellular trafficking of SR proteins. Several SR proteins are able to shuttle between the nucleus and the cytoplasm, while many others are restricted in the nucleus (Caceres et al., 1998). Shuttling SR proteins have been implicated in mediating mRNA nuclear export due to their ability to interact with the crucial RNA export factor TAP (Huang et al., 2003). Importantly, the RS domain in shuttling SR proteins undergoes dephosphorylation, which is required for the interaction of TAP with the N-terminal sequences of shuttling SR proteins (Huang et al., 2004; Lai and Tam, 2004). Since nonshuttling SR proteins do not have the ability to interact with TAP and they do not leave the nucleus, how they avoid potential interference with RNA export remains unknown. Mammalian cells must have a mechanism to allow smooth transition of mRNA-containing ribonucleoprotein complexes (mRNPs) from splicing to export, especially when shuttling and nonshuttling SR proteins are both involved in processing of the same precursor mRNA.

Here we report the development of a somatic complementation system derived from conditional SR protein

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knockout mice to study the function and regulation of SR proteins in vivo. We found that two prototypical SR proteins, ASF/SF2 and SC35, are each required for cell viability in mouse embryo fibroblasts (MEFs). Interestingly, contrary to its requirement in other cellular models, the RS domain of ASF/SF2 is not essential for cell survival in MEFs. To understand whether shuttling is obligatory for individual shuttling SR proteins to function in vivo and why nonshuttling SR proteins do not interfere with nuclear export of postslicing mRNPs, we analyzed mRNP maturation in MEFs complemented by various mutant SR proteins. We observed that shuttling and nonshuttling SR proteins are segregated during mRNP maturation in an orderly fashion. We further showed that this sorting process is regulated by differential dephosphorylation of the RS domain and that the nonshuttling SR protein SC35 carries an unprecedented phosphatase-resistant domain, which is transferable and acts in a dominant manner. These results reconcile many biochemical and cell biological observations and suggest a model for the functional requirements and regulation of SR proteins in RNA metabolism.

## Results

### A Somatic Genetic System for Functional Study of SR Proteins In Vivo

SR proteins are general splicing factors involved in both constitutive and regulated splicing in higher eukaryotic cells. Despite extensive biochemical characterization, the mechanism of SR protein action in a cellular context remains elusive, emphasizing the importance of developing genetically tractable systems. We previously reported the construction of conditional knockout mice for two SR proteins, SC35 and ASF/SF2 (Wang et al., 2001; Xu et al., 2005). Induced null mutation of these SR proteins in the mouse caused early embryonic lethality, demonstrating their critical roles in development. These results are consistent with an earlier report that ASF/SF2 was essential for cell viability in chicken DT40 cells (Wang et al., 1998). To determine their requirement in normal fibroblasts, we derived mouse embryo fibroblasts (MEFs) from conditional knockout mice and inactivated the "floxed" endogenous gene by infecting the cells with a retrovirus expressing the Cre recombinase. Cre virus infection induced cell death in MEFs carrying double floxed alleles, but not in wt MEFs (Figure 1A). These results demonstrate that both SC35 and ASF/SF2 are required for cell viability in normal fibroblasts.

To develop a genetic complementation system, we immortalized the MEFs from homozygous conditional knockout mice with large T antigen and introduced a tet-off construct to express HA-tagged, exogenous SR proteins (Figure 1B). In conditional ASF/SF2 knockout MEFs, for example, the exogenous gene was constitutively expressed at a level comparable to that of the endogenous gene, and after Cre virus infection, the expression of the endogenous gene was completely eliminated (Figure 1C). We established tet-inducible cell lines via single cell cloning for both SC35 and ASF/SF2, in which the endogenous genes were deleted and functionally replaced by the corresponding exogenous genes. Upon addition of Doxycycline (Dox, a synthetic

tetracycline analog) to the culture media, HA-tagged ASF/SF2 was undetectable after 5 days (Figure 1D). A similar result was obtained with the SC35 tet-off line (data not shown).

In response to SR protein depletion, cell growth declined by 2 days (Figure 2A). Cell growth arrest was evidenced by dramatic reduction in Br-dU labeling (Figure 2B) and enlarged cell size (Figures 2C and 2D). Cell death was detectable starting at day 4 or 5, and by day 7, most cells detached and died. These data show that both SC35 and ASF/SF2 play a nonredundant role in vivo and that each is required for cell viability in MEFs. This lethal phenotype could be fully rescued by reexpressing the appropriate SR protein via retrovirus (Figure 2A), establishing a genetic complementation system. Differences in cell populations between wt and ASF/SF2-rescued MEFs were likely due to the selection for cells properly expressing the exogenous gene. Isolated clones from rescued cells proliferated indistinguishably from wt MEFs (data not shown).

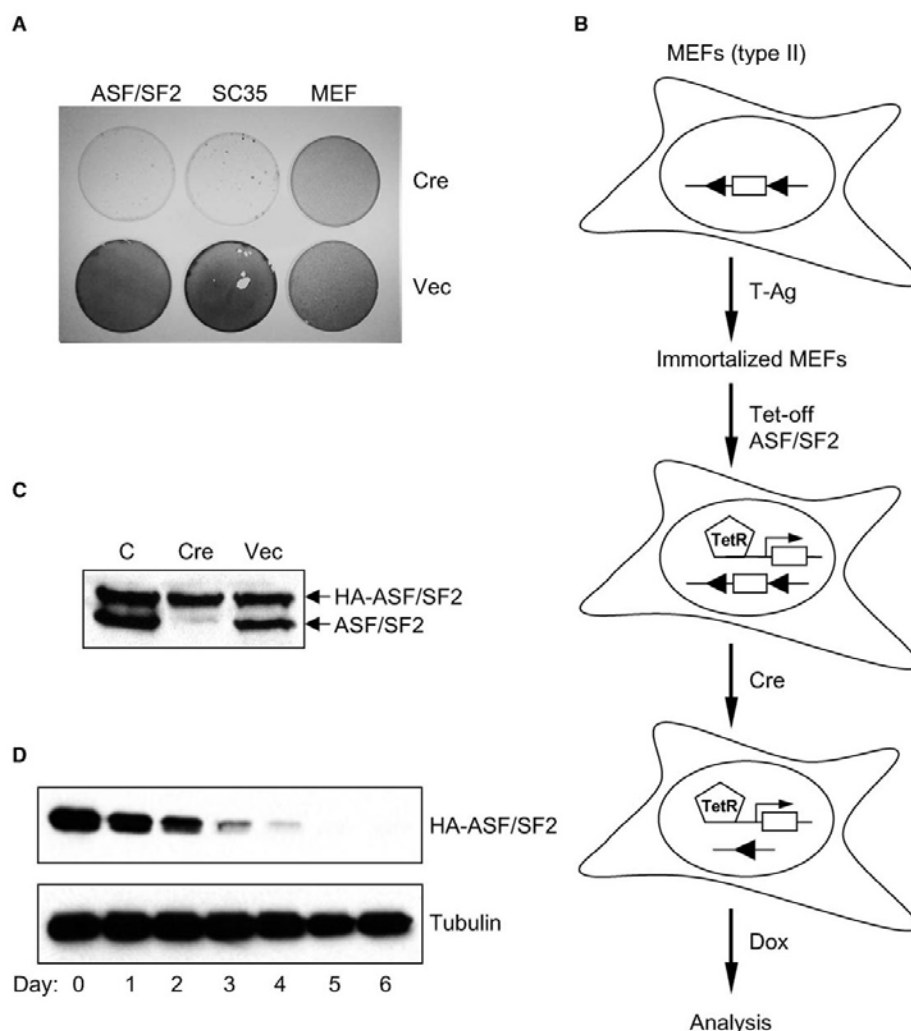
### Requirement for RRRMs but Not the RS Domain in MEFs

Using this somatic genetic system, we tested the complementation of ASF/SF2-depleted cells by different SR proteins or by different ASF/SF2 mutants. Clearly, each SR protein is uniquely required in MEFs because ASF/SF2-depleted MEFs could be complemented by ASF/SF2 but not by related SR proteins such as SC35 and SRp40 (Figure 3A). Numerous in vitro studies have shown that RNA recognition motifs (RRMs) are vital for SR proteins to function in splicing. As expected, mutations in a key RRM motif of ASF/SF2 (the FF-DD mutant, see Caceres and Krainer [1993]) abolished its ability to complement cell survival, demonstrating that the critical function of ASF/SF2 in vivo relies on its ability to bind RNA.

Most biochemical experiments suggest that the RS domain is essential for splicing, and a vital role of the RS domain in cell survival was also demonstrated by genetic complementation in chicken DT40 cells and in *Drosophila* (Dauwalder and Mattox, 1998; Wang et al., 1998). However, Krainer and colleagues reported that ASF/SF2 lacking the RS domain was still active in splicing of certain pre-mRNA substrates in vitro, indicating that the RS domain may not always be required (Zhu and Krainer, 2000). Furthermore, the RS domain is dispensable in SR-protein-mediated splice site selection (Caceres and Krainer, 1993). Thus, it is possible that the RS domain is functionally required in a substrate and cell-type-dependent manner. To test this hypothesis, we tested a mutant ASF/SF2 lacking the RS domain ( $\Delta$ RS) and found that it was sufficient to complement the lethal phenotype in MEFs (Figure 3A). Although the cells complemented by  $\Delta$ RS grew slightly slower than wt MEFs or MEFs complemented by wt ASF/SF2, this result demonstrates that the RS domain is not always required, even though its cognate SR protein is essential for cell viability. A similar finding was recently reported that documented the dispensability of the RS domain in the essential U1-70K protein in plants (Salz et al., 2004).

**Modulation of Divergent RRRMs by the RS Domain**  
ASF/SF2 appears to be conserved in *Drosophila*. The fly homolog dASF, however, is clearly functionally distinct





**Figure 1. SR Proteins Are Essential for Cell Viability**

(A) Wt MEFs or MEFs derived from conditional ASF/SF2 and SC35 knockout mice were infected with a Cre-expressing retrovirus or a control virus. Attached cells were stained with crystal violet. The few colonies remaining after Cre virus infection were due to inefficient deletion of the endogenous gene, which was confirmed by genotyping isolated colonies.

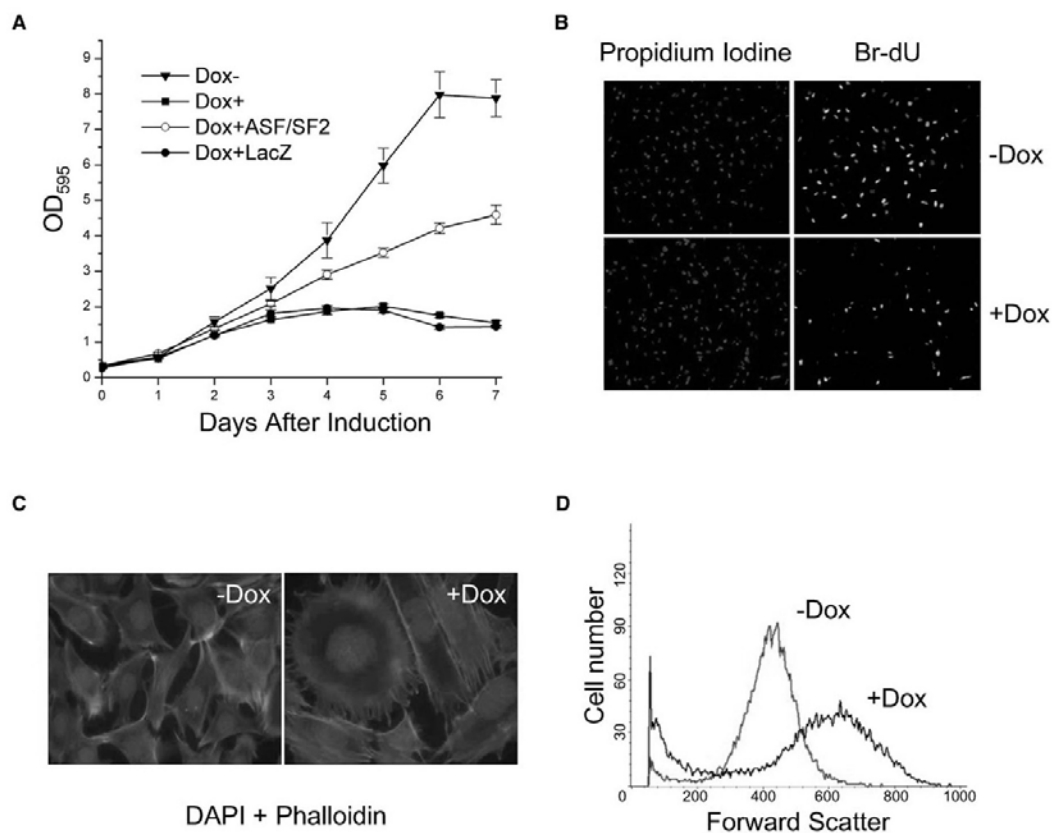
(B) Construction of the somatic genetic complementation system. MEFs were first immortalized using SV40 large T antigen. A tet-off system was engineered into the cells to drive the expression of an exogenous gene under tetracycline control. The endogenous gene was then deleted by a Cre-expressing retrovirus. Cloned cell lines were characterized before and after the addition of the tetracycline analog Dox.

(C) Expression of endogenous and exogenous ASF/SF2 in MEFs. Western blotting analysis using an anti-ASF/SF2 antibody showed that the exogenous ASF/SF2 transduced by the retrovirus was expressed at a comparable level to the endogenous ASF/SF2. After Cre virus infection, endogenous ASF/SF2 expression was progressively diminished. Cells rescued by exogenous ASF/SF2 grew indistinguishably from wt MEFs.

(D) Downregulation of exogenous ASF/SF2 by Dox. Expression was effectively eliminated by Dox in 5 days.  $\beta$ -tubulin was used as a loading control.

from its mammalian counterpart in several important aspects. The *Drosophila* protein did not support constitutive splicing in S100 but was fully capable of altering splice site selection in alternative splicing assays in vitro (Allemand et al., 2001). This functional distinction from mammalian ASF/SF2 was attributed to the RS domain in dASF, which could not be modified by the SR protein kinase SRPK1 in vitro and was unable to mediate

nucleocytoplasmic shuttling of dASF. In this regard, it remained to be determined whether dASF is orthologous to ASF/SF2 in mammalian cells despite their sequence similarities. By functional complementation, we found that dASF is a true ASF/SF2 ortholog (Figure 3B). Interestingly, however, the mutant dASF lacking its RS domain failed to complement, suggesting that the RRRMs in dASF are divergent enough that the activity



**Figure 2. ASF/SF2 Is Required for Cell Growth**

(A) Impaired cell proliferation after Dox-induced suppression of exogenous ASF/SF2. The lethal phenotype could be rescued by reexpressing ASF/SF2, thereby establishing the genetic complementation assay. Error bars represent standard deviations of data from three independent experiments.

(B) S-phase block in ASF/SF2-depleted MEFs revealed by Br-dU labeling.

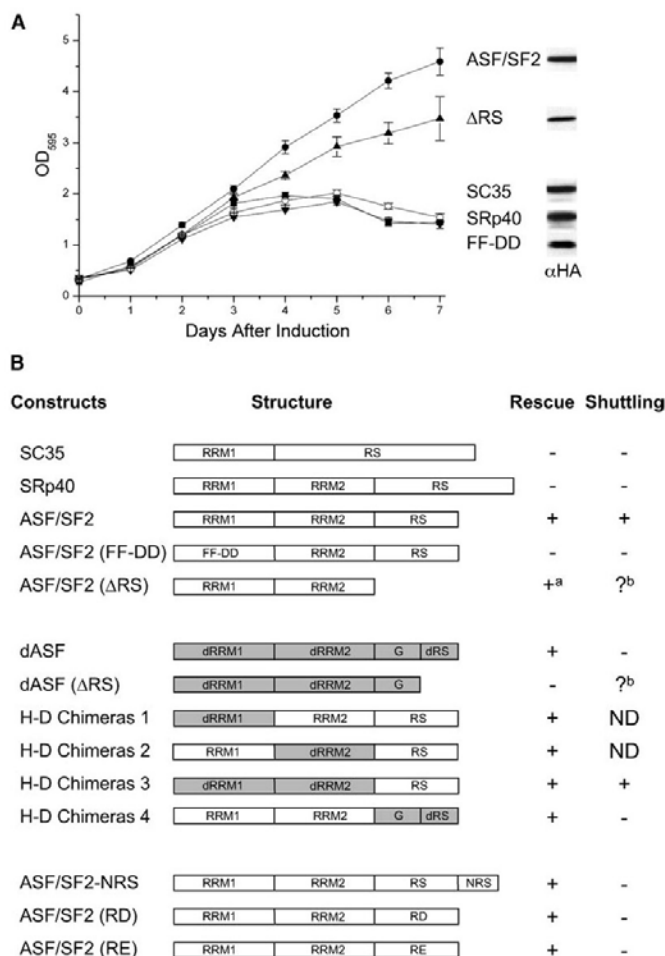
(C and D) Cell growth arrest induced by ASF/SF2 depletion *in vivo* revealed by cell staining (DNA by DAPI and actin by phalloidin) and by cell sorting.

of dASF in mammalian cells must be strengthened by the presence of its RS domain. The unique properties associated with the RS domain of dASF appeared unimportant because substitutions of the dASF RS domain with either the RS domain of ASF/SF2 (Figure 3B) or synthetic RS repeats (data not shown) were able to complement ASF/SF2-depleted MEFs.

#### Essential SR Protein Function Not Tied to the Ability to Shuttle

The ability of dASF to complement ASF/SF2-depleted MEFs also suggests that the shuttling property of individual SR proteins may not be obligatory for their functions *in vivo*. We confirmed that ASF/SF2, but not SC35 or dASF, shuttles in MEFs by the heterokaryon array (Figures 4Aa–4Af). The failure to shuttle was due to the property of the RS domain of dASF because the fusion protein consisting of the RRM from ASF/SF2 and the RS domain from dASF resulted in a nonshuttling SR

protein, but the fusion protein consisting of the RRM from dASF and the RS domain from ASF/SF2 retained the ability to shuttle (Figures 4Ag–4Aj). Interestingly, both fusion proteins were fully functional in rescuing ASF/SF2-deficient MEFs (Figure 3). Thus, the ability to shuttle is uncoupled from the ability to complement. This result is consistent with an earlier observation that the RS domain from the shuttling ASF/SF2 was functionally interchangeable with the RS domain from the nonshuttling SR proteins SC35 and SRp40 in DT40 cells (Wang et al., 1998). To further determine the functional requirements for SR proteins to shuttle, we took advantage of a nuclear retention signal (NRS) identified in nonshuttling SC35 (Cazalla et al., 2002). Fusion of this NRS to full-length ASF/SF2 prevented shuttling (Figures 4Ak and 4Al), yet the fusion protein was fully functional in complementing ASF/SF2-depleted MEFs (Figure 3B). Together, we conclude that the shuttling property is not critical for ASF/SF2 to function *in vivo*.



**Figure 3. Genetic Complementation of ASF/SF2-Depleted MEFs by Wt and Mutant SR Proteins**

(A) Requirement for specific RRRMs, but not the RS domain, in complementation. Exogenous genes were introduced into MEFs by retrovirus. Dox was added at the beginning of infection. Cell density was determined by quantifying extracted crystal violet at OD<sub>595</sub>. The expression level of individual exogenous genes was determined by anti-HA Western blotting. Complementation by wt ASF/SF2 was used as a positive control. Error bars represent standard deviations of data from three independent experiments.

(B) Constructs tested by complementation of ASF/SF2-depleted MEFs. The structures of wt and SR proteins are each diagrammed. ΔRS, RS domain deleted ASF/SF2. FF-DD, mutant ASF/SF2 containing a double phenylalanine to aspartic acid substitution in RRM1. Domains from *Drosophila* ASF (dASF) are illustrated in filled boxes. Chimeras are ASF/SF2 hybrids consisting of corresponding domains from human (H) or *Drosophila* (D) ASF/SF2. NRS, nuclear retention signal from SC35. RD and RE, mutant ASF/SF2 in which RS repeats in the RS domain were replaced by RD or RE repeats. Summarized on the right are the activity of individual proteins in complementing ASF/SF2-depleted MEFs and the ability to shuttle between the nucleus and the cytoplasm as determined in Figure 4. Note a, MEFs complemented by ΔRS grew slower, indicating partial complementation. Note b, the ability to shuttle is unknown, as the shuttling assay could not be performed on indicated constructs because of the presence of the mutant protein in both the nucleus and the cytoplasm. ND, not determined.

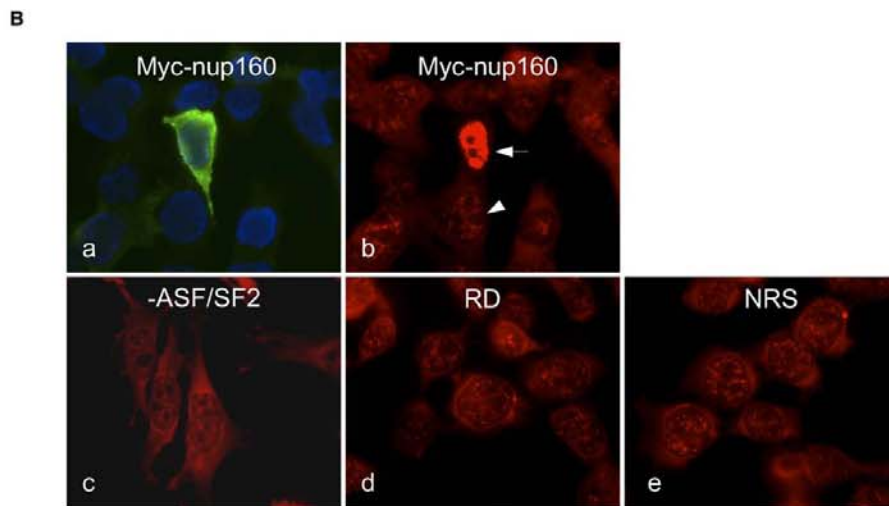
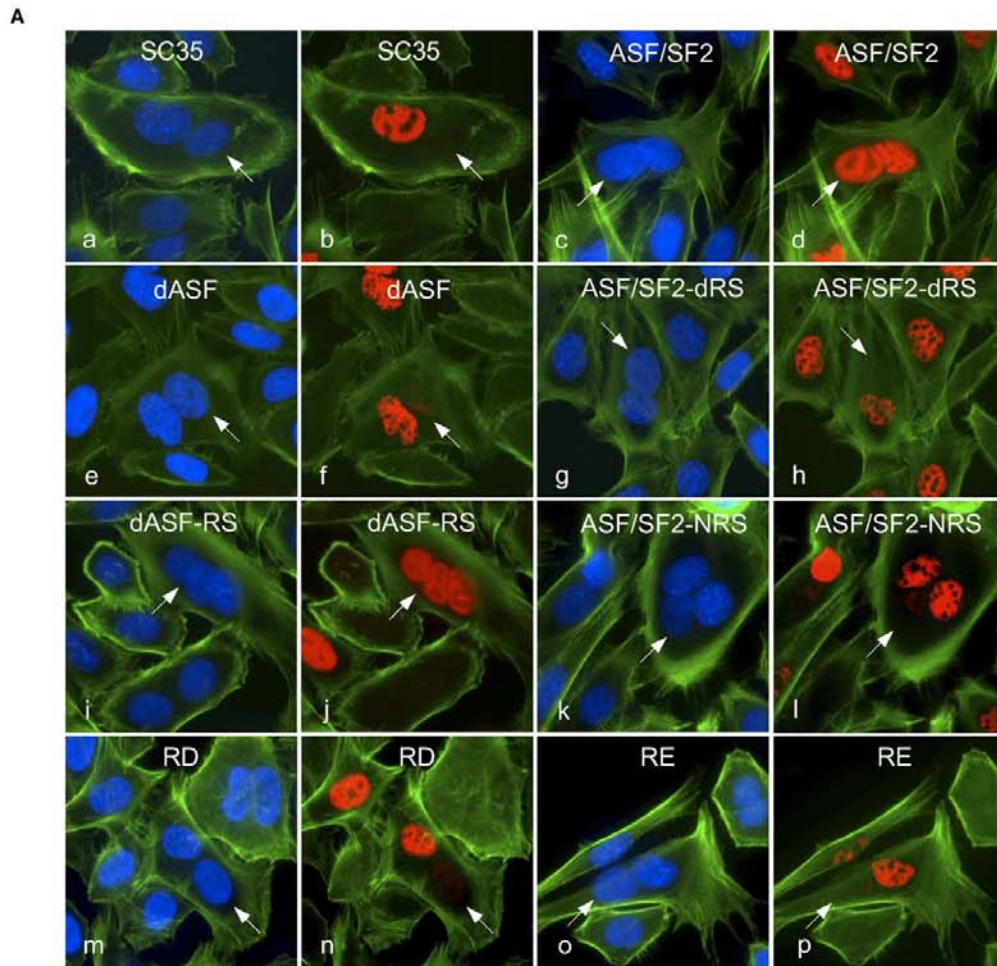
#### Functional Substitution of RS Repeats by RD and RE Mutants

Shuttling SR proteins such as ASF/SF2 and 9G8 have been shown to remain associated with mRNPs from splicing to export, suggesting that they may play a role in the mRNA export process (Huang et al., 2003). It was further demonstrated that these shuttling SR proteins interact with a critical mRNA export mediator TAP in a dephosphorylation-dependent manner (Huang et al., 2004; Lai and Tam, 2004). Our current finding that shuttling was not obligatory for SR proteins to function in vivo raised several interesting questions regarding (1) how nonshuttling SR proteins would avoid blocking mRNA maturation from splicing to export, (2) whether prevention of a shuttling SR protein from dephosphorylation would cause mRNA to accumulate in the nucleus, and (3) whether blocking the ability to shuttle would impair mRNA export.

To address these questions, we tested the function of a number of ASF/SF2 dephosphorylation mutants in MEFs. The RD and RE mutants of ASF/SF2 were previously reported, in which the serine (S) residues in the

domain were replaced by the negatively charged aspartic acid (D) or glutamic acid (E) residues (Cazalla et al., 2002). These mutants have been shown to be active in splicing, indicating that the negatively charged amino acids are sufficient to mimic a hyperphosphorylation state of the RS domain. To extend these in vitro splicing results, we tested the RD and RE mutants in vivo and found they were fully functional in complementing ASF/SF2-depleted MEFs (Figure 3B). These observations strongly suggest that a phosphorylation/dephosphorylation cycle is not critical for individual SR proteins to function in vivo. Interestingly, both RD and RE mutations also prevented ASF/SF2 from shuttling between the nucleus and the cytoplasm (Figures 4Am-4Ap), again consistent with the functionality of the other ASF/SF2 shuttling mutants in complementation.

To determine whether depletion of ASF/SF2 alone or prevention of its dephosphorylation and shuttling would impair RNA export, we carried out in situ hybridization using Texas-Red-labeled oligo-dT. As a control, we transfected wt MEFs with a dominant-negative



nucleoporin mutant nup160 (aa 317–697), which was previously shown to block mRNA export (Vasu et al., 2001). Poly(A)<sup>+</sup> mRNA accumulated in the nucleus of the cells expressing the mutant pore protein, in contrast to the normal distribution of poly(A)<sup>+</sup> mRNA in the cytoplasm as well as in nuclear speckles in untransfected MEFs (Figures 4Ba and 4Bb). In ASF/SF2-depleted MEFs, we detected no sign of mRNA accumulation (Figure 4Bc). This was also true for ASF/SF2-depleted MEFs complemented by the RD mutant or by ASF/SF2 fused to the SC35 NRS, both of which were no longer able to shuttle (Figures 4Bd and 4Be). Together, these observations strongly suggest that depletion of a single shuttling SR protein or impairment of its ability to shuttle had little impact on mRNA export in MEFs.

#### SR Proteins during mRNP Maturation from Splicing to Export

The finding that depletion of a single shuttling SR protein did not cause a detectable effect on mRNA export may be explained by functional redundancy among SR proteins. However, the lack of impact on mRNA export due to the RD and RE mutants, which might be expected to impose some dominant-negative effects on mRNP maturation, was somewhat surprising. To address this paradigm as well as the related issue of why nonshuttling SR proteins do not interfere with mRNP maturation during the transition from splicing to export, we conducted an analysis of mRNP maturation by following a fractionation scheme developed by Piñol-Roma and colleagues (Mili et al., 2001). This approach has recently been used to document the association of TAP with hypophosphorylated shuttling SR proteins in postsplicing mRNPs (Huang et al., 2004; Lai and Tam, 2004). In wt and mutant ASF/SF2-complemented MEFs, individual exogenous genes were expressed at comparable levels, and *in vivo* phosphate labeling showed that all potential phosphorylation sites in ASF/SF2 were mutated in the RD mutant (Figure 5B).

Using this fractionation scheme, MEFs complemented by wt and various SR protein mutants were separated into cytosolic (S), nuclear soluble (NS), and nuclear matrix-associated insoluble (NI) fractions. mRNPs in these fractions were immunoprecipitated by a monoclonal antibody against hnRNP A1, a shuttling RNA binding protein associated with mRNPs at all RNA maturation stages from splicing to export (Mili et al., 2001). Consistent with previous characterization of mRNPs at these three stages, nuclear matrix-associated mRNPs contained a mixture of pre-mRNA and spliced mRNA, whereas nuclear soluble mRNPs were associated only with spliced mRNA (data not shown). Western blotting analysis of immunoprecipitated mRNPs showed that nuclear matrix-associated mRNPs were associated

with a mixture of hyper- and hypophosphorylated ASF/SF2, while nuclear soluble mRNPs mainly contained hypophosphorylated ASF/SF2 (Figure 5C, lanes 2 and 3), as previously reported (Lai and Tam, 2004). Interestingly, ASF/SF2 was efficiently released from exported mRNPs in the cytoplasm (Figure 5C, lane 1).

Strikingly, the RD mutant was present in the NI fraction but was largely absent from mRNPs in the S and NS fractions, indicating that the mutant SR protein was released early during mRNP maturation (Figure 5C, lanes 4–6). A similar observation was also made with the RE mutant (data not shown). These observations provide a plausible explanation for the finding that the RD and RE mutants could fully complement the ASF/SF2-depleted MEFs without blocking mRNA export. To test whether normal nonshuttling SR proteins behave in a similar way, we tested SC35 and found that it was also released (Figure 5C, lanes 7–9).

We next tested the NRS-retained ASF/SF2 and found that it was strongly attached to the matrix-associated mRNPs, but little, if any, of this protein was associated with the nuclear soluble and cytosolic mRNPs (Figure 5C, lanes 10–12). This observation is consistent with its conversion from a shuttling to a nonshuttling SR protein. In contrast, when the RS domain was removed from ASF/SF2, the mutant protein was constantly attached to mRNPs, as evidenced by the same ratio between the  $\Delta$ RS and hnRNP A1 proteins at all RNP maturation stages (Figure 5C, lanes 13–15). Although the association of  $\Delta$ RS with cytosolic mRNP may be contributed by direct binding of the cytoplasmic  $\Delta$ RS protein to exported mRNA, this observation is reminiscent of phosphorylation-dependent release of the SR-like RNA binding protein Npl3p in yeast (Gilbert et al., 2001). Together, our results suggest distinct recycling pathways for shuttling and nonshuttling SR proteins during mRNP maturation in mammalian cells. While shuttling SR proteins remain associated with mRNPs to facilitate mRNA export, nonshuttling SR proteins are sorted away from postsplicing mRNPs, perhaps during an early cotranscriptional splicing step.

#### A Key Role of RS Domain Dephosphorylation for Sorting SR Proteins in the Nucleus

Although how nonshuttling SR proteins are released from postsplicing mRNPs remains to be defined, our existing data suggest a relationship between nuclear trafficking of SR proteins and the phosphorylation state of the RS domain. For example, mimicking a hyperphosphorylation state of the RS domain by the RD and RE mutants prevented shuttling and induced early release from mRNPs. The ability of the NRS to retard shuttling may also relate to a hyperphosphorylation state. To test this hypothesis, we prepared nuclear extracts

Figure 4. Analysis of Nucleocytoplasmic Shuttling of Wt and Mutant SR Proteins and Impact of Shuttling Mutants on mRNA Export (A) Prevention of ASF/SF2 shuttling by mimicking a hyperphosphorylated state in the RS domain. MEFs expressing various mutant SR proteins were fused to HeLa cells, and shuttling was determined by anti-HA staining (red). Nuclei from mouse and human cells were determined by DAPI (blue), and fusion was monitored by actin staining (green). Mouse nuclei were identified by punctate nuclear staining. Human nuclei are indicated by arrows. (B) Detection of mRNA export defects by oligo-(dT)<sub>50</sub> *in situ* hybridization. The expressed Nup160 mutant was detected by anti-myc immunostaining (green in [Ba]); and poly(A)<sup>+</sup> mRNA was localized by oligo-(dT)<sub>50</sub> *in situ* hybridization (red in [Bb]–[Be]). Accumulation of poly(A)<sup>+</sup> mRNA in Nup160 transfected nuclei is indicated by an arrow. Normal poly(A)<sup>+</sup> mRNA distribution (diffuse in the cytoplasm and punctate in the nucleus) is indicated by an arrowhead.

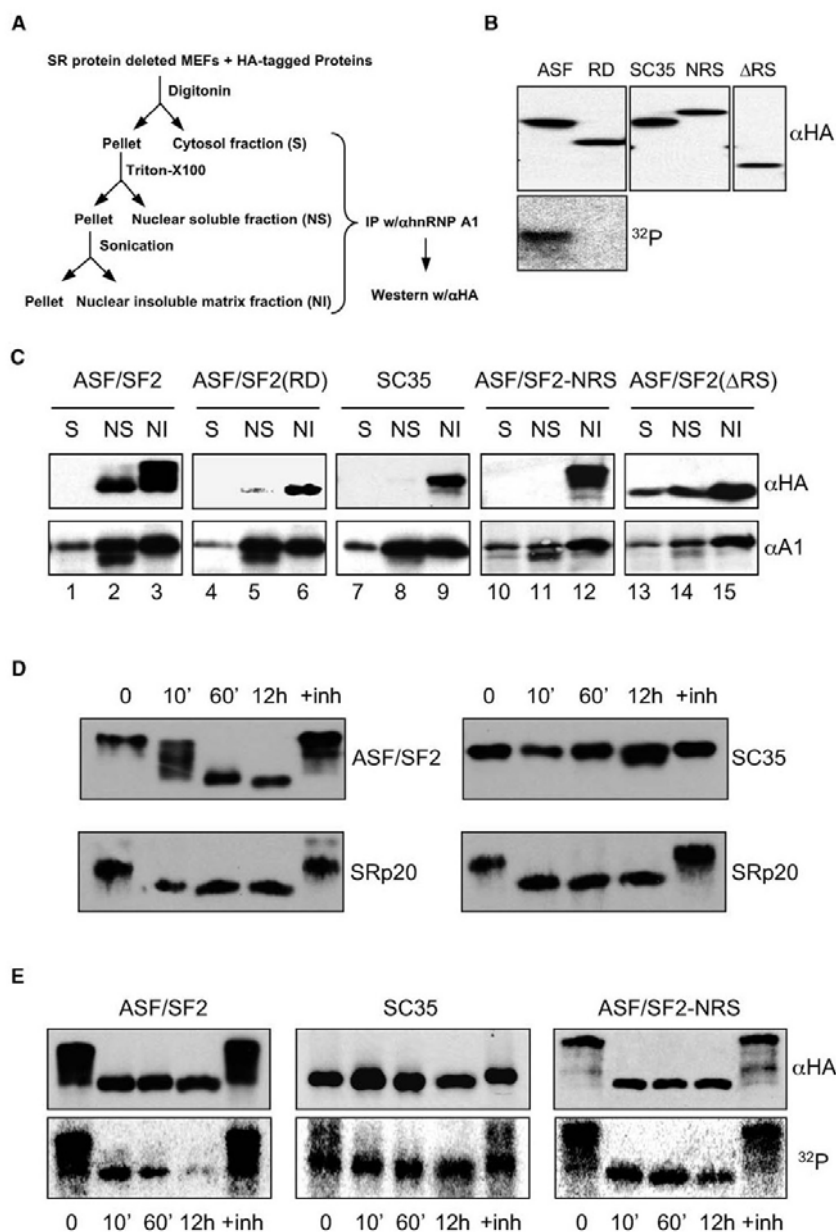


Figure 5. Distinct Pathways for SR Protein Recycling In Vivo and Differential Regulation of SR Proteins by Dephosphorylation

(A) Fractionation of ribonucleoprotein (RNP) complexes. S, cytosolic RNPs; NS, nuclear soluble RNPs; NI, nuclear insoluble, matrix-associated RNPs.

(B) The level of exogenously expressed SR proteins in total cell lysates determined by anti-HA immunoblotting. The lower panel shows in vivo labeled wt and the RD mutant.

(C) Fractionated RNPs were captured using anti-hnRNP A1 followed by immunoblotting with anti-HA (upper panels) and anti-hnRNP A1 (lower panels) antibodies.

(D) Nuclear extracts from indicated MEFs were incubated for various periods of time. HA-tagged SR proteins were detected by Western blotting. SRp20 was detected in the same extracts as an internal control. A cocktail of phosphatase inhibitors (+inh) was used to block the activity of endogenous phosphatases.

(E) In vivo-labeled SR proteins were immunoprecipitated and treated with calf intestinal phosphatase (CIP) for various periods of time as indicated.  $\beta$ -glycerophosphate was used to block the activity of CIP (+inh). Proteins were resolved in SDS-PAGE and detected by anti-HA Western blotting (upper panel). The level of phosphorylation was quantified by autoradiography (lower panel).



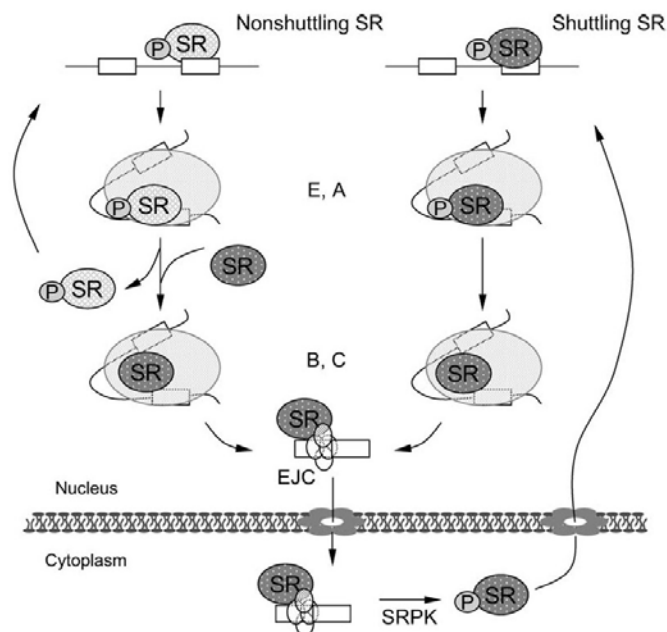


Figure 6. A Model for SR Protein Action and Recycling In Vivo

Shuttling and nonshuttling SR proteins are indicated by different fill patterns. "P" indicates a hyperphosphorylated state of SR proteins. Phosphorylated SR proteins first bind to nascent pre-mRNA transcripts (each unit may be from separate transcripts or from different regions in the same multi-intron-containing transcript) to initiate spliceosome assembly. For a shuttling SR protein (the pathway on the right), dephosphorylation may take place during the transition from prespliceosome (E and A complexes) to mature spliceosome (B and C complexes). Such a shuttling SR protein may remain associated with post-splicing RNPs and contribute to mRNA export in conjunction with the exon junction complex (EJC). Exported RNPs may be disassembled in the cytoplasm, likely aided by the action of the cytoplasmically localized SR-protein-specific kinases (SRPKs). SRPK-mediated phosphorylation also promotes reimport of the shuttling SR protein. When a nonshuttling SR protein is involved in initial spliceosome assembly (the pathway on the left), the SR protein is released, and the RS domain likely mediates the release. The released SR protein recycles within the nucleus for following rounds of splicing. The latter function may be fulfilled by another SR protein to promote splicing and transition from splicing to export. Such a function may require an available SR protein in a relatively hypophosphorylated state, which may result in preferential selection for a shuttling SR protein in the nucleoplasm.

from MEFs expressing various SR protein mutants and analyzed HA-tagged SR proteins by Western blotting after incubation of the extracts at 30°C for various periods of time to detect the action of endogenous phosphatases (Figure 5D). ASF/SF2 and SRp20, both of which are shuttling SR proteins, underwent efficient and progressive dephosphorylation, as indicated by a mobility shift in SDS-PAGE. This shift was due to dephosphorylation rather than protein fragmentation, because little mobility shift was detected in the presence of a cocktail of phosphatase inhibitors. In contrast, SC35 appeared largely resistant to endogenous phosphatases, which was clearly active in dephosphorylating SRp20 in the same extract (Figure 5D).

To further demonstrate the differential sensitivity of SR proteins to dephosphorylation, we performed *in vivo*  $^{32}\text{P}$  labeling of SR proteins and analyzed dephosphorylation of immunoprecipitated SR proteins by calf intestinal phosphatase (CIP). Individual precipitated SR proteins were quantified by anti-HA immunoblotting, while their degree of phosphorylation was measured by autoradiography (Figure 5E). Under the same treatment conditions, ASF/SF2 was readily dephosphorylated, which appears to progress in two phases. In the first 10 min, ~70% of incorporated phosphates were removed, and the remainder was slowly turned over during overnight incubation (Figure 5E, left panel). Dephosphorylation could be prevented by the phosphatase inhibitor  $\beta$ -glycerophosphate. In contrast, SC35 was highly resistant to dephosphorylation by CIP, and

~50% of incorporated phosphates remained associated with the protein even after overnight incubation (Figure 5E, middle panel) (note that the minor band above SC35 in the autoradiograph likely corresponds to a coimmunoprecipitated phosphoprotein because it was not detectable by the anti-HA antibody even when a large amount of sample was loaded on the gel).

As shown above, the NRS from SC35 was able to prevent ASF/SF2 from shuttling in a dominant and transferable fashion. We next addressed whether the function of the NRS was related to resistance to dephosphorylation. The ASF/SF2-NRS fusion protein was similarly labeled *in vivo* by  $^{32}\text{P}$ , immunoprecipitated by the anti-HA antibody, and treated with CIP (Figure 5E, right panel). This experiment showed that the NRS also transferred phosphatase resistance to ASF/SF2: while wt ASF/SF2 was largely dephosphorylated by CIP after overnight incubation, the ASF/SF2-NRS fusion retained ~20% of total phosphate after a similar treatment. Thus, the NRS appears to be a portable phosphatase-resistance domain from SC35.

Considered together, our analysis reveals that shuttling ASF/SF2 and nonshuttling SC35 are differentially regulated by dephosphorylation during mRNP maturation. Although this finding remains to be extended to all SR proteins, and other sequence motifs in SR proteins may use distinct mechanisms for sorting and recycling, our observations made with a variety of SR protein mutants strongly suggest that many shuttling and nonshuttling SR proteins may be differentially

regulated by dephosphorylation, and regulated sorting of SR proteins may be critical for mRNP transition from splicing to export in mammalian cells. Based on our current findings, we propose a model for SR protein sorting as depicted in Figure 6, which provides plausible explanations to a range of interesting but puzzling observations made in previous studies.

## Discussion

**Central Requirement of SR Proteins for Cell Viability**  
SR splicing factors have been shown to play diverse roles in RNA metabolism, including constitutive and alternative splicing, mRNA export, RNA stability, and translation (Huang and Steitz, 2005). More recently, an SR protein has been linked to genomic stability via its role in suppressing R loop formation during transcription (Li and Manley, 2005). Given such important and diverse roles of SR proteins in gene expression, it is not surprising that SR proteins are critical for cell survival. Consistently, genetic deletion of ASF/SF2 in chicken DT40 cells is also lethal (Wang et al., 1998). However, RNAi-mediated knockdown of individual SR proteins does not seem to induce cell death in insects and nematodes (Longman et al., 2000; Park et al., 2004). It is unclear whether different functional consequences on cell viability result from different roles of SR proteins in different organisms or from the knockdown versus knock-out approaches.

The central question is in identifying which functional aspects of SR proteins are essential for cell viability. Because of the essential role of SR proteins in splicing, cell death may result from a broad effect on RNA processing. However, few targets have been identified for any specific SR protein in vivo, which has been widely assumed to be due to a functional redundancy among SR proteins. Functional redundancy, however, does not preclude the possibility that a reduction in the overall splicing efficiency causes some general metabolic defects, leading to cell cycle arrest and cell death. An argument against this possibility is the observation that genetic depletion of a SR protein does not seem to cause a general disruption of cell metabolism in non-proliferating cardiac myocytes (Ding et al., 2004; Xu et al., 2005). We may also consider the possibility that the requirement for cell survival may be due to defects after splicing or even unrelated to RNA metabolism. Shuttling SR proteins have been implicated in mRNA export, stability control, and translation (Huang and Steitz, 2005). However, we found that the shuttling property of a single SR protein is not essential for cell survival. Recently, it was shown that ASF/SF2 plays a role in maintaining genomic stability (Li and Manley, 2005). Genomic instability may trigger cell cycle arrest and apoptosis in DT40 cells. However, genomic instability does not explain the cell-lethal phenotype in ASF/SF2-depleted MEFs, because MEFs treated with a lethal dose of radiation could not proliferate but remain healthy looking without apoptosis and cell death for at least 2 weeks. Considered together, the requirement of SR proteins for viability of proliferating cells may be due to alterations of a specific and critical set of splicing events.

## Function of the RS Domain in Splicing

The RS domain in SR proteins plays a critical role in protein-protein and protein-RNA interactions in splicing. However, the RS domain is not required for in vitro splicing with certain pre-mRNA substrates (Zhu and Krainer, 2000). We found that the RS domain of ASF/SF2 is not essential for cell survival in MEFs. These observations raise an intriguing question of whether the essential role of SR proteins in vivo is due to their functions in alternative, but not constitutive, splicing. Consistent with this possibility, the RS domain is known to be dispensable for alternative splicing in vitro. Additionally, ASF/SF2-depleted MEFs could be rescued by the *Drosophila* homolog dASF, which was previously shown to be active in alternative splicing but not in constitutive splicing (Allemand et al., 2001). In theory, constitutive splicing events normally involve strong splicing signals and multiple splicing enhancers. SR proteins may be collectively important for constitutive splicing, but, because of their functional redundancy, the requirement for any specific SR protein is less crucial. For alternative splicing, on the other hand, the role of SR proteins becomes critical because alternative splicing events are often associated with weak splice sites and less positive regulatory elements. Consequently, many alternative splicing events may respond to specific SR proteins qualitatively (unique requirement) or quantitatively (dosage effect).

Interestingly, while the RS domain is dispensable for ASF/SF2 to function in vivo, an RS domain is required for dASF to complement ASF/SF2-depleted MEFs. The simplest explanation to this finding is that the dASF RRM is distinct from ASF/SF2 and thus suboptimal in binding ASF/SF2 targets. As a result, an RS domain is required to provide an extra contact to a nearby RNA sequence or a protein in the spliceosome to stabilize protein-RNA and/or protein-protein during splicing. Thus, a "nonspecific" RS domain may strengthen divergent RRMs in splicing. Many splicing enhancers may serve as binding sites for SR proteins (Fairbrother et al., 2002; Zhang and Chasin, 2004). The RRMs in SR proteins may only recognize a range of *cis*-acting RNA motifs, but, with the help of an RS domain, the range may be significantly extended. Consequently, the substrate spectrum for individual SR proteins is enlarged, thereby contributing to their functional redundancy in splicing.

## Do Shuttling SR Proteins Play an Essential Role in mRNA Export?

A puzzling observation from our current study is the lack of an effect on mRNA export upon depletion of ASF/SF2 or prevention of its ability to shuttle. A role for shuttling SR proteins in mRNA export has been demonstrated for SRp20 and 9G8 in injected oocytes (Huang et al., 2003). Like 9G8, ASF/SF2 has been shown to directly interact with TAP (Huang et al., 2004; Lai and Tam, 2004). Assuming ASF/SF2 plays a similar role in mRNA export, the lack of detectable mRNA export defects in ASF/SF2-depleted MEFs may be due to one of the following possibilities: (1) shuttling SR proteins may be able to stimulate mRNA export, but they are not essential for the process; (2) shuttling SR proteins may be involved in nuclear export of specific mRNAs, which could not be detected by oligo-dT in situ hybridization; or (3) shuttling SR proteins may share redundant functions in



mRNA export. Because of these possibilities, the role of shuttling SR proteins in mRNA export remains to be investigated.

#### Regulated Sorting of SR Proteins in the Nucleus

One of the major findings in this report is the regulated sorting of shuttling and nonshuttling SR proteins in distinct recycling pathways, which explains why nonshuttling SR proteins do not interfere with downstream mRNP maturation steps. As depicted in Figure 6, early release of nonshuttling SR proteins may be related to SR protein remodeling during splicing. It has been shown that phosphorylation is required for spliceosome assembly and dephosphorylation is crucial for catalysis (Mermoud et al., 1992; Kohtz et al., 1994; Mermoud et al., 1994; Cao et al., 1997). Previously, a thio-phosphorylated SR protein was used to initiate spliceosome assembly, and the stalled spliceosome was rescued by another SR protein capable of dephosphorylation, indicating that a cycle of phosphorylation and dephosphorylation is not obligatory for a single SR protein to function in each round of the splicing reaction (Xiao and Manley, 1998). It is unclear how a thio-phosphorylated SR protein in the stalled spliceosome could be rearranged to give room to another SR protein to continue the splicing reaction. According to the proposed sorting and exchange model, a phosphatase-resistant SR protein may be replaced by a phosphatase-sensitive one, which may also take place naturally in the nucleus. Clearly, the RS domain provides a point for regulation by phosphorylation and dephosphorylation. As shown recently, the RS domain appears to be critical for switches in protein-protein and protein-RNA interactions within the spliceosome (Shen and Green, 2004). Such RS domain-mediated switches may also explain the puzzling observation that at least one RS domain, in either U2AF65 or U2AF35, is required for the function of the U2AF heterodimer in *Drosophila* because one RS domain may be sufficient for rearrangement of U2AF in the spliceosome (Rudner et al., 1998).

It is striking that nonshuttling SC35 carries a domain that confers remarkable and unprecedented resistance to phosphatases. Currently, little is known about specific phosphatases for SR proteins in mammalian cells. The phosphatase Glc7p was recently identified in budding yeast, which directly acts on Npl3p, an SR-like RNA binding protein implicated in mRNA export (Gilbert and Guthrie, 2004). It will be interesting to determine whether this PP1-type phosphatase is responsible for dephosphorylation of SR proteins in mammalian cells and, if so, whether ASF/SF2, SC35, and other SR proteins show differential sensitivity. In any case, our current results clearly indicate that SR proteins may be regulated by differential dephosphorylation, and that the phosphorylation-state-dependent sorting mechanism may be critical for an orderly transition of mRNPs from splicing to export.

#### Experimental Procedures

##### Plasmids

To express hemagglutinin (HA)-tagged ASF/SF2 by retrovirus, an adaptor containing the HA tag bracketed by the BclI and XbaI sites was first ligated to the N terminus of the ASF/SF2 coding sequence PCR amplified from the pCEP4-ASF/SF2 plasmid (Lai et al., 2001). The tagged unit was then amplified by PCR using specific primers

carrying the BclI and BamHI sites, and the resulting fragment was inserted into the BamHI site in the retroviral vector pBaBe-Hygro. The pRevTRE-ASF/SF2 plasmid was constructed by subcloning HA-tagged ASF/SF2 cDNA into the pRevTRE vector (Clontech). pBaBe-Hygro-RD, RE, RS10, NRS mutants were generated by replacing ASF/SF2 cDNA in pBaBe-Hygro-HA-ASF/SF2 with individual sequences from pCGT7-SF2-RD, SF2-RE, SF2-RS10, and SF2/NRS-SC35 (Cazalla et al., 2002). pBaBe-Hygro-HA-dASF was generated similarly by using the *Drosophila* ASF sequence from pTrcHis-dASF (Allemand et al., 2001). Individual human/*Drosophila* chimeric ASF/SF2 constructs were constructed by using proper restriction fragments from the human and fly genes. Cre cDNA was PCR amplified and cloned into the EcoRI and XhoI sites in the pBaBe-Puro vector.

##### Retrovirus Infection

To produce retrovirus, individual constructs were transfected by calcium phosphate into LinX cells (Hannon et al., 1999). Supernatant was collected 72 hr after transfection and cell debris removed by centrifugation at 300 × g for 5 min. Viral titers were estimated by the colony formation assay after drug selection. To infect MEFs, three-fourths of the viral supernatant (estimated MOI of 10) was mixed with one-fourth fresh culture media and 10 μg/ml of polybrene, and, after spinning at 500 × g for 1 hr, the mixture was applied to freshly seeded MEFs. Stable cell lines were selected by adding proper drugs (4 μg/ml puromycin, 300 μg/ml hygromycin, and 800 μg/ml G418) to culture media 48 hr after infection.

##### Construction and Characterization of tet-Inducible MEFs

Primary murine embryonic fibroblasts were derived from "floxed" ASF/SF2 and SC35 mice (Wang et al., 2001; Xu et al., 2005). Cells were immortalized by SV40 large T antigen. The established MEF lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, and appropriate antibiotics. To introduce the tet-off system to the cell, immortalized MEFs were coinfecting with retroviruses carrying RevTet-Off-IN (Clontech) and RevTRE-ASF/SF2. Stable cell lines were cloned by serial dilution after drug selection with 300 μg/ml hygromycin and 800 μg/ml G418. Tet-regulated expression was monitored 3–5 days after adding 1 μg/ml Doxycycline (BD Bioscience) to culture media. Clones showing tight tet control were selected for deletion of the endogenous gene by a Cre-expressing virus followed by puromycin selection. Isolated cells were subjected to single cell cloning, and Cre-induced gene deletion was verified by PCR genotyping and Western blotting. To detect the expression of the exogenous gene, total lysate corresponding to ~4 × 10<sup>4</sup> cells was loaded on a 10% SDS-PAGE gel, transferred to nitrocellulose, and probed by peroxidase-conjugated anti-HA antibody 3F10 (Roche).

Established MEF cell lines were cultured in 6-well plates. After the addition of Dox, cell growth was monitored by taking one plate each day, washing twice in PBS, and fixing with 0.5% glutaraldehyde in PBS. Fixed cells were stained with 0.1% crystal violet overnight at 37°C. Crystal violet was extracted from individual wells with 1M acetic acid, measured at OD<sub>595</sub>. For cell sorting analysis, cells were harvested, resuspended in PBS, and analyzed on a LSR Flow Cytometer (Becton Dickinson). The Br-dU uptake assay was performed using a kit from Roche.

##### Heterokaryon Assay

MEFs expressing HA-tagged proteins were mixed with HeLa cells in the 2:3 ratio followed by inoculation on coverslips. After attachment, cells were incubated for 2 hr in the presence of 50 μg/ml cycloheximide. Afterward, the concentration of cycloheximide was increased to 100 μg/ml and incubation continued for another 30 min. Cell fusion was induced by polyethylene glycol (PEG) as previously described (Pinol-Roma and Dreyfuss, 1992). After fusion, cells were incubated for 2 hr in the presence of 100 μg/ml cycloheximide and then fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Fixed cells were permeabilized in 0.5% Triton X-100 for 5 min, incubated for 1 hr at room temperature with an anti-HA antibody HA.11 (Covance) in PBS plus 5% FBS, washed with PBS, and stained for 1 hr with Alexa594-conjugated Donkey anti-mouse IgG (1:500; Molecular Probes) and Alexa488-conjugated phalloidin

(1:500; Molecular Probes) in PBS plus 5% FBS. Coverslips were mounted with a mounting solution containing DAPI and imaged under a Zeiss Axioskop microscope.

#### Immunopurification of mRNP Complexes from MEFs

Preparation of mRNPs was according to Mili et al. (2001). Briefly, cells were collected in 1.0 ml RSB-100 (10 mM Tris-HCl [pH 7.4], 100 mM NaCl, 2.5 mM MgCl<sub>2</sub>). Digitonin (Calbiochem) was added at a final concentration of 40 µg/ml, and cells were incubated on ice for 5 min. After centrifugation at 2000 × g for 8 min, the supernatant was collected as the soluble cytosolic fraction. The pellet was resuspended in RSB-100 containing 0.5% Triton X-100 and incubated on ice for 5 min. The soluble nuclear fraction was then collected after centrifugation at 2000 × g for 8 min. The pellet was finally resuspended in the same buffer and disrupted by sonication (Heat Systems XL2005) twice, 5 s each time. The sonicated material was layered onto a 30% sucrose cushion in RSB-100 and centrifuged at 4000 × g for 15 min. The supernatant was collected as the nuclear insoluble fraction. Individual fractions (500 µl) were used for mRNP isolation by adding 25 µl protein A-Sepharose (Pharmacia) pre-conjugated with 2 µg purified anti-hnRNP A1 (4B10) antibody. After incubation at 4°C for 1 hr with rocking, protein A beads were washed with RSB-100 containing 0.5% Triton X-100 and then with RSB-100 alone. Purified mRNP complexes were eluted in 20 µl SDS loading buffer and analyzed in a 10% SDS-PAGE gel.

#### Poly(A)<sup>+</sup> RNA In Situ Hybridization

Poly(A)<sup>+</sup> RNA was detected by in situ hybridization with Texas Red-oligo(dT)<sub>50</sub> as described (Vasu et al., 2001). As a positive control, a nucleoporin mutant nup160 (aa 317–697) in pCS2MT was transfected into wt MEFs to detect mRNA accumulation in the nucleus. MEFs were grown on a coverslip for ~16 hr before fixation with 3% formaldehyde in PBS for 20 min on ice and permeabilization with 0.5% Triton X-100 in PBS. After incubation for 5 min in 2 × SSC, cells were prehybridized for 1 hr at 37°C with 50% formamide in 2 × SSC containing 1 mg/ml BSA, 1 mg/ml yeast tRNA (Invitrogen), 1 mM vanadyl ribonucleoside complexes (Sigma), and 10% dextran sulfate. Permeabilized cells were hybridized for 16 hr at 37°C with Texas Red-oligo(dT)<sub>50</sub> (MWG Biotech) at the concentration of 50 µg/µl in the same buffer, washed three times in 2 × SSC (37°C, 5 min each), and then refixed as above. The expression of the mutant nup160 was detected with a FITC-anti-myc antibody (1:500; Calbiochem).

#### Phosphatase Treatment and In Vivo <sup>32</sup>P Labeling

MEFs complemented by ASF/SF2 and SC35 were additionally infected with a retrovirus expressing HA-tagged SRp20 as an internal control. Nuclear extracts were prepared from these MEFs. Nuclear extracts corresponding to 5 × 10<sup>6</sup> cells were diluted in 20 µl buffer (20 mM HEPES (pH 7.4), 100 mM KCl, 1 mM DTT) and then incubated at 30°C for different periods of time. As a control, nuclear extracts were incubated for 1 hr in the presence of 1% phosphatase inhibitor cocktail (Sigma). The reaction was stopped by adding 20 µl of the SDS-PAGE loading buffer, and samples were analyzed by Western blotting.

For in vivo labeling, MEFs cultured in 100 mm dishes were washed with phosphate-free DMEM (MP Biomedicals) and labeled for 4 hr in phosphate-free DMEM plus 10% FBS and 0.25 mCi/ml <sup>32</sup>Pi (MP Biomedicals). After labeling, cells were washed with PBS three times and then subjected to disruption by sonication in RSB-100 containing 0.5% Triton X-100 and 1% phosphatase inhibitor cocktail. HA-tagged SR proteins were immunopurified by an anti-HA antibody under the same condition used for purification of hnRNP complexes. Purified SR proteins were incubated at 30°C for various periods of time with 0.5 U/µl calf intestine phosphatase (NEB) in the phosphatase buffer (100 mM NaCl, 50 mM Tris-HCl (pH 7.9), 10 mM MgCl<sub>2</sub>, 1 mM DTT). HA-tagged proteins were detected by Western blotting, and the level of phosphorylation was quantified on the typhoon imaging system (Amersham Pharmacia Biotech).

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## **CHAPTER IV**

### **The SR Family of Splicing Commitment Factors is Required for Transcriptional Elongation in Mammalian Cells**

**Abstract**

It has been well documented that transcription can affect processing of nascent transcripts, and conversely, components of the splicing machinery can stimulate transcription<sup>1-3</sup>. However, the mechanism for linking these two fundamental processes in gene expression has remained elusive. Here, we report that SR proteins, a family of RNA binding proteins required for committing pre-mRNA to the splicing pathway<sup>4</sup>, are critical for transcription elongation in mammalian cells. Analysis of nascent transcripts revealed gene-specific blockage of transcription elongation in SR protein-depleted cells, and remarkably, the defect could be functionally rescued using a purified SR protein, suggesting a direct role of SR proteins in this process. The elongation defects triggered extensive double-stranded DNA breaks, leading to ATM-mediated activation of p53, induced p21 expression, and cell cycle arrest. These findings suggest that timely recognition of splicing signals by SR proteins in nascent transcripts is a driving force for transcription elongation in vertebrates.

Increasingly evidence suggests that gene transcription is temporally coupled with pre-mRNA processing during gene expression in mammalian cells <sup>1</sup>. In most experimental systems, the transcription machinery appears to recruit various RNA processing factors to allow co-transcriptional processing of nascent transcripts <sup>5,6</sup>. In addition, many transcription factors, including Pol II itself, may have a dual role in transcription and splicing, suggesting that these essential gene expression steps are essentially an integrated process for proper gene expression in eukaryotic cells <sup>2,3</sup>. Interestingly, an early report indicated that the splicing machinery co-purified with a transcription elongation factor appeared to have a stimulating effect on transcription elongation, suggesting that splicing may be reciprocally coupled with transcription <sup>7</sup>. However, it remains undefined whether the entire splicing complex or a specific component(s) in the complex is involved in transcription elongation. In fact, the existing data do not rule out the possibility that a transcription factor(s) in the co-purified splicing complex might be responsible for the observed effect. Thus, the reciprocal coupling between transcription and splicing still remains conceptually possible, but experimentally unproven.

SR proteins are a class of RNA binding proteins capable of kinetically committing pre-mRNA to the splicing pathway and facilitating spliceosome assembly in multiple steps <sup>4,8</sup>. Consistent with their role as essential splicing factors in higher eukaryotic cells, SR proteins are essential for viability of proliferating cells, such as chicken DT40 cells <sup>9</sup> and primary mouse embryo fibroblasts (MEFs) (Figure IV-1A; <sup>10</sup>. Puzzling, however, is the observation that inactivation of single SR proteins in vivo does not seem to cause widespread defects in splicing of most cellular genes, which is thought to be due

to functional redundancy among SR proteins in constitutive splicing<sup>11,12</sup>. To determine potential global effect on gene expression in SR protein-depleted MEFs, we examined the production of nascent transcripts by <sup>3</sup>H-labeling in MEFs after a specific SR protein was effectively depleted but the cells were still viable. We detected a significant attenuation in gene transcription (Figure IV-1B). Analysis of oligo-dT selected polyA<sup>+</sup> mRNA indicated that Pol II-mediated transcription was impaired in SR protein-depleted MEFs (Figure IV-1C). We also detected by RT-PCR down-regulation of several Pol III transcripts in SC35-depleted MEFs, consistent with the severe effect in the <sup>3</sup>H-labeling experiments in response to in vivo depletion of SC35 than SF2/ASF (Figure IV-1B, data not shown).

To further confirm that the defect resulted from defects in transcription rather than accelerated RNA turnover, we carried out in situ analysis of Bromo-uridine labeled nascent transcripts, revealing diminishing nascent Pol II and Pol III transcripts, which are diffusely localized in the nucleus (Figure IV-1D). In comparison, Pol I transcripts appear relatively unaffected in the nucleolus (Figure IV-1D). The reduction of nascent transcripts in the nucleoplasm was further illustrated by selective inhibition of Pol I transcripts with a low dose of Actinomycin. D (Act. D)<sup>13</sup>. These data indicate that the immediate effect of SR protein depletion in vivo is neither due to inefficient splicing nor induced RNA decay and suggest that SR proteins might play a critical role in transcription in mammalian cells.

The impairment of transcription in the nucleoplasm might be indirectly due to catastrophic events that ultimately lead to the malfunction of the core transcriptional machinery in SR protein-depleted cells. If this were the case, we would expect



widespread defects in the assembly of Pol II-containing transcription complexes on endogenous genes. To directly test this possibility, we utilized a custom array consisting ~20 tiled mouse genomic loci and conducted chromatin immunoprecipitation (ChIP) with antibodies against Pol II and various modified histones associated with gene activation using the newly developed ChIP-DSL technology <sup>14</sup>. Surprisingly, we detected no defect in histone modifications (e.g. Me<sup>3</sup>H3K4) and the assembly of Pol II-containing complexes on gene promoters in both SC35 and SF2/ASF-depleted MEFs (Figure IV-2A). Instead, we observed the accumulation of unphosphorylated Pol II in multiple gene in the tiling array, 3 representatives of which are illustrated in Figure IV-2A (arrows).

The accumulation was more prevalent in SC35-deleted MEFs compared to SF2/ASF-depleted MEFs, consistent with the severer effect of SC35 depletion than SF2/ASF on transcription (Figure IV-1B). Because dynamic Pol II phosphorylation is known to play a critical role in transcription elongation <sup>15</sup>, the accumulation of unphosphorylated Pol II in the transcribed region strongly suggests a specific transcription elongation defect in SR protein-depleted cells. Furthermore, although we have not yet identified SF2/ASF-responsive genes, which will require a larger tiling array, the data suggest gene-specific responses to in vivo depletion of different SR proteins. One of the common functional consequences in response to a defect in transcription elongation is the induction of alternative splicing <sup>16,17</sup>. Consistent with this possibility, we indeed detected induced inclusion of the alternative exon in the PTB gene <sup>18</sup> when the steady level of total PTB transcript was reduced in SC35-depleted MEFs compared to wt and SF2/ASF-depleted MEFs (Figure IV-2B and 2C).

The accumulation of unphosphorylated Pol II in the transcribed region strongly suggests but does not directly prove the elongation defect in SR protein-depleted MEFs. To directly analyze the nascent transcript, we labeled cells with Br-uridine<sup>13</sup>. Nascent PTB transcripts could be detected by RT-PCR in anti-BrdU antibody immunoprecipitated RNA from Br-uridine-labeled cells, but not from uridine-labeled or Br-uridine labeled cells inhibited with a high dose of Act. D, demonstrating the specificity in immunoprecipitation (Figure IV-3A). We next performed the nuclear run-on assay based on the use Br-UTP as diagrammed in Figure IV-3B. To directly assay for transcription elongation, we prepared nuclei from both wt and SC35-depleted cells according to the standard nuclear run-on protocol<sup>19</sup>. Analysis of Br-UTP labeled nascent PTB transcripts by RT-PCR showed attenuated elongation of the PTB transcript beyond the Pol II pausing region in response to SC35 depletion (Figure IV-3C and 3E, grey bars), demonstrating that SC35 is indeed required for transcription elongation *in vivo*.

Although SC35 depletion impaired transcription elongation, the effect could be indirectly due to functional defects of some critical transcription elongation factors in SC35-depleted cells or directly due to the requirement for the SR protein in the transcription elongation process. To distinguish between these possibilities, we attempted rescuing the elongation defect using purified SC35. Remarkably, we found that purified SC35 was sufficient to relieve the blockage at the Pol II pausing region, restoring the elongation of the nascent PTB transcript (Figure IV-3C and 3E, filled bars). The rescue is specific for SC35 because other purified SR proteins, such as SF2/ASF and 9G8, had little effect (Figure IV-3D). Addition of purified SC35 to wt nuclei had little

stimulating effect, suggesting that endogenous SC35 in the elongating complex is sufficient for transcription elongation (Figure IV-3F). Together, these results demonstrate a direct and specific role of SC35 in transcription elongation within the PTB locus.

Previously, Manley and colleagues demonstrated that *in vivo* depletion of SF2/ASF induced double-stranded DNA breaks in chicken DT40 cells due to delayed resolution of R-loop transiently formed beyond the elongating Pol II complex<sup>20</sup>. Our current observation extends this model by showing a direct role of SR proteins in transcription elongation (Figure IV-4). Because the previous study on SF2/ASF was carried out on the transformed chicken DT40 cells, we determined whether the induced double-stranded DNA breaks might represent a universal response to SR protein depletion in normal mammalian cells. By immunostaining wt and SR protein-depleted cells with an antibody against phosphorylated  $\gamma$ H2AX, which is known to become accumulated near DNA break sites in the nucleus, we indeed observed strong foci after withdraw of SC35 or SF2/ASF (Figure IV-4A). Cells are known to respond to double-stranded DNA breaks by the activation of the ATM pathway to arrest cell cycle progression before the completion of DNA repair<sup>21</sup>. We determined whether ATM was activated in SR protein-depleted cells and found by immunocytochemistry that ATM was indeed activated as indicated by site-specific phosphorylation of itself (Figure IV-4B) and one of its downstream substrates SMC1 (Figure IV-4C). As expected, activated ATM phosphorylated p53 at Ser-18 (Figure IV-4D), which then induced p21 overexpression (Figure IV-4E).

Considered together, our data suggest a general model for functional and mechanistic coupling between transcription and splicing (Figure IV-4). As shown previously, SR proteins are recruited to sites of transcription in the nucleus via their interaction with the carboxy domain of the largest subunit of the Pol II polymerase<sup>22</sup>. The recruitment allows efficient recognition of splicing signals in emerging transcripts for co-transcriptional assembly of splicing complexes<sup>2,3</sup>. Here, we provide the first experimental evidence that SR proteins required for committing pre-mRNA to the splicing pathway are in fact critical for transcription in the first place. In wt cells, the interaction of SR proteins with emerging splicing signals may allow resolution of R-loop in a timely manner and a defect in this process exposes single-stranded DNA attackable by nucleases, leading to double-stranded DNA breaks in the stalled regions. In this regard, the function of SR proteins may be viewed as a functional equivalent to the single-stranded DNA binding protein PRA essential for elongation of DNA replication forks<sup>23</sup>. The induced double-stranded DNA breaks activate the ATM pathway to arrest cell cycle progression, an immediate functional consequence observed in SR protein-depleted cells<sup>20</sup>. Because of widespread defects in transcription, SR protein-depleted cells are therefore committed to irreversible total mortality. The transcription defects induced by SR protein depletion may be particularly detrimental to proliferating cells relative to terminally differentiated non-dividing cells, which may explain the dispensability of SC35 in non-dividing cardiomyocytes<sup>11</sup>. The experimental evidence documented here demonstrates that transcription and RNA splicing are not only temporally coupled but are actually intertwined events that are mutually dependent on

one another and that the SR splicing commitment factors function as key integrators in these processes that are essential for proper gene expression in eukaryotic cells.

## **Experimental procedures**

### **Cell culture**

SF2/ASF and SC35 tet-repressible MEFs were derived as previously described<sup>10</sup>. The MEFs were normally cultured in DMEM plus 10% FBS and 1mM sodium pyruvate. The exogenous SR protein was completely depleted in ~4 days in the presence of 1ug/ml of Doxycycline (Dox), but treated cells remained viable up to 10 days. Live cells were quantified by fixation with 0.5% glutaraldehyde in PBS followed staining with 0.1% crystal violet overnight at 37C. All assays were performed on cells treated with Dox for 5 days.

### **Nascent RNA analysis by tritium and Br-uridine labeling**

In vivo tritium labeling was performed as described<sup>24</sup>. Briefly, <sup>3</sup>H-uridine (15Ci/mmol) was added to cells in 10cm dish at a concentration of 0.5uCi/ml for 2 hrs. Cells were washed 4 times with ice-cold PBS followed by addition of 10 ml cold 5% trichloroacetic acid (TCA). After 5 min, TCA soluble fraction was harvest (TCA soluble pool) and remaining was rinsed twice with 5% TCA and the acid-precipitated material was hydrolyzed by incubation with 10ml 10% TCA at 70°C for 1 hr. Aliquots (1 ml) of both soluble and TCA-precipitated/hydrolyzed materials were counted in Beckman scintillation counter. To select <sup>3</sup>H-labeled mRNA, cells were harvested in Trizol (Invitrogen) and total RNA was purified. PolyA<sup>+</sup> mRNA was selected using the

Oligotex mRNA extraction kit (Qiagen). To label nascent RNA with Br-uridine, cells growing on coverslips were incubated with 2mM Br-uridine (Sigma-Aldrich) for 1 hr. Cells were next fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, stained for 1 hr with anti-Br-dU antibody (1:200; Sigma-Aldrich), and developed for 1 hr with Alexa594-conjugated Donkey anti-mouse IgG (1:500; Molecular Probes). Actin was simultaneously labeled with Alexa488-conjugated phalloidin. To inhibit Pol I activity, cells were incubated with 0.04ug/ml actinomycin D (Act. D) for 3 hrs before adding Br-uridine<sup>13</sup>. For qPCR analysis, the following PCR primers were used: PTB exon 1-3 (F: 5'-TTG GGT CGG TTT CTG CTA TTC-3', R: 5'-GGC AGA GCT, GCT CAT GAT G-3'), PTB exon 8-10 (F: 5'-AGA CTA, CAC TCG ACC TGA CCT GC-3', R: 5'-GGG TGT GAC TCT CTC AGG GTT C-3'), PTB exon 14-15 F: 5'-GTG TCA GAG GAC GAC CTC AAG-3', R: 5'-CAT GGT TGT GCA GTT CAA TC-3'), 18S rRNA (F: 5'-TGC GAA TGG CTC ATT AAA TC-3', R: 5'-GCC CGT CGG CAT GTA TTA G-3'), GAPDH (F: 5'-TAC ATG TTC CAG TAT GAC TC-3, R: 5'-CCA CGA CAT ACT CAG CAC-3).

### **Tiling array analysis by ChIP-DSL**

The ChIP-DSL assay was according to Kwon et al.<sup>14</sup>. Based on the ChIP-DSL method, ~20 randomly selected mouse genomic loci were tiled every 0.5 kb excluding repeats from 10 kb upstream the transcription start to 10 kb downstream the polyadenylation site. About  $1 \times 10^6$  cells were used for each ChIP-DSL experiment using anti-Me<sup>3</sup>H3K4 (07-473, Upstate Biotechnology) and anti-RNAP (8WG16, Covance). Both input (5% of total DNA) and antibody-enriched DNA were co-hybridized to the

tiling array. After data normalization, the signal corresponding to each probe was plotted against UCSC mouse genome assembly (May 2004 freeze) as the log<sub>2</sub> ratio of enriched over input.

### **Nuclear run-on and RT-PCR assays**

Preparation of nuclei and run-on reaction were carried out according to Ausubel et al.<sup>25</sup> with the following modifications. Instead of using <sup>32</sup>P-labeled NTPs, we used a NTP mix (1.8mM ATP, 0.5mM CTP and GTP, 0.375mM UTP) plus Bromo-UTP (0.125mM). The run-on reactions were performed at 25<sup>0</sup>C for 15 min on ~5 x10<sup>6</sup> purified nuclei in the run-on buffer (50mM Tris-HCl pH7.4, 10mM MgCl<sub>2</sub>, 150mM NaCl, 25% glycerol, 0.5mM PMSF and 25U/ml RNasin) plus the NTP/Br-UTP cocktail. After the reaction, total RNA was purified using the Qiagen RNeasy mini kit. BrU-labeled RNA from 100 ug total RNA was selected with 2ul anti-BrdU antibody (B2531, Sigma) bound to 15 ul Proten L beads (Pierce) in the RSB-100 buffer (10mM Tris-HCl pH 7.4, 100mM NaCl, 2.5mM MgCl<sub>2</sub> and 0.4% Triton X-100). BrU-RNA bound to beads was extracted and reverse transcribed in a 20ul RT reaction containing 1/3 of purified BrU-labeled RNA and 125ng random hexmers and 2 ul RT product was used for PCR analysis using <sup>32</sup>P-labeled primers. Each forward primer (10pmol) was labeled with 1 ul <sup>32</sup>P- ATP (800Ci/mmol). The following primers were used: PTB-a (F: 5'-TGG TGC TGT GCT GTT TCT TC-3', R: 5'-TGA GAG TGC AGG ACC CTC TT-3'), PTB-b (F: 5'-CCA GGA GAA ACT CGG TTG AA-3', R: 5'-TCA AGA CGG AAG ACG GAA AC-3'), PTB-c (F: 5'-CCC AGG TCA CTG GTA TGC TT-3', R: 5'-GGA CTA AGG CAG GCT CCT CT-3'), PTB-d (F: 5'-AGA GGA GGC TGC CAA CAC TA-3', R: 5'-GTC CAG

GGT CAC TGG GTA GA-3'), PTB-e (F: 5'-CTG GCT GAC TTA CGG GAT GT-3', R: 5'-TGT GCT TGA AGC TTT GGT TG-3'), and PTB-f (F: 5'-CCA CAG GAA CAG GCT AGG ATA G-3', R: 5'-TCT ATT CCG TGT GGT CAC AGA C-3'). PCR was performed for 32 cycles in a 20ul reaction containing 1X Promega Taq buffer plus 2.5mM MgCl<sub>2</sub>, 0.5pmol labeled forward primer and 0.5pmol unlabeled reverse primer. 4ul PCR product was analyzed on 6% TBE-buffered polyacrymide gel and then quantified on a Phosphoimager (Molecular Dynamics).

### **Immunostaining and Western blotting**

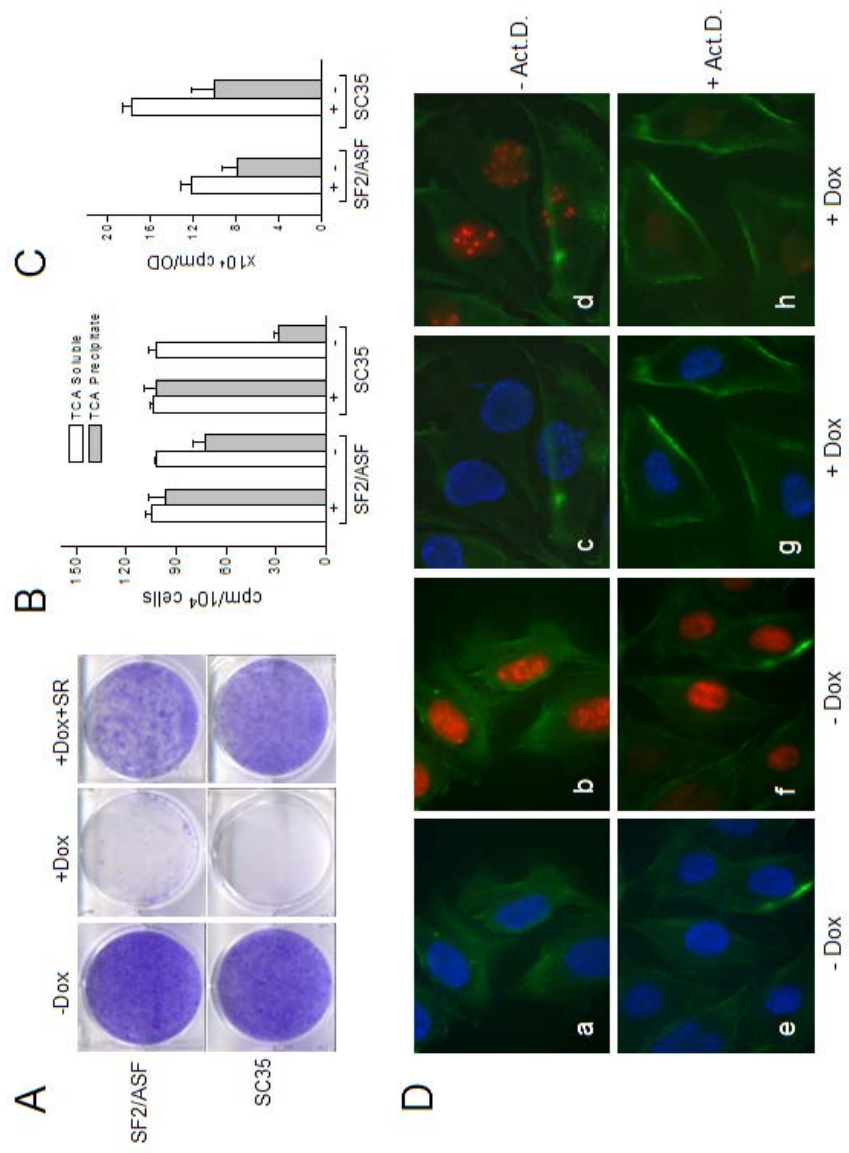
MEFs were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Fixed cells were permeabilized in 0.5% Triton X-100 for 5 min, incubated for 1 hr at room temperature with primary antibodies in PBS plus 5% FBS, washed in PBS, and stained for 1 hr with Alexa594-conjugated secondary antibody (1:500; Molecular Probes) and Alexa488-conjugated phalloidin. Western blotting was performed with total cell lysate corresponding to  $\sim 5 \times 10^4$  cells. Antibodies used in immunostaining or Western include anti- $\gamma$ H2AX (613401, Biolegend), anti-total p53 (FL-393, Santa Cruz Biotechnology), anti-phospho-p53 Ser18 (16G8, Cell Signaling). Anti-phosphoATM and anti-phosphoSMC1 were gifts of R. Gatti (UCLA).



## ACKNOWLEDGEMENTS

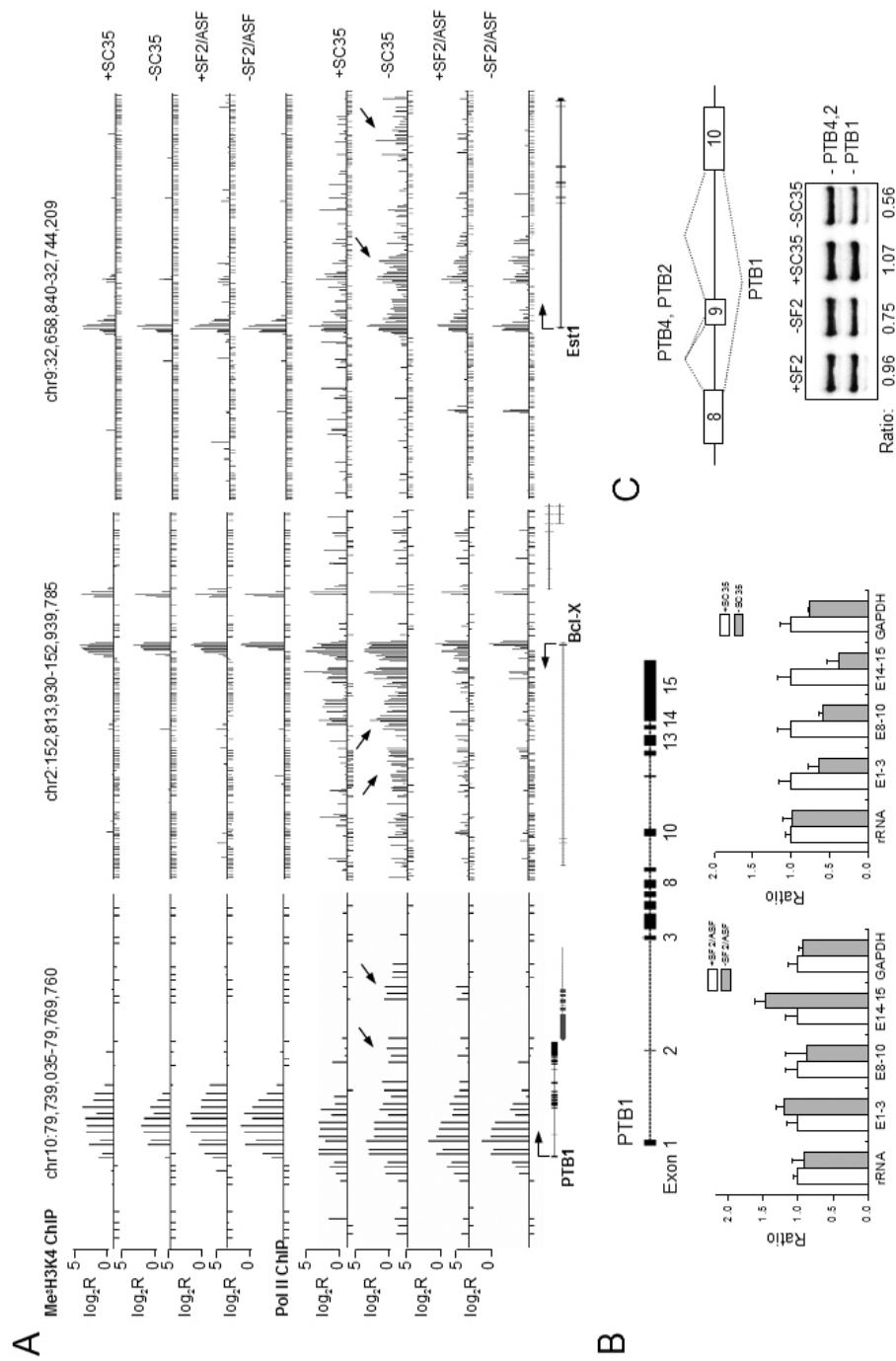
The text of Chapter IV, in part or in full, is a reprint of a manuscript in preparation. I was the primary researcher and the first author in this manuscript. The co-authors listed in this manuscript assisted and collaborated with the research which forms the basis for this chapter. I thank my co-authors Gabriela Coutinho-Mansfield, Dong Wang and Xiang-Dong Fu for permission to use this publication in my dissertation.

**Figure IV-1.** SR proteins are required for transcription in mouse embryo fibroblasts. (A) SR proteins are essential for viability of MEFs. The MEFs were derived from conditional SC35 and SF2/ASF knockout mice. The endogenous gene was deleted by a Cre-expressing virus and the cells were complemented by the exogenous gene expressed from a tet-off promoter<sup>10</sup>. Cells were stained 10 days after the addition of Dox. (B) Reduction of nascent transcripts in SR protein-depleted cells (5 days after the Dox treatment) as determined by <sup>3</sup>H-labeling. (C) Reduction of <sup>3</sup>H-labeled PolyA<sup>+</sup> mRNA. <sup>3</sup>H-labeled mRNA was counted in equal OD of purified Poly<sup>+</sup> mRNA. (D) Selective inhibition of transcription in the nucleoplasm of SR protein-deleted MEFs. In the presence of a low dose of Act. D, Pol I transcription was selectively suppressed in the nucleoli, which allowed more direct visualization of reduced transcription in the nucleoplasm in response to SR protein depletion.



**Figure IV-1**

**Figure IV-2.** Evidence for the transcription elongation defect induced by SR protein depletion in vivo. (A) Tiling array analysis showed no defect in complex assembly at gene promoters as indicated by Me<sup>3</sup>H3K4 and Pol II in SR protein-depleted MEFs. Instead, some unphosphorylated Pol II became accumulated (arrows) in the transcribed region of some (three illustrated), but not all genes, suggesting Pol II pausing induced by SR protein depletion in vivo. Gene structures are illustrated at the bottom. (B) qPCR analysis of PTB mRNA in SR protein-depleted MEFs using three different PCR primer pairs. Ribosomal RNA and GAPDH mRNA were analyzed as controls. (C) Alternative splicing of the PTB gene in SR protein-depleted MEFs. The results showed SC35 depletion-induced exon inclusion, consistent with the transcription elongation defect in specific response to SC35 depletion.



**Figure IV-2**

**Figure IV-3.** A direct role of SC35 in transcription elongation. (A) RT-PCR analysis of the nascent PTB transcript. Total RNA was extracted from wt MEFs labeled with Br-uridine, uridine, or Br-uridine plus Act. D, selected on beads coated with anti-BrdU antibody, and analyzed by RT-PCR. (B) Diagram for the nuclear run-on assay based on the use Br-UTP. (C) Nuclear run-on assay on the nascent PTB transcript. The gene structure and the Pol II tiling array result as in Figure IV-2A are shown to provide references for the positions of small PCR amplicons (a to f). A representative set of RT-PCR data is shown. Each set of three represents RNA from nuclei of wt MEFs (-Dox), SC35-depleted MEFs (+Dox), and SC35-depleted MEFs plus recombinant SC35 (+Dox+rSC35). RT-PCR reactions were performed in the linear range according to titration experiments. (D) Complementation of nuclei from SC35-depleted MEFs with another recombinant SR proteins SF2/ASF and 9G8, showing that related SR proteins were insufficient to rescue the elongation defects. (E) Quantification of the nuclear run-on assay. Data are presented from three biological repeats of the experiment in panel C. (F) Addition of recombinant SC35 to nuclei from wt MEFs, showing that the extra SC35 did not generally stimulate transcription elongation.

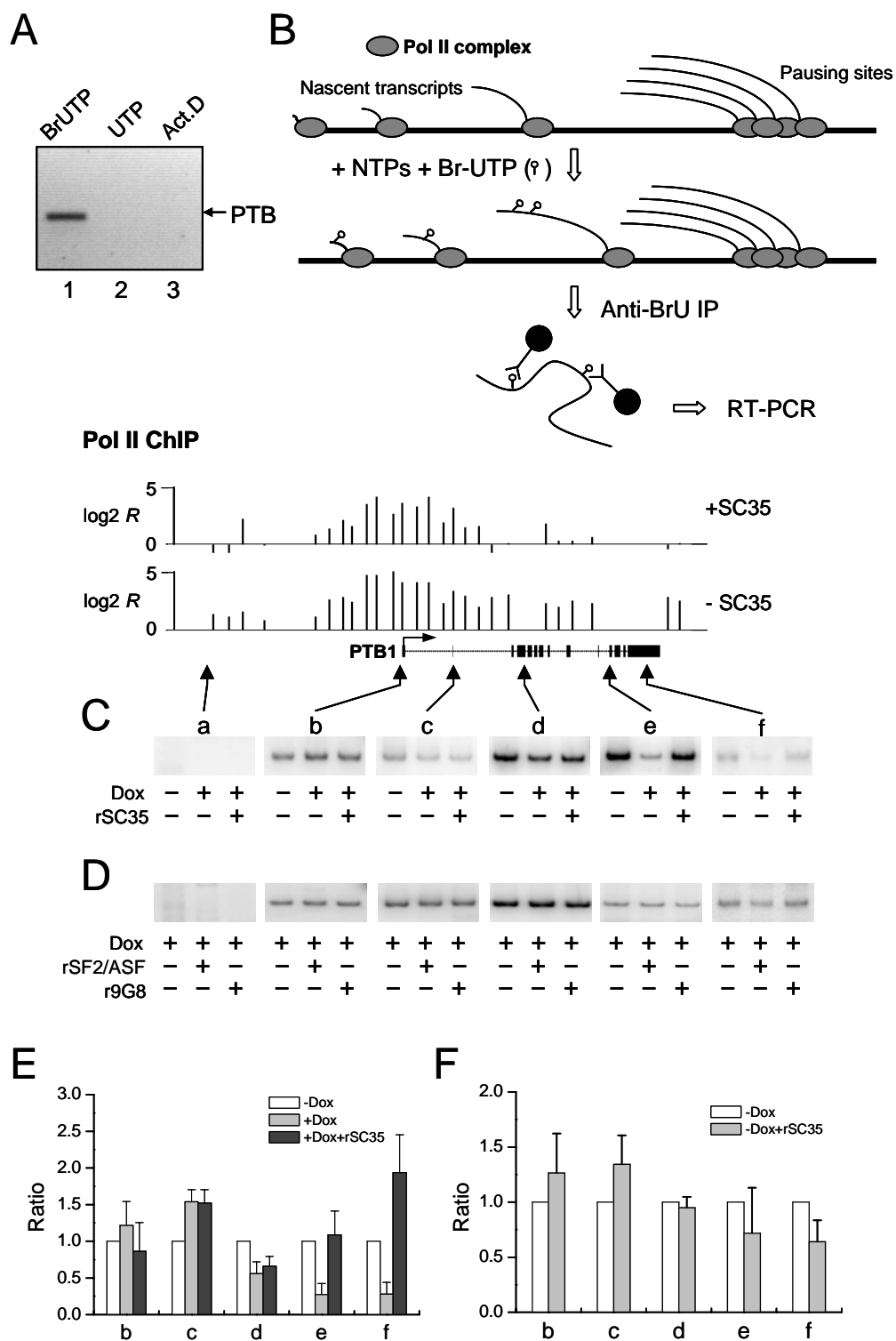


Figure IV-3

**Figure IV-4.** Model for coupling between transcription and splicing by SR proteins. In wt cells, SR proteins may initially scan cis-acting RNA elements in nascent transcripts, and once other splicing signals emerge, synergistic interactions may take place to stimulate spliceosome assembly co-transcriptionally. This may also allow displacement of RNA from template DNA in a timely fashion so that the transcribing Pol II complex can move forward. In the absence of a specific SR protein, the displacement may be retarded, resulting in the formation of extended R-loop, which becomes attackable by nucleases. Resulting double-stranded DNA breaks could be marked by phosphorylated  $\gamma$ H2AX (A). This triggered the activation of the ATM pathway in SR protein-deficient cells as indicated by ATM phosphorylation at Ser1981 and ATM-mediated phosphorylation of SMC1 at Ser966 (B). The activated ATM then induced p53 phosphorylation at Ser18 (C), and the activated p53 induced p21 expression (D), leading to cell cycle arrest as the immediate response to SR protein depletion in vivo as previously demonstrated in chicken DT40 cells<sup>26</sup> as well as in SR protein-depleted MEFs<sup>27</sup>.



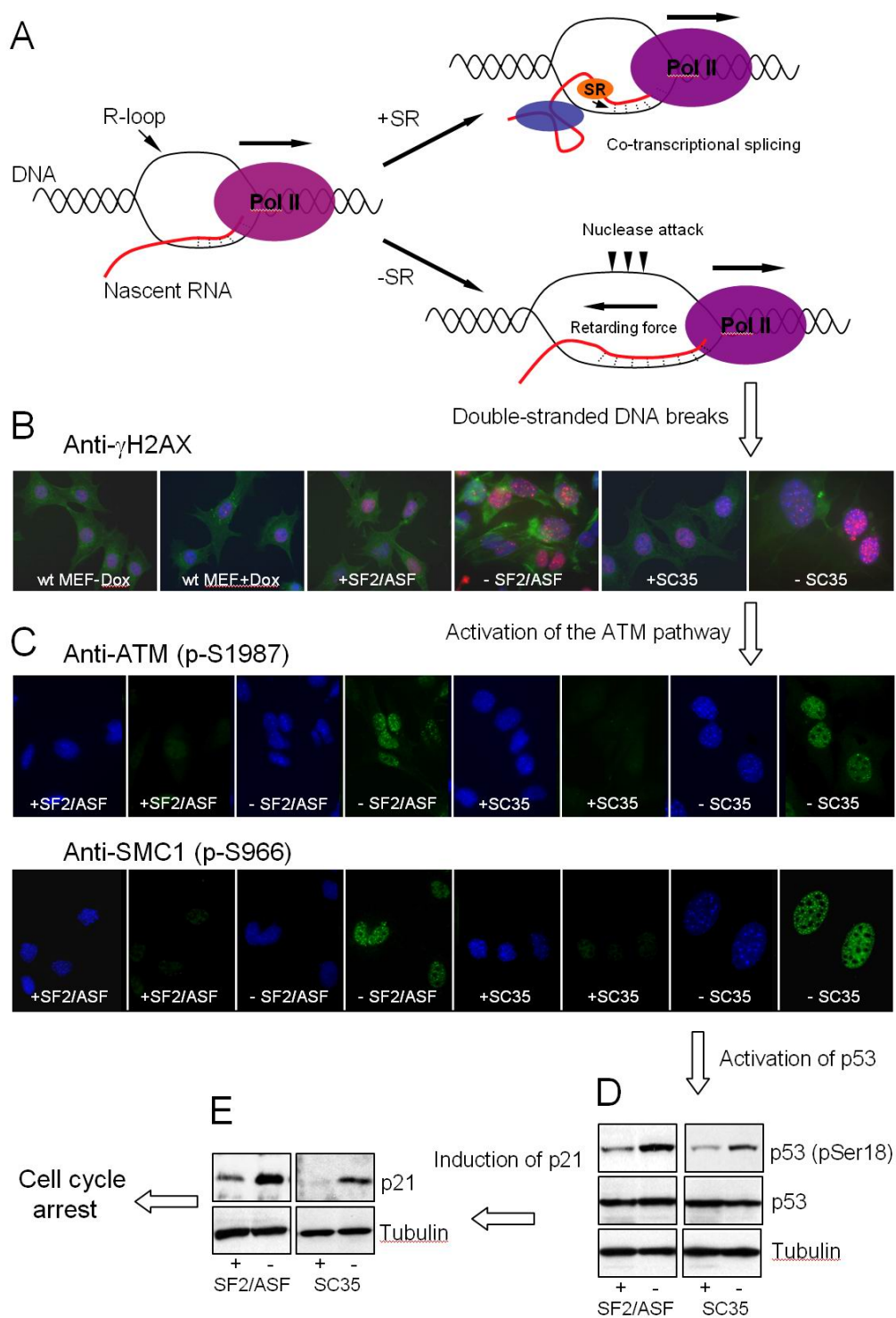


Figure IV-4

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## **CHAPTER V**

### **Splicing Regulator SC35 Is Essential for Genomic Stability and Cell Proliferation during Mammalian Organogenesis**

**Abstract**

The SR family of splicing regulators was initially characterized for their critical roles in constitutive and regulated splicing. Recent studies have implicated them in different aspects of gene expression processes, including transcription, RNA stability, mRNA transport and translation. While knockout studies have demonstrated their essential functions during animal development, the pathway(s) leading to specific cellular phenotype remains poorly understood. We report here that the SR protein SC35 controls cell proliferation during pituitary gland development, but is completely dispensable in terminal differentiated mature cardiomyocytes in mice. We show that loss of *SC35* in mouse embryonic fibroblasts (MEFs) induces G2/M cell cycle arrest and genomic instability, resulting at least in part from p53 hyperphosphorylation and hyperacetylation. While p53 hyperphosphorylation appears related to ATM activation, its hyperacetylation has been attributed to the increased expression of the acetyltransferase *p300* and the aberrant splicing of the deacetylase *Sirt1*. These findings reveal the involvement of SC35 in specific pathways to regulate cell proliferation during mammalian organogenesis and suggest its potential function in tumorigenesis.

## Introduction

More than half of the human genes undergo alternative splicing in humans, suggesting that regulated splicing plays a fundamental role in cellular physiology and organ development <sup>1</sup>. In addition, pre-mRNA splicing is not only an essential intermediate step in gene expression, but also connects upstream transcriptional events and downstream mRNA export, degradation, and even translation <sup>2</sup>. Given the importance of pre-mRNA splicing in gene expression and in various developmental and disease processes <sup>3</sup>, we are still at the beginning in understanding the role of distinct splicing regulators in cell proliferation and cell cycle progression that leads to specific cellular and developmental phenotypes.

Our current study focuses on the role of the SR family of splicing regulators. SR proteins are a family of RNA binding proteins that are characterized by one or two RNA recognition motifs and a signature RS domain enriched with arginine and serine repeats, hence the name for this family. SR proteins play critical roles in both constitutive and alternative splicing <sup>4-7</sup>. It is generally assumed that they play important house keeping roles in most, if not all, cell types. Indeed, knockout of individual SR genes in mice resulted in early embryonic lethality <sup>8-10</sup>. Similarly, using chicken DT40 cells or mouse embryo fibroblasts (MEFs) derived from conditional knockout embryos, ablation of the SR protein ASF/SF2 gave rise to a cell lethal phenotype <sup>11, 12</sup>. In the case of DT40 cells, cell mortality induced by ASF/SF2 depletion appears to be caused by multiple defects, including genomic instability triggered by elevated double strand DNA break, and subsequent apoptosis <sup>13-15</sup>.

Here, using conditional mouse knockout and inducible somatic genetic complementation systems, we report that the SR protein SC35 plays a critical role in cell proliferation, but surprisingly, it is dispensable in terminal differentiated mature cardiomyocytes in the heart. In conditional knockout MEFs, we found that depletion of SC35 induced cell cycle arrest at the G2/M phase. Remarkably, such a defect in cell cycle progression could be partially relieved by inactivation of the *p53* tumor suppressor gene, indicating a key role of *p53* in the SC35-mediated cell proliferation pathway. Analysis of the *p53* activation mechanism revealed hyperphosphorylation of *p53* at the site known to be modified by activated ATM<sup>16</sup>, consistent with double strand DNA break observed in SC35-deficient MEFs. We also detected *p53* hyperacetylation, which was linked to the increased expression of the *p53* acetyltransferase *p300*<sup>17-21</sup> and the aberrant splicing of the *p53* deacetylase *Sirt1*<sup>22-25</sup>. These observations provide evidence for a general role of SR proteins in maintaining genomic stability and cell proliferation in vertebrates. The ability of SR proteins to modulate *p53* functions in cell growth control now raises the possibility that SR protein overexpression detected in various tumor types<sup>26-28</sup> may directly contribute to cancer progression.

## Results

### Reduced Pituitary Gland Cell Proliferation in SC35 Conditional Knockout Mice

To begin to understand specific function of SC35 during mammalian development, we genetically engineered tissue-specific SC35 ablation in the well-characterized anterior pituitary gland primordium at embryonic day 9.5 (e9.5) using the *Pitx/Cre* transgenic mouse<sup>29</sup>. Histological analyses revealed growth arrest of the anterior pituitary gland at

e10.5 and e15.5, but Cre expression-negative posterior pituitary gland was unaffected (Figure V-1A, data not shown). The result demonstrates the essential role of SC35 in pituitary development, but the severe anterior pituitary gland growth arrest phenotype precluded further analysis of the SC35 function during pituitary cell differentiation and proliferation at later developmental stages. We therefore generated another transgenic line, *Pit-1-Cre-hpAP*, to express the Cre recombinase at the later stages of pituitary starting e13.5 using a 15.5kb *Pit-1* promoter. This promoter contains all regulatory information of the *Pit-1* gene and is capable of driving specific transgene expression in all three *Pit-1* cell lineages: the somatotrophs, lactotrophs and thyrotrophs (Figure V-1B)<sup>30,31</sup>. The dicistronic transgene expresses both the Cre recombinase and a separate human placenta alkaline phosphatase (hpAP) using an internal ribosomal entrance sequence (IRES) (Figure V-1B)<sup>32</sup>. Spatiotemporal expression pattern of the transgene was analyzed initially based on the hpAP enzymatic activity, demonstrating the early transgene activity at e13.5 that coincided with the initial expression of the endogenous *Pit-1* gene and the onset of the pituitary gland cell type differentiation (Figure V-1C). Immunohistochemical analysis using a Pit-1 specific antibody on the adjacent section showed that the hpAP activity colocalized with the *Pit-1* expression pattern (Figure V-1C) and strong transgene expression persisted to the adult pituitary (Figure V-1D). The enzymatic activity of Cre was further confirmed based on the expression of  $\beta$ -galactosidase using the ROSA26 Cre reporter mice (data not shown)<sup>33</sup>.

Conditional deletion of *SC35* using the *Pit-1-Cre-hpAP* transgenic mouse produced apparently normal adult mice in Mendelian frequency and the mutant mice exhibited comparable body weight to wild-type littermate controls (data not shown).



Gross analyses of the pituitary gland, however, revealed that the SC35-deficient gland was only one fifth to one tenth of the normal size compared to wt littermate controls (Figure V-1D). The severe hypoplastic pituitary phenotype could be potentially due to defects in *Pit-1* cell lineages, including increased cell death, reduced cell proliferation, and/or lack of cell differentiation. Because loss of SR proteins has been shown to induce apoptosis in DT40 cells<sup>15</sup>, we examined potential increase in programmed cell death in the mutant pituitary gland by using terminal transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay. We could not detect any significant increase of apoptotic cells in the mutant pituitary gland either during embryonic stages (e13.5, e14.5, e15.5 and e17.5) or postnatal stages (p2, p8, p15, p21, p28 and p35) (data not shown). These results indicate that cell death may not account for the severe hypoplastic phenotype caused by SC35 deletion.

We next determined whether SC35 ablation could prevent cell differentiation. Each cell type in the *Pit-1* lineage expresses a distinct terminal hormonal marker (*e.g.* GH from somatotrophs, PRL from lactotrophs and  $\beta$ -TSH from thyrotrophs). A small population of terminal differentiated *Pit-1*-dependent cell lineages was clearly detectable based on immunohistochemical analysis of the mutant adult pituitary gland (Figure V-2A, data not shown). By contrast, the *Pit-1*-independent gonadotrophs (LH positive) and corticotrophs (ACTH positive) were not affected (Figure V-2A). The increased density of LH-positive and ACTH-positive cells was likely due to the reduction of the *Pit-1* lineage cells. The presence of GH-secreting somatotrophs is consistent with the observation that the mutant mice had normal body weight. To ascertain that the remaining *Pit-1* lineage cells were indeed Cre-positive, we examined the expression of the transgene in mutant

mice, and found strong hpAP activity in the adult pituitary (Figure V-1D, and data not shown), therefore ruling out the possibility that the remaining population of the *Pit-1* lineage cells were due to selective expansion of Cre-negative cells.

The results described above thus suggest the possibility that SC35 is required for cell proliferation. The pituitary precursor cells undergo rapid proliferation between e9.5 and e13.5 in developing embryos. The second wave of pituitary cell proliferation begins after birth. The proliferating cells were labeled with a thymidine base analog, 5-bromo-2-deoxyuridine (BrdU), which is selectively incorporated into DNA during the S-phase of cell cycle. After two hours labeling, pituitary histological sections were prepared and analyzed for the BrdU-incorporation rate. The mutant pituitary precursor cells had a very similar proliferation rate to wild-type littermate controls between e13.5 and e17.5 (data not shown), which is consistent with the fact that few *Pit1*-expressing cells are proliferating during these embryonic stages. In contrast, the BrdU incorporation rate was significantly reduced in SC35 mutant mice at p5 (~41% reduction), p11 (~50% reduction), and p28 (~58% reduction) (Figure V-2B). Together, these results provide definitive evidence for the requirement of SC35 in cell proliferation during pituitary organogenesis.

### **SC35 is dispensable in terminal differentiated mature cardiomyocytes**

The requirement for SR splicing factors in cell proliferation may result from specific defects in cell growth pathways or from general meltdown of gene expression. To distinguish between these possibilities, and more importantly, to closely examine the SC35 requirement in post-mitotic cells, we chose to delete SC35 in terminal

differentiated mature cardiomyocytes using the tamoxifen-inducible gene targeting strategy<sup>34</sup>. We previously showed that conditional knockout of SC35 during early cardiogenesis in mouse resulted in severe cardiac hypertrophy<sup>35</sup>. However, it remains to be determined whether the disease phenotype is due to developmental problems during cardiomyocyte differentiation/maturation or to cell autonomous defects in mature cardiomyocytes. In this study, we crossed the conditional SC35 knockout mouse with the transgenic mouse in which the expression of Cre was driven by the cardiomyocyte-specific myosin heavy chain (MHC) promoter in a tamoxifen-inducible fashion<sup>34</sup>. By administration of tamoxifen at the postnatal stage of 3 weeks, SC35 deletion was induced in adult in efficiency comparable to MLC2v-Cre mediated ablation in embryos (Figure V-3A)<sup>35</sup>. Significantly, the SC35 mutant mice exhibited no difference in survival from wt littermate controls during the period of more than 80 weeks of observation (Figure V-3B). Histological analysis of hearts from 80-week-old mice detected no cardiac hypertrophy in the mutant animals (data not shown). Furthermore, echocardiographic analysis of SC35 deficient mice revealed no functional defects in heart performance in comparison with wt littermate controls (Figure V-3C). Using the same strategy, we induced deletion of another SR protein ASF/SF2 in adult heart and observed total mortality in two months (Figure V-3B), thus unequivocally demonstrating the differential requirement for different SR proteins in adult heart. We conclude that *SC35* is dispensable in mature post-mitotic cardiomyocytes. These results contrast dramatically to the situations in developing heart<sup>35</sup> and pituitary (Figure V-1 and 2), suggesting that SC35 may play a specific role in the regulation of cell proliferation, rather than providing a general function for cell survival.

### **SC35 plays a major role in cell cycle progress through the G2/M phase in MEFs**

We next studied the SC35 function in cell proliferation using the mouse embryonic fibroblasts (MEFs). The type II conditional knockout MEFs (SC35<sup>II/II</sup>) exhibited normal cell proliferation compared to wt MEFs (data not shown). To analyze the SC35 function in cell cycle progression, we took a somatic genetic strategy described earlier in creating conditional *ASF/SF2* knockout MEFs<sup>12</sup> by generating an SC35-null MEF line in which the endogenous *SC35* gene was deleted and the cell was complemented by an exogenous, HA-tagged SC35 (HA-SC35) under an inducible Tet-off promoter (Figure V-4A). In the absence of doxycycline (Dox), the MEFs grew in a similar rate to wt MEF controls (data not shown). Upon addition of Dox, exogenous HA-SC35 became diminished in four days (Figure V-4A), and cell proliferation was arrested almost instantly (Figure V-4B). SC35-depleted MEFs were rounded up in a week to 10 days and then died (data not shown). Analogous to the situation in SC35-deficient pituitary, however, we did not detect any significant increase of apoptotic cells based on both TUNEL and staining for Annexin-V and active caspase-3 (data not shown), indicating that programmed cell death was not a major event in MEFs, which is different from the situation in ASF/SF2-depleted chicken DT40 cells<sup>14,15</sup>. As expected, retroviral expression of the wild type full-length SC35 completely rescued the proliferation defect in the presence of Dox (Figure V-4C and 4D). Interestingly, while the C-terminal nuclear retention signal (NRS)<sup>36</sup> was dispensable, the RS domain was essential for SC35 to function *in vivo* (Figure 4C and 4D), which contrasts the non-essential role of the RS domain of ASF/SF2 in MEFs<sup>12</sup>.

Having established the somatic genetic complementation system for SC35, we next addressed its requirement in cell proliferation and cell cycle progression. By BrdU-labeling, we found that the S-phase cell population was dramatically reduced from 72% to 9% in the presence of Dox, (Figure V-5A), suggesting a major reduction in cell proliferation as seen in the developing pituitary gland (Figure V-2B). The dramatic reduction in BrdU labeling suggests potential G1/S cycle arrest triggered by the activation of the S-phase checkpoint in SC35-deficient MEFs. To examine this possibility, we determined the cell cycle profile and found that in vivo SC35 depletion induced G2/M arrest (Figure V-5B). Furthermore, the M-phase cell population was increased from 40% to 53% based on cell staining with an antibody against phosphorylated histone H3 at serine-10, a marker for mitotic cells, which was correlated with an overall increase in cell size (Figure V-5C). These observations indicate that the reduction of S-phase cells is an indirect effect of cell cycle arrest at G2/M.

To directly demonstrate the SC35-deficient MEFs can successfully progress through the S phase, we analyzed cell cycle progression by synchronizing the mutant cells at G1 phase followed by monitoring cell cycle progression by FACS after serum stimulation (Figure V-5D). After three days of serum starvation, cells were relatively arrested at the G1 phase; upon serum addition, these cells were progressively transited through S-phase and the majority of cells were arrested at the G2/M phase (Figure V-5E). These results suggest that the DNA replication machinery is competent in SC35-depleted MEFs and that SC35 may play a critical role in the regulation of the G2/M cell cycle transition.

These observations are reminiscent of the phenotype observed in chicken DT40 cells in which the cell cycle defect has been attributed to double-strand DNA breaks induced by depletion of ASF/SF2<sup>13</sup>. To determine whether double-strand DNA breaks also take place in normal cells, such as MEFs, and are a general phenotype in response to *in vivo* depletion of SR proteins, we stained the cells with an antibody against phosphorylated histone  $\gamma$ H2AX at serine-139 before and after SC35 depletion, and detected DNA damage-induced foci in SC35-depleted MEFs (Figure V-5F). A similar result was also obtained with ASF/SF2-depleted MEFs (data not shown). Together, these results suggest a critical and universal role of SR proteins in maintaining genomic stability in vertebrates.

### **SC35-mediated cell proliferation is dependent on p53**

The essential function of SC35 in cell proliferation in cell and animal models raised the possibility that SC35 is required for cell cycle progression of all proliferating cells. To test this hypothesis, we used siRNA to knockdown SC35 in the tumorigenic HCT116 colorectal carcinoma cells (Figure V-6A). While non-specific control siRNA and specific siRNA against U2AF65 had no detectable effect on cell proliferation based on BrdU-labeling, siRNA against SC35 impaired cell proliferation in those cells. These results suggest a universal requirement for SC35 in cell proliferation.

Because DNA damage is known to activate p53, and more importantly, activated p53 has been shown to be essential for cell cycle arrest and apoptosis in response to DNA damage<sup>37, 38</sup>, we asked whether p53 was required for SC35-dependent cell cycle progression. For this purpose, we took advantage of an isogenic HCT116 cell line in

which p53 was inactivated by homologous recombination<sup>37</sup>. Strikingly, we found that p53 inactivation was able to partially bypass the cell cycle block (Figure V-6B). This observation prompted us to examine whether deletion of p53 *in vivo* could rescue the SC35 early embryonic lethal phenotype, we crossed the SC35 heterozygous mice with p53 null mice. Mice with the p53<sup>-/-</sup>/SC35<sup>+/-</sup> genotype were obtained at the expected Mendelian efficiency, but p53<sup>-/-</sup>/SC35<sup>-/-</sup> embryos died at early gestation stages, indicating that the p53 pathway is only part of the SC35-dependent program in development (Ding and Fu, unpublished results). Therefore, SC35 may regulate cell proliferation in certain cell types in a p53-dependent manner, but the overall requirement for SC35 in animal development clearly involves both p53-dependent and independent pathways.

### **Mechanisms of p53 activation in response to SC35 depletion**

The p53 network is essential for a variety of stress-induced cellular responses, including cell cycle arrest, senescence and apoptosis. To gain insights into the p53-dependent and SC35-mediated cell cycle arrest, we analyzed the expression profile of 96 key genes involved in the p53 pathway using a mouse SuperArray (Figure V-7A). As predicted, a number of p53 target genes were significantly up regulated, including Cdkn1a (p21<sup>cip1</sup>) and Sfn (14-3-3 $\sigma$ ). By Western blotting, we confirmed that p21<sup>cip1</sup> protein was indeed dramatically elevated in SC35-depleted MEFs (Figure V-7B). We also observed induction of another cyclin-dependent kinase inhibitor p27<sup>kip1</sup>, a well-characterized target for the retinoblastoma (Rb) protein (Figure V-7B). These findings suggest that SC35 depletion may have triggered multiple pathways to inhibit cell cycle progression.

The SuperArray profile also revealed up-regulation of several p53 modifiers,

including p300 acetyltransferase, Chk1 (Chk1) and Csnk2 $\beta$  (CK2 $\beta$ ) kinases (Figure V-7A). This observation suggests that p53 may be activated in SC35-deficient MEFs by some specific post-translational modification events. To determine the activation mechanisms, we examined the expression of p53 and its post-translational modifications (Figure V-7C). While the overall p53 protein level was similar between wt and SC35-deficient MEFs, we found that phosphorylation at serine-18 (pS18) was elevated, which is somewhat expected because serine-18 is the major site for phosphorylation by activated ATM family of kinases in response to double-strand DNA breaks<sup>16, 39-42</sup>, which were abundantly observed in SC35-depleted MEFs (Figure V-5C). Interestingly, besides p53 hyperphosphorylation, our survey of p53 post-translational modifications also revealed hyperacetylation of p53 at lysine-379 (acK379) (Figure V-5C). This post-translational event may be attributed to increased expression of *p300* (Figure V-7A and D), which has been shown to act as a specific acetyltransferase towards the lysine-379 residue<sup>17-21</sup>.

As p53 acetylation is regulated by both acetyltransferases and deacetylases, we further characterized this hyperacetylation event by examining the p53 deacetylase *Sirt1*<sup>22-25</sup> and detected a significant reduction in the full-length SirT1 protein with a concurrent appearance of a truncated protein form in SC35-depleted MEFs compared to wt control cells (Figure 7E). This observation raised the possibility that SC35 depletion may have induced alternative splicing of the *Sirt1* transcript. RT-PCR analyses indeed confirmed this, revealing a smaller *Sirt1* transcript, and direct sequencing of this PCR product showed specific skipping of exon 8 from the primary *Sirt1* transcript (Figure V-7F, data not shown). This particular alternative splicing event appears to be specific to SC35



depletion *in vivo* because a parallel analysis of ASF/SF2 depletion in MEFs did not have the same effect on *SirT1* splicing (data not shown). Therefore, different SR proteins may be required for cell cycle progression by affecting different pathways. In the case of SC35, the p53 pathway appears to be activated in a general response to DNA damage. In addition, loss of SC35 also triggered the transcriptional activation of a p53 acetyltransferase *p300* and aberrant splicing of the p53-specific deacetylase *SirT1*, which together resulted in p53 hyperacetylation. This post-translational event in conjunction with double strand DNA break-induced p53 hyperphosphorylation may constitute some key events that lead to the observed cell cycle arrest (Figure V-7G).

## Discussion

The SR family of splicing factors has been extensively studied at the level of biochemistry for their diverse roles in RNA metabolism and gene expression<sup>2</sup>. However, few studies have linked their activities to specific pathways to elucidate their biological functions *in vivo* because of the lethal phenotype observed at the cellular and animal levels. Loss of a specific SR protein may be sufficient to induce some general effects that ultimately cause cell mortality. Manley and colleagues recently established such a general cellular response to SR protein deficiency, demonstrating a remarkable genomic instability phenotype induced by DNA damage and linked the phenotype to a specific defect in solving the so-called R-loop in ASF/SF2-deficient chicken DT40 cells<sup>13,43</sup>. We observed similar DNA damage events in normal diploid MEFs depleted of both SC35 and ASF/SF2, thus establishing the generality of the DNA damage-induced phenotype in vertebrates. Importantly, we observed that the DNA damage was not sufficient to prevent

cell cycle progression through the S-phase, in spite of the increased expression of p21<sup>cip1</sup> and p27<sup>kip1</sup>. Instead, SC35-depleted MEFs were arrested in the G2/M phase, apparently resulting from the activation of some major cell cycle regulators, such as p21<sup>cip1</sup> and p27<sup>kip1</sup>. The functional link between SC35 and cell cycle control is fully consistent with the essential function of SC35 in proliferating thymus<sup>10</sup> and pituitary (present study), but not in terminal differentiated mature cardioomyocytes<sup>35</sup>. The fact that ASF/SF2 plays a critical role in adult cardioomyocytes, which is in contrast to the dispensability of SC35 in adult heart, suggests additional and unique requirement for this SR protein in heart performance as we recently demonstrated during physiological analysis of ASF/SF2-deficient heart<sup>9</sup>.

Although SR proteins are involved in some common pathways, it is also clear that SR proteins are not functionally redundant *in vivo* and each SR protein may be involved in specific pathways via a unique spectrum of targets in a given cell type. The development of conditional knockout cellular models permits in-depth analysis of the functional requirement for SR proteins *in vivo*. It has been shown that ASF/SF2 ablation in DT40 cells trigger apoptosis<sup>15</sup>. Activation of the apoptotic pathway, however, may be specific for lymphocytes and other cell types, but not for developing heart and pituitary as documented in previous<sup>35</sup> and present studies. Furthermore, we did not see any sign of programmed cell death in MEFs depleted of SC35 or ASF/SF2. Consistent with the activation of the DNA damage-responsive pathway, we found hyperphosphorylation of p53 at serine-18, a major site responsive to activation of the ATM pathway<sup>16, 39-42</sup>. Interestingly, in addition to the phosphorylation event induced by depletion of both SC35 and ASF/SF2, we detected a p53 hyperacetylation event that was uniquely triggered by

depletion of SC35. The activation of the acetyltransferase *p300* coupled with the down-regulation of the p53-specific deacetylase *Sirt1* provided a plausible explanation to the observed p53 hyperacetylation event. Although it is currently unclear how *p300* is activated at the level of transcription as revealed from our SuperArray analysis of the p53 pathway, a specific defect in *Sirt1* splicing detected by RT-PCR and Western blotting seems to account for the down regulation of this negative regulator for p53. Therefore, while the direct effect of SC35 on *Sirt1* splicing remains to be demonstrated, the finding is fully consistent with the biochemical activity of SC35 as a splicing regulator, thereby providing a critical functional link between regulated splicing and cell cycle control in mammalian cells.

The combined effect of p53 hyperphosphorylation and hyperacetylation may directly and potently regulate its downstream targets, including *p21<sup>cip1</sup>*, to induce cell cycle arrest. Since we also observed the up-regulation of *p27<sup>kip1</sup>*, it is likely that SC35 may also induce other cell cycle regulators besides those involved in the p53 pathway. Despite potential contributions by other pathways, however, we found that inactivation of p53 was able to partially relieve the cell cycle block in HCT116 colon carcinoma cells, suggesting that activated p53 is functionally responsible, at least in part, to the observed cell cycle arrest induced by SC35 depletion. However, it is also clear that the activation of the p53 pathway is insufficient to account for the full spectrum of SC35 functions *in vivo* because SC35 null mutation in the p53 null background still causes embryonic lethality (Ding and Fu, unpublished results). Likewise, it has been shown that overcoming genomic instability induced by ASF/SF2 depletion is insufficient to prevent cell death in chicken DT40 cells<sup>13</sup>. Together, these observations suggest that SR

proteins contribute to multiple critical pathways in the regulation of cell proliferation.

The involvement of SC35, and likely other SR proteins, in some major regulatory pathways for cell proliferation and cell cycle progression is fully corroborated with increasing evidence that the SR family of splicing factors and regulators may directly contribute to the oncogenic process. Indeed, elevated expression of SR proteins has been detected in multiple types of tumors<sup>26-28, 44</sup>. In most cases, however, their exact roles in tumorigenesis are unknown, although a recent study showed that ASF/SF2 is specifically involved in regulated splicing of the Ron proto-oncogene and the splicing defect appears to directly contribute to elevated cellular motility and invasiveness<sup>45</sup>. This and other specific regulatory events may thus play an important part in tumor progression *in vivo*. Furthermore, the ability of SR proteins to affect genomic stability and integrity may provide a mechanism to facilitate tumor development, selection, and expansion. Here, we show that SR proteins are involved in the regulation of the p53 pathway, which is a well-known tumor suppressor gene. Since ablation of specific SR proteins can activate p53, it is conceivable that overexpression of SC35 and other SR proteins in tumors may directly or indirectly suppress p53 in addition to their effects on modulating other critical cellular genes at the level of splicing, which may together enhance cell proliferation in a mis-regulated fashion. This newly elucidated function of SR proteins in antagonizing some tumor suppressor genes, such as p53, formally suggest a critical role of this family of splicing regulators in tumorigenesis.

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## **Experimental Procedures**

### **Generation of *Pit-Cre/AP* transgenic mice**

Standard molecular biology procedures were used to generate the *Pit-Cre/hpAP* transgene construct. In brief, the 15.5kb endogenous *Pit-1* promoter and enhancer region was cloned in front of the mini  $\beta$ -globin intron region and the full-length Cre cDNA followed by human placenta alkaline phosphatase (hpAP) and poly(A<sup>+</sup>) signal. The hpAP cDNA is fused with IRES sequences, which allows Cap-independent translation<sup>32, 46</sup>. The transgenic mice were generated by direct injection of fertilized eggs with the linearized transgene construct.

### **Alkaline phosphatase and $\beta$ -galactosidase staining**

The transgene expression was analyzed by monitoring the hpAP enzymatic activity as described<sup>32, 46</sup>. In brief, staged embryos were dissected and fixed in 10% buffered formalin at 4°C for 30 min. The cryosections from each embryo were post-fixed for 30 min. and rinsed with PBS twice before heat inactivation of endogenous alkaline phosphatase activity<sup>32</sup>. The hpAP activity was then assayed at room temperature overnight using BM purple substrate from Roach. The Cre recombinase function were

analyzed by crossing the transgenic mice with the ROSA26 reporter mice followed by X-gal staining for  $\beta$ -galactosidase activity essentially the same as described<sup>33</sup>.

### **Ablation of SC35 in the pituitary and heart**

The conditional *SC35* knockout allele was generated by flanking both axons of the entire *SC35* locus with two loxP sites as described<sup>10</sup>. Homozygous type II *SC35* mice were crossed with the *Pit-Cre/hpAP* transgenic mice to generate pituitary specific deletion of the *SC35* gene. To ablate *SC35* in adult heart, the conditional *SC35* knockout mice were similarly crossed to the tamoxifen-inducible *MHC-Cre* transgenic mice<sup>34</sup>. To induce gene deletion, tamoxifen was intraperitoneally injected into mice of postnatal three weeks once a day for 5 days as described<sup>34</sup>. Deletion efficiency was determined by Southern blotting and echocardiographic analysis was performed as previously described<sup>35</sup>.

### **Cell type determination by immunohistochemistry, BrdU-labeling and TUNEL assays**

All pituitary specific antibodies used and the immunohistochemistry procedure were as described<sup>47</sup>. BrdU labeling and TUNEL assay were performed as described according to the manufacture's suggestion<sup>47</sup>. Briefly, staged pregnant females and newborn pups were labeled for two hours with BrdU/PBS at 0.1mg/g body weight. Incorporated BrdU was detected on 10um cryostat sections as recommended (ICN, Irvine). All sections were counter-stained with DAPI before photographing.

### **Plasmids, Tet-off inducible system and TAT-Cre protein transduction**

The *pRevTRE-SC35* plasmid was constructed by subcloning a HA-tagged *SC35* cDNA into the *pRevTRE* vector (Clontech), which was used to produce retrovirus.

T-antigen immortalized MEFs carrying floxed SC35 alleles were co-infected with RevTRE-SC35 and RevTet-OFF-IN viruses followed by single cell cloning and removal of the endogenous SC35 gene to establish the inducible SC35 expression cell line as described for ASF/SF2 (Lin et al., 2005). The SC35 tet-off cell line was verified by PCR genotyping (primers F: 5'AAA ATG TCT TGC CAT CTC CCT CCC C3', R1: 5'GGT CTT GGT TAT TTG GCC AAG AAT CAC3', R2: 5'TCC ATG GAC CGA TGG ACT GAG TTT GT3'; PCR condition: 94°C 30", 65°C 30", 72°C 60" with 35 cycles) and characterized by Western blotting. Complementation was carried out using wt and mutant SC35 expressed as Myc-tagged fusion protein from retrovirus.

### **Cell Proliferation and Cell Cycle analyses**

Cells were cultured in 12-well plates. The culture medium was changed 24 hours before the MTT assay; 100ul MTT stock solution (5mg/ml in PBS) was added to each well containing 1.0ml medium and the plate was incubated at 37°C for 1 hour. After removing the medium, the converted dye was solubilized with 1ml of acidic isopropanol (0.04 M HCl in absolute isopropanol) and measured at 570nm with background subtraction at 670nm within 1 hour after adding isopropanol. BrdU uptake studies were also performed using an in situ cell proliferation kit (Roche). The siRNA microinjection assay was essentially the same as previously described<sup>29</sup>.

To profile cell cycle in SC35-depleted MEFs, Doxycycline was included in the media for 5 days. Induced cells and similarly treated wt controls were harvested, washed with PBS, and fixed in 70% ethanol/PBS for 30 minutes at 4°C. Cells were washed with PBS and stained with PI for 30 minutes at room temperature before FACS analysis on a LSR Flow Cytometer (Becton Dickinson).

### **Western blotting and Immunofluorescence staining**

Whole cell protein was extracted in SDS loading buffer for PAGE. After blotting onto nitrocellulose, the membrane was probed with  $\alpha$ HA (Roche). To detect p53 modifications, the cells were lysed in a Flag-lyses buffer (Luo et al., 2001) and analyzed by Western blotting using anti-total p53 (FL-393, Santa Cruz Biotechnology), anti-phospho-p53 serine-18 (16G8, Cell Signaling), and anti-Acetyl-p53K379 and anti-acetyl-p53K317 (2271-PC-050, Trevigen). Anti-SirT1 was from Upstate (07-131).

For immunofluorescence staining, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min. at room temperature and permeabilized in 0.5% Triton X-100 for 5 min. The fixed cells were incubated for 1 hour at room temperature with anti-phospho-Histone  $\gamma$ H2AX-ser139 (2F3, Biolegend) in PBS plus 1% FBS. After washing, the cells were developed for 1 hour at room temperature with alexa594-conjugated Donkey anti-mouse IgG (1:500; Molecular Probes) and alexa488-conjugated Phalloidin (1:500; Molecular Probes). Coverslips were mounted with a mounting solution containing DAPI and imaged under a Zeiss Axioskop microscope.



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The text of Chapter V, in part or in full, is a reprint of the manuscript that has been submitted to *Molecular and Cellular Biology*. I was one of the primary researchers and co-authors in this manuscript. My contributions included derivation of inducible cell lines, characterization of cell growth defect and analysis of gene expression profile. I thank my co-authors Ran Xiao, Ye Sun, Jian-Hua Ding, Dave W. Rose, Michael G. Rosenfeld, Xiang-Dong Fu and Xue Li for permission to use this publication in my dissertation.

**Figure V-1. Pituitary agenesis in SC35 conditional knockout mice.** **A.** Early deletion of SC35 by the *Pitx-Cre* transgene resulted in severe anterior pituitary (A) agenesis. The Cre-negative posterior pituitary gland (P) is not affected. **B.** Schematic diagram of *Pit-1-Cre-hpAP* dicistronic transgene. **C.** Expression of the transgene (AP staining purple) and Pit-1 protein (green immunostaining) on adjacent sections of a pituitary gland from the transgenic mouse. **D.** Hypoplastic adult pituitary gland in conditional SC35 knockout mediated by the *Pit-1-Cre-hpAP* transgene.

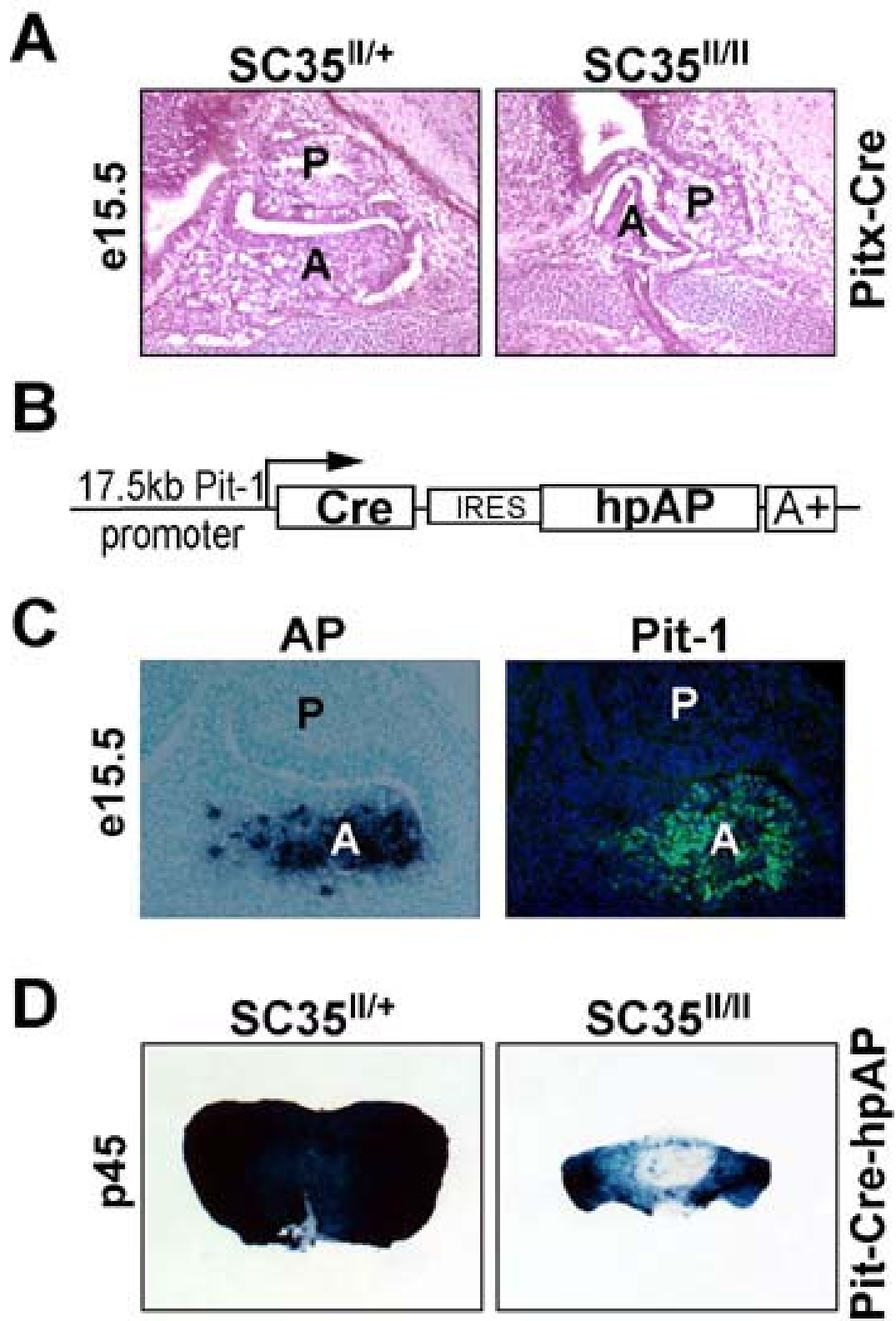


Figure V-1

**Figure V-2. SC35 is required for pituitary cell proliferation, but not cell differentiation.** **A.** Immunohistochemical staining of adult pituitary gland using cell-type specific hormonal markers. The *Pit-1*-dependent lineages, somatotrophs (growth hormone, GH), lactotrophs (prolactin, PRL) and thyrotrophs ( $\beta$  unit thyroid stimulating hormone,  $\beta$ TSH), were markedly reduced. The *Pit-1* independent lineages, gonadotrophs (luteinizing hormone, LH) and corticotrophs (adrenocorticotrophic hormone, ACTH), were unaffected, which resulted in the apparent increase of these *Pit-1* independent cell lineages in SC35-deficient pituitary. **B.** Reduced cell proliferation detected by BrdU immunohistochemical staining (red) in postnatal (p) pituitary gland at day p5 (~41% reduction), p11 (~50% reduction) and p28 (~58% reduction).

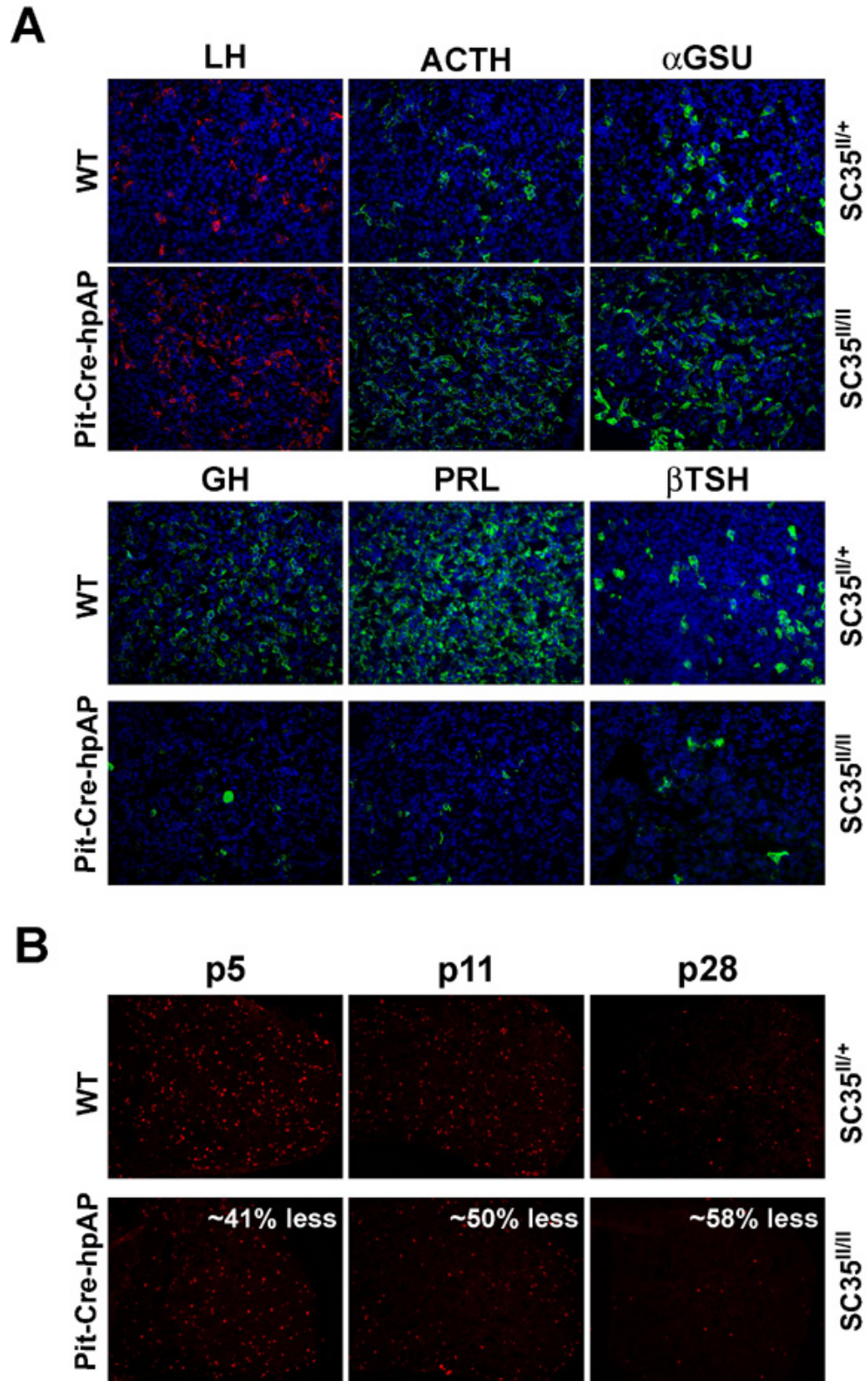


Figure V-2

**Figure V-3. SC35 is non-essential in terminal differentiated mature cardiomyocytes.**  
**A.** Genomic Southern blotting analyses of tamoxifen-induced *SC35* deletion in adult heart. Control DNA was from conditional *SC35* knockout mice mediated by *MLC2v-Cre*<sup>35</sup>. **B** The Kaplan-Meier survival plot after tamoxifen-induced deletion of *SC35* and *ASF/SF2* in the adult heart. **C.** Echocardiographic analysis of *SC35* deficient mice.

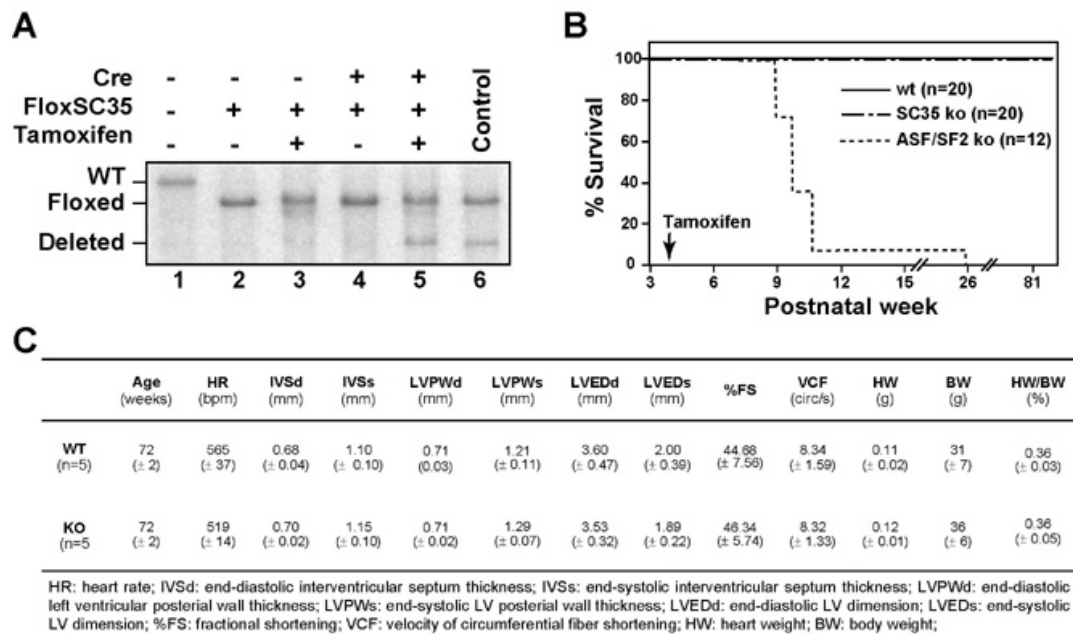


Figure V-3

**Figure V-4. Genetic complementation of SC35 knockout MEFs.** **A.** Construction of a tet-repressible SC35 expression unit and time course analyses of HA-SC35 expression after addition of doxycyclin (Dox). **B.** Growth arrest induced by SC35 depletion in MEFs. **C.** and **D.** Functional rescue of SC35-depleted MEFs by wt and mutant SC35.



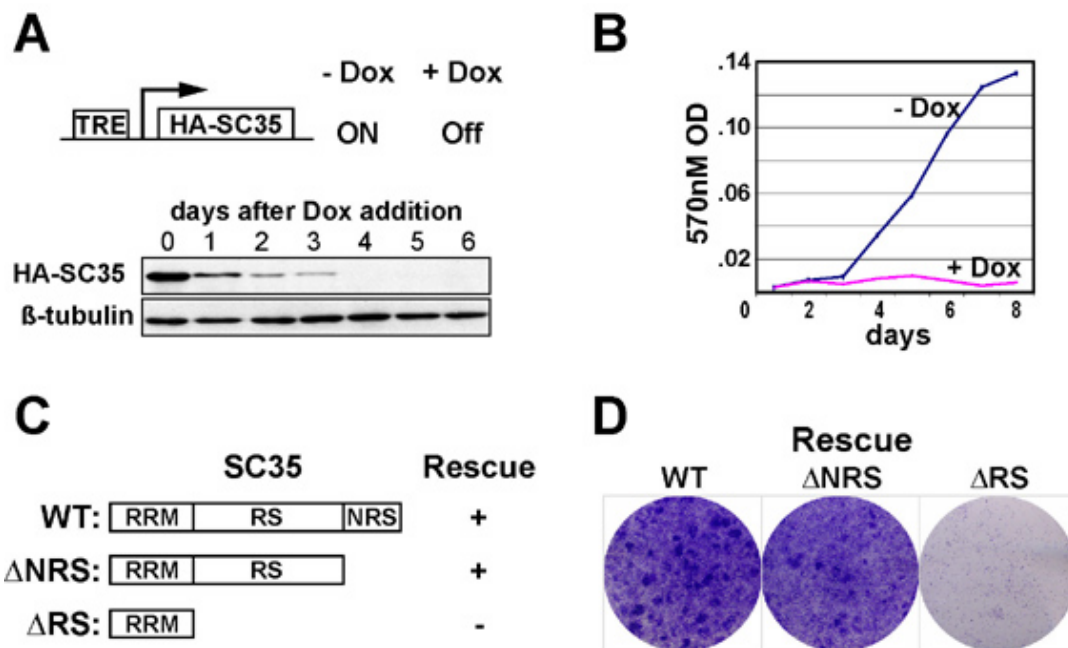


Figure V-4

**Figure V-5. G2/M cell cycle arrest induced by SC35 deficiency in MEFs.** **A.** Analysis of proliferating cells by BrdU-labeling before and after SC35 depletion. **B.** FACS analysis of cell cycle in wt and SC35-depleted MEFs, showing a reduction in S-phase cells and a dramatic increase in G2/M phase cells. **C.** Analysis of M-phase cells based on staining for the mitotic phosphorylated histone H3 marker. **D** and **E.** Experimental design (**D**) and time course FACS analyses (**E**) of cell cycle progression of SC35 deficient MEFs. **F.** Immunocytochemical analyses of SC35-depleted cells by staining with phalloidin (for actin) to detect general cell morphology and an antibody against phosphorylated  $\gamma$ H2AX to detect induced foci accumulated on broken DNA. Cells stained for actin and for phosphorylated  $\gamma$ H2AX were photographed at 40X and 100X, respectively. Nuclei were labeled with DAPI.

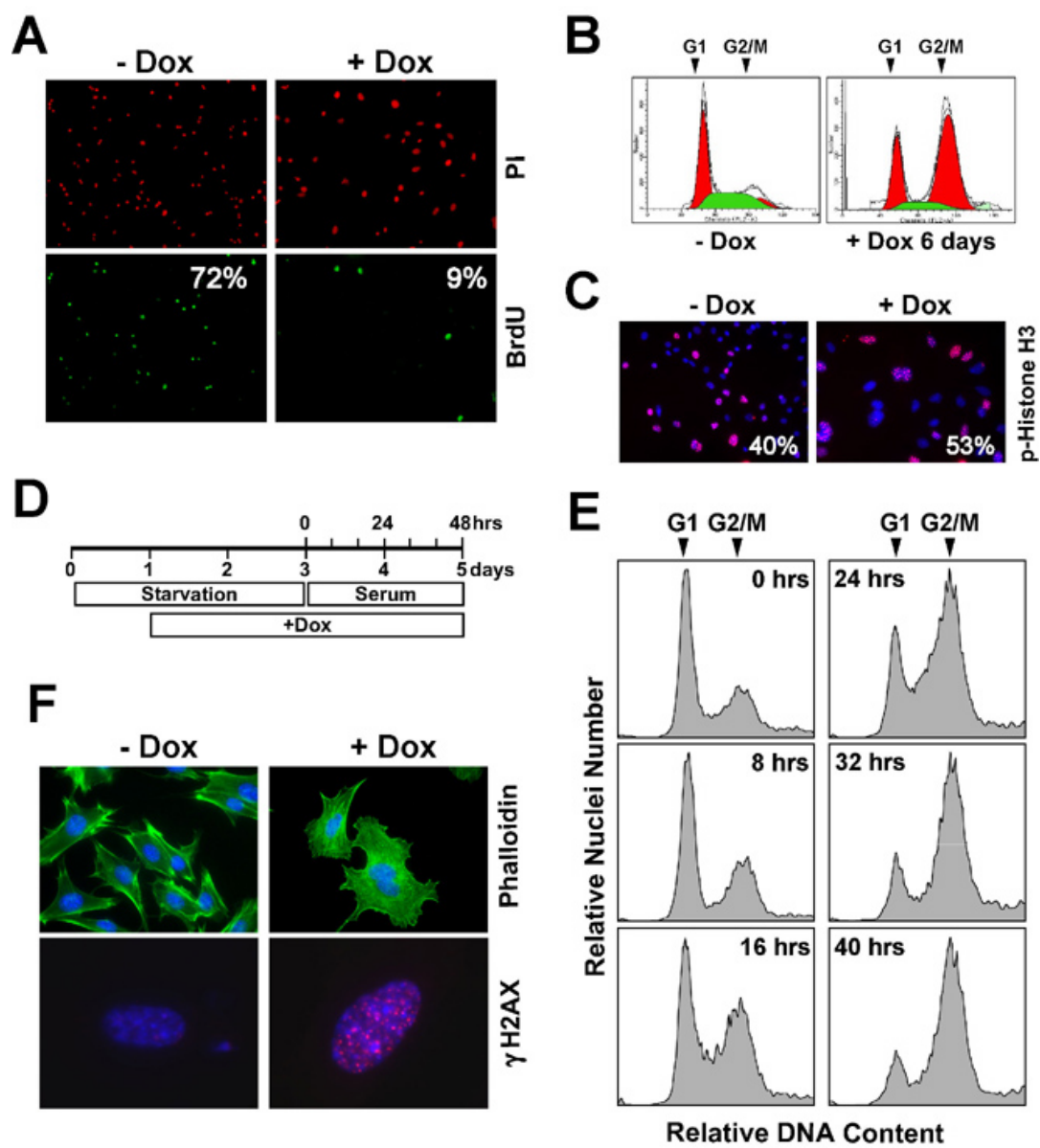


Figure V-5

**Figure V-6. SC35 is required for p53-dependent cell proliferation. A. and B.** Microinjection of siRNA against SC35 and U2AF65 in wt HCT116 (**A**) and HCT116p53<sup>-/-</sup> (**B**) colorectal carcinoma cells. A non-specific (ns) siRNA was also microinjected as a control. Cell proliferation was analyzed by BrdU-labeling.

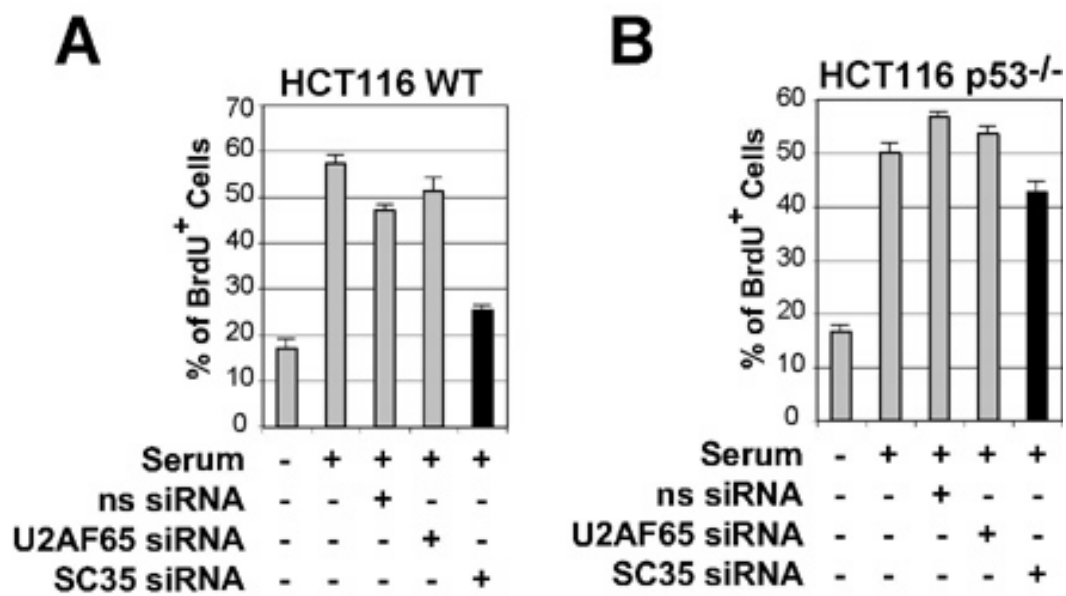


Figure V-6

**Figure V-7. Analysis of the p53 pathway in SC35-depleted MEFs.** **A.** Analysis of the p53 pathway on a SuperArray containing most known p53 target genes. Up- and down-regulated genes in SC35-deficient MEFs are marked red and green, respectively. **B.** Western blotting analyses of the cyclin-dependent protein kinase inhibitors, p21<sup>cip1</sup> and p27<sup>kip1</sup>. **C.** Western blotting analysis of p53 post-translational modifications using specific anti-p53 antibodies as indicated. **D.** Confirmation of induced p300 expression in SC35-depleted MEFs by RT-PCR. **E.** *SirT1* down-regulation and identification of an aberrant form of SirT1 in SC35-depleted MEFs by Western blotting. **F.** RT-PCR analysis of *SirT1* alternative splicing (arrowhead) using a pair of specific primers around exon 8 (arrows). **G.** Proposed mechanisms of SC35 depletion-induced cell cycle progression via the activated p53 pathway.

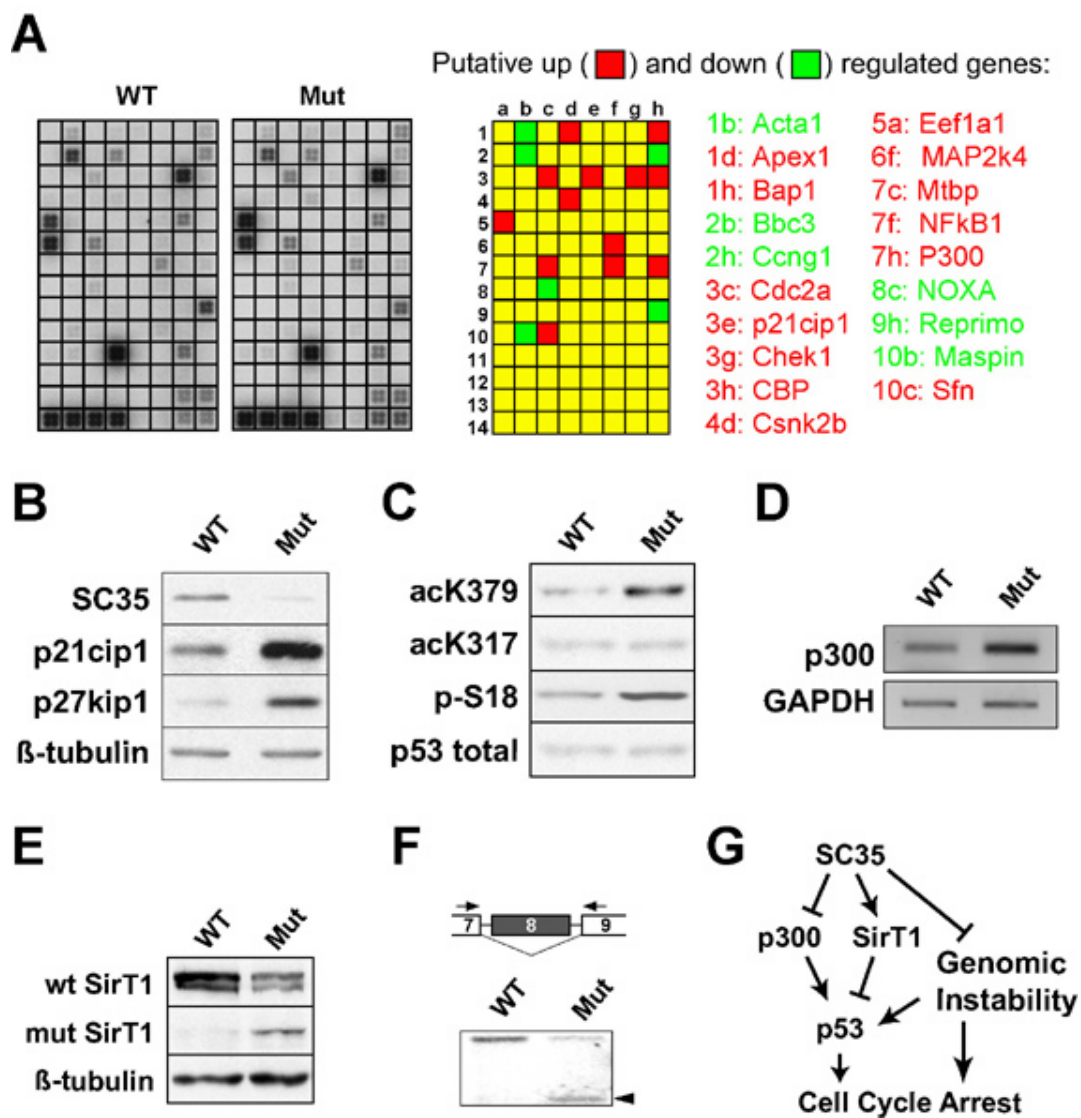


Figure V-7

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## **CHAPTER VI**

### **Conclusions**

## **Project summary**

Five and a half years have passed since I started my Ph.D. study in Dr. Xiang-Dong Fu's laboratory. My first project was the characterization of mutually exclusive splicing on the FGFR2/KGFR gene. In the second year of my Ph.D. research, as suggested by my mentor Dr. Fu, I redirected my focus to SR proteins. To do gene ablation assays, I took the advantages of conditional ASF/SF2 and SC35 KO mice generated in our lab. With help from other lab members and collaborators, I established a powerful cellular genetic system by *ex vivo* culturing MEFs derived from the KO mice to characterize the functions of SR splicing factors in living mammalian cells. This effort finally gained considerable rewards. We demonstrated that both SR proteins are required in proliferating fibroblasts, and that the RNA binding motif is essential for SR proteins function *in vivo*. Using several phosphorylation mutants of SR proteins, we identified a possible sorting mechanism for mRNP transition from splicing to export. This finding resolves the puzzle of how SR proteins with different shuttling properties are recycled in the cell. The rest of my research projects on SR proteins can be divided into two major directions: cell cycle arrest caused by SR protein depletion and transcription related functions of SR proteins. In one of our two manuscripts (Chapter V), we described an interesting finding that SC35 is required for mouse organ development and survival of dividing cells but dispensable in non-proliferating cells such as cardiomyocytes. A possible mechanism is that SC35 depletion plays an important role in activation of the p53 pathway and cell cycle arrest in KO cells. In the other manuscript (Chapter IV), we analyzed gene expression in SR protein depleted cells and found a significant defect in transcription elongation of specific genes. This elongation defect can be reconstituted by

adding back a purified SR protein, suggesting a direct role of SR proteins in this process. We further demonstrated that the elongation defect triggered extensive double-stranded DNA breaks, leading to ATM-mediated activation of p53, induced p21 expression, and cell cycle arrest. These findings suggest that timely recognition of splicing signals by SR proteins in nascent transcripts is a driving force for transcription elongation in vertebrates, and might be critical for preventing transcription related DNA damage.

## **Questions and future directions**

### **In vivo roles of SR proteins in negatively regulating alternative splicing**

Members of the SR family of splicing factors are among the best-characterized splicing regulators, and have been extensively studied by biochemical analyses. SR proteins behave simply *in vitro*, functioning as splicing activators that promote exon inclusion upon binding to exonic splicing enhancers (ESEs) <sup>1</sup>. However, further studies indicate that different SR proteins may influence splice site selection in both positive and negative fashions (see Chapter II). In our preliminary studies of alternative splicing changes in SR KO mouse heart muscles, we detected similar numbers of exon inclusion/exclusion events (G. Mansfield, et al., unpublished data). This suggests that alternative splicing regulated by SR proteins *in vivo* is more complicated than expected. Possible mechanisms for negative selection of exons by SR proteins are discussed in Chapter II. Briefly, we proposed three models: (A) An SR protein may bind to an ESE located in flanking exons of an alternative exon, enhancing splicing of this exon and skipping the weak alternative exon. Depletion of the specific SR protein reverses this skipping pattern. (B) The function of an ESE-bound SR protein may be blocked by

another ESE-bound SR protein with weaker activity in splicing activation. Removal of the SR protein with a stronger binding but weaker splicing activity may promote exon inclusion. (C) The same cis-acting ESE may be recognized by both positive and negative SR proteins. In this case, ASF/SF2 and SC35 could play negative roles, causing these exons to have more inclusion in KO cells.

To further investigate possible mechanisms, we plan to examine SR protein effects in a mini-gene expression system. This system contains both CMV and T7 promoters driving a cloned mini-gene with an alternative exon and flanking intron/exons. Unbiasly selected exons from individual genes, which are more included with SR protein depletion, will be cloned into this expression system. Exons with more skipping in KO cells will be cloned as controls. These mini gene constructs will first be transfected into our inducible MEFs before or after SR proteins depletion. Using RT-PCR, we can detect the splicing pattern change in the mini gene. Once we validate that mini-genes mimic splicing patterns characteristic of their endogenous genes in transfected cells, further co-transfection with mini-gene constructs and SR protein expression vectors will be carried out to see whether there are opposite effects on the mini-gene splicing with SR protein overexpression. *In vitro* mini-gene transcripts from T7 promoter can be used in cell-free splicing systems with purified SR proteins to determine if the effect is direct. Outcomes of the above assays may deliver some hints about how negatively regulated exons are controlled by an SR protein. Further assays may be needed to find out which proposed model fits specific exons.

### **Regulatory mechanism(s) of SR proteins in transcription elongation**

### R-loop hypothesis

As described in Chapter IV, we observed elongation defects in specific genes in SC35 KO MEFs. That defect can be rescued by adding recombinant SC35 to the nuclear run-on system, suggesting a direct role of SC35 in transcriptional elongation. However, the mechanism of SC35 regulation of the transcriptional complex remains to be elucidated. One possibility is R-loop formation in SR protein depleted cells, as previously proposed<sup>2,3</sup>. RNA/DNA hybrids (R-loops) formed with nascent transcripts was detected in ASF/SF2 depleted chicken DT-40 cells. R-loop structure, in yeast, is suggested to cause genomic instability and elongation defects<sup>4</sup>. However, we should be more careful to apply this model to explain the elongation defect of our system. More direct endogenous evidence is needed to support the hypothesis that R-loop is sufficient to cause an elongation defect in mammalian cells, since the majority of R-loop studies have been characterized in bacteria and yeast. Furthermore, it is still unknown how R-loop structure causes impairment of transcription. This might be due to potential side effects of the assay system. In addition, it is plausible that a pausing transcription complex has more chance to form RNA/DNA hybrids than a well-processing complex. Therefore, R-loop structure might be a consequence of a paused polymerase rather than a cause. Further assays need to be done to rule out or support such possibilities.

### SR proteins and R-loop formation

R-loop structure formed by *in vivo* depletion of SR proteins is suggested to cause double strand DNA breaks (DSB) and genomic instability<sup>2</sup>. In this scenario, SR proteins may play a critical role in packaging of novel transcripts, and prevent them from interacting with DNA template. However, this model still has some unsolved questions.



For example, when polymerase transcribes an intron-less template like a histone or snRNA gene, does SR protein still bind to the nascent RNA and perform similar functions? Alternatively, when a nascent transcript has a long intron, which is common in mammalian genomes, do SR proteins bind to a specific region, or general regions, to prevent it from attaching to DNA? Although the answer to such questions is still unknown, we can hypothesize based on available knowledge. RNA binding specificities of SR proteins are regulated by RS domain phosphorylation. Therefore, when a novel transcript emerges, a hypo-phosphorylated SR protein may bind to it with a non-specific manner to prevent annealing to DNA templates. In the later stages of RNA processing, when other RNA binding proteins are present and SR protein binding is not necessary, the SR protein might be hyper-phosphorylated by specific kinases and re-located to a specific ESE to promote splicing. It is worthy of notice that such model extends the exon scanning model, where SR proteins are recruited to nascent RNA first by non-specific binding and then anchored to a specific ESE by moving along the transcript.

#### How are SR proteins loaded on nascent transcripts?

It is still not very clear in the field how SR proteins are recruited to splice sites. Pol II CTD is a proposed landing site for SR proteins to access transcripts<sup>5-7</sup>. Spector and colleagues found that SR proteins bind CTD in a RS domain phosphorylation dependent manner. Therefore, the RS domain might be critical for SR protein recruitment<sup>7,8</sup>. In our complementation system, the RS domain of ASF/SF2 seems dispensable for its cellular functions, but the RS domain of SC35 is required. Since RS domains in both SR proteins are not necessary in regulated splicing *in vitro*, we suspect that the RS domain may have a function beyond splicing *in vivo*. Thus, inability to be recruited to CTD might be a

possible reason that the SC35 RS domain depletion mutant loses the ability to function *in vivo*. The recruitment of ASF/SF2 to CTD may be different due to the structural differences (SC35 has one RRM while ASF/SF2 has two, and their binding properties are distinct). It is worth mentioning that recruitment of another SR protein SRp20 to CTD does not require its RS domain, suggesting SR proteins may be recruited to transcription complexes by multiple mechanisms. Additional run-on assays and co-localization/binding assays using RS domain mutants are needed to address this question.

Phosphorylation is a key regulatory modification in both splicing and transcription/elongation. How phosphorylation regulates splicing has been discussed in detail in Chapters II and III. In transcription, extensive phosphorylation on CTD is required for promoter clearance (phosphor-Serine5 in YSPTSPS heptamer) and the transition from initiation to elongation (phosphor-Serine2). Phosphorylation of CTD is also required for recruiting factors involved mRNA processes like capping and splicing<sup>9</sup>. Inhibition of CTD phosphorylation by CTD kinase inhibitor DRB prevents it from interacting with SR proteins<sup>7</sup>. Therefore, to dissect the pathway of SC35 recruitment, DRB can be used in run-on assays to see if CTD phosphorylation is critical for reconstitution of transcriptional elongation using recombinant SC35. It will be interesting to determine whether there is decreased association of SR protein with novel transcripts, R-loop formations and/or DNA damages in DRB treated cells.

#### SR proteins and transcription elongation

While evidence for the involvement of splicing factors in transcriptional elongation is limited, recent reports based on transcription regulation of HIV-1 TAR gene revealed a link between splicing factors and CTD kinase complex P-TEFb<sup>10, 11</sup>.

Phosphorylation of CTD Ser2 by P-TEFb plays a critical role in releasing polymerase complexes from general pausing shortly after leaving the promoter region<sup>12,13</sup>. Therefore, it is possible that a selective set of splicing factors is required for transcriptional elongation due to their role in promoting P-TEFb recruitment to elongating transcriptional complexes. That hypothesis leads us to think of SR proteins as candidates, since they are recruited to transcription sites and seem to be required for elongation in our MEF system. It will also be important to determine whether and how SR proteins may selectively bind to P-TEFb and/or other elongation factors.

Besides bona fide elongation factors, SR proteins also interact with other factors related to elongation. One example is DNA topoisomerase I (TopoI). TopoI has been reported as a SR protein kinase whose activity can be significantly enhanced when bound to DNA<sup>14,15</sup>. TopoI is required in bacteria for polymerase elongation and prevention of R-loop formation and DNA recombination<sup>16-18</sup>. Recently, mammalian TopoI has been reported to be required for elongation of RNA polymerase II on chromatin templates<sup>19</sup>. So, it seems plausible that a functional relationship might exist between TopoI and SC35 in regulating transcription and suppressing R-loop formation. Protein interaction and functional assays are needed to address this issue.

#### Elongation defect and DNA damage

Transcription is correlated with DNA mutation and recombination, a phenomena called transcription-associated recombination (TAR)<sup>20, 21</sup>. Transcription-dependent R-loop formation is the most possible mechanism behind DNA mutation induced by depletion of SR proteins<sup>22</sup>. However, the R-loop formation model can not exclude other possibilities that transcription is employed to induce genomic instability. For example,

transcription has been recently reported to synergistically increase 4-nitroquinoline-N-oxide or methyl methanesulfonate-induced recombination in yeast <sup>23</sup>. In this system, it does not seem to involve the formation of R-loops and DNA mutations may thus be induced by other mechanisms. Moreover, using plasmid-borne direct-repeat constructs, Prado and Aguilera recently provided evidence that a collision between transcription and replication promotes TAR <sup>24</sup>. Pol II transcription encountering a head-on oncoming replication causes a replication fork pause (RFP) that is linked to a significant increase in recombination. These results support the idea that a paused transcription complex can be mutagenic because it acts as a roadblock to an upcoming replication fork. It is important to further pursue these mechanistic questions to understand induced genomic instability in SR protein depleted cells. The inducible genetic systems developed in our lab provide an ideal experimental platform to future investigate fundamental questions on the relationship between regulated transcription/splicing and maintenance of genomic stability.

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