Title
A characterization of the role of phosphorylation in regulating pre-mRNA splicing

Permalink
https://escholarship.org/uc/item/3p51243

Author
McKay, Susannah Leigh

Publication Date
2010

Peer reviewed|Thesis/dissertation
A Characterization of the role of phosphorylation in regulating pre-mRNA splicing

A Dissertation submitted in partial satisfaction of the Requirements for the Degree Doctor of Philosophy in

Biology

by

Susannah Leigh McKay

Committee in charge:
Prof. Tracy L. Johnson, Chair
Prof. Xiang-Dong Fu
Prof. Jim Kadonaga
Prof. Amy Pasquinelli
Prof. Lorraine Pillus
Prof. Jim Wilhelm

2010
The Dissertation of Susannah Leigh McKay is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

Chair

University of California, San Diego

2010
DEDICATION

This work is dedicated to my parents, Drs. David and Mary Fae, to my sisters, Amy and Jill, and to my husband Daniel who have supported me so much through all of these years.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signature Page</td>
<td>iii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iv</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>v</td>
</tr>
<tr>
<td>List of Figures</td>
<td>vi</td>
</tr>
<tr>
<td>List of Tables</td>
<td>x</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>xi</td>
</tr>
<tr>
<td>Vita and Publications</td>
<td>xii</td>
</tr>
<tr>
<td>Abstract of the Dissertation</td>
<td>xiii</td>
</tr>
<tr>
<td><strong>Chapter 1. Introduction</strong></td>
<td>1</td>
</tr>
<tr>
<td><strong>Chapter 2. A bird's-eye view of post-translational modifications in the spliceosome and their roles in spliceosome dynamics</strong></td>
<td>19</td>
</tr>
<tr>
<td><strong>Chapter 3. RRM recognition motif phosphorylation regulates RNA binding protein activity: Yeast Cus2 phosphorylation and RNA binding</strong></td>
<td>59</td>
</tr>
<tr>
<td><strong>Chapter 4. Genetic Dissection of a role for yeast U2 snRNP components in transcription: Analysis of the Cus2:TAT-SF1 homology</strong></td>
<td>92</td>
</tr>
<tr>
<td><strong>Chapter 5. Discussion</strong></td>
<td>133</td>
</tr>
<tr>
<td>References</td>
<td>149</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td></td>
</tr>
<tr>
<td>-----------------</td>
<td></td>
</tr>
<tr>
<td><strong>Chapter 1</strong></td>
<td></td>
</tr>
<tr>
<td>Figure 1.1. The splicing reaction .................................................. 3</td>
<td></td>
</tr>
<tr>
<td>Figure 1.2. Alternative splicing is the main source of protein diversity .... 5</td>
<td></td>
</tr>
<tr>
<td>Figure 1.3. Alternate U2 Structures .................................................. 14</td>
<td></td>
</tr>
<tr>
<td><strong>Chapter 2</strong></td>
<td></td>
</tr>
<tr>
<td>Figure 2.1. Dynamic RNP rearrangements occur throughout the spliceosome assembly pathway ................................................................. 20</td>
<td></td>
</tr>
<tr>
<td>Figure 2.2. Ubiquitination regulates Brr2’s role in formation of a catalytically-active spliceosome .......................................................... 23</td>
<td></td>
</tr>
<tr>
<td><strong>Chapter 3</strong></td>
<td></td>
</tr>
<tr>
<td>Figure 3.1. Splicing factors phosphorylated at conserved tyrosine residues within RRM-RNP motifs ............................................................. 66</td>
<td></td>
</tr>
<tr>
<td>Figure 3.2. Cus2 and U2 snRNA structures .............................................. 68</td>
<td></td>
</tr>
<tr>
<td>Figure 3.3. Cus2 RRM1 phospho-mutants exhibit genetic interactions with U2-IIc ................................................................. 72</td>
<td></td>
</tr>
<tr>
<td>Figure 3.4. Cus2 RRM1 alanine mutants exacerbate the splicing defect caused by the U2-G53A allele ......................................................... 74</td>
<td></td>
</tr>
<tr>
<td>Figure 3.5. Cus2 RRM1 mutants show altered binding of the U2 snRNA .......... 76</td>
<td></td>
</tr>
<tr>
<td>Figure 3.6. Prp5-GNT mutation is suppressed by Cus2 RRM1 mutants .......... 78</td>
<td></td>
</tr>
<tr>
<td>Supplemental Figure 3.1. Recombinant Cus2 is phosphorylated by several kinases in vitro ................................................................. 91</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 4

Figure 4.1. Cus2 does not exhibit genetic interactions with p-TEFb-associated cyclin homologs ................................................................. 101
Figure 4.2. Cus2 does not suppress bur2Δ 6-AU sensitivity .................... 105
Figure 4.3 Cus2 overexpression does not complement the Spt-phenotype of an spt4Δ strain ........................................................................ 107
Figure 4.4. U2 snRNA does not exhibit genetic interactions with p-TEFb-associated cyclin homologs ......................................................... 110
Figure 4.5. U2 snRNA does not exhibit genetic interactions with transcription elongation factor mutants or alter their transcription-related phenotypes ................................................................. 113
Figure 4.6. U2 snRNA does not suppress known phenotypes of bur2Δ cells ........................................................................................................ 116
Figure 4.7. U2 snRNA does not exhibit transcription-related phenotypes..... 119
Figure 4.8. The Bur complex does not exhibit physical interactions with Cus2 or U2 snRNA ........................................................................................................ 122
Supplemental Figure 4.1. U2 snRNA does not affect INO1 transcription..... 132

Chapter 5

Figure 5.1. bur2Δ has an in vivo splicing defect that cannot be repaired by alternate forms of U2 snRNA ........................................................................................................ 140
Figure 5.2. CUS2 deletion suppresses phenotypes of Prp19 and Prp2 alleles ................................................................................................. 145
LIST OF TABLES

Table 2.1. Post-translational modifications of splicing factors ..................... 46
Table 3.1. List of yeast strains used in this study ........................................ 88
Table 3.2. List of plasmids used in the study ................................................. 88
Table 3.3. List of primers used in this study .................................................. 90
Table 4.1. List of yeast strains used in this study ........................................... 130
Table 4.2. List of plasmids used in the study ............................................... 131
ACKNOWLEDGEMENTS

I would like to acknowledge my dissertation committee for their assistance with this thesis and for working with me to achieve my goals.

I would like to thank Tracy Johnson for her mentorship. She’s been supportive of my growth, whether it academic, personal, or professional, every step of the way.

I would like to acknowledge all the members of the Johnson lab past and present who have helped me at every step of the way throughout graduate school including Christina Chung, Felizza Gunderson, Julia Claggett, Herve Tiriac, Azad Munshi, Kim Elliot, and all of our fantastic undergraduates, particularly Patricia Tu and Caitlin Rodriguez.

My friends and family have been amazingly supportive throughout graduate school. I am lucky to have been surrounded by so many kind, smart, and fun people. I could not have done it without all of you.

VITA

2000 Bachelor of Arts, Rice University, Houston
2000-2003 U.S. Peace Corps Volunteer Teacher, Republic of Vanuatu
2010 Doctor of Philosophy, University of California, San Diego

PUBLICATIONS


FIELDS OF STUDY

Major Field: Molecular Biology

Study of spliceosome dynamics
Professor Tracy L. Johnson
ABSTRACT OF THE DISSERTATION

A Characterization of the role of phosphorylation in regulating pre-mRNA splicing

by

Susannah Leigh McKay
Doctor of Philosophy in Biology

University of California, San Diego, 2010
Professor Tracy L. Johnson, Chair

Pre-mRNA splicing is a key process in gene regulation that involves the removal of noncoding sequences from nascent RNA transcripts to form a mature message that can be translated into a protein. The spliceosome is a large, dynamic macromolecular complex which is responsible for the identification and removal of these introns. To achieve its role in splicing, the Spliceosome undergoes a series of dynamic rearrangements involving protein-protein, protein-RNA, and RNA-RNA interactions. How these rearrangements are regulated is not well understood and may involve posttranslational
modifications of the protein factors. The work presented in this thesis provides evidence that many splicing factors are in fact posttranslationally modified by phosphorylation, acetylation, ubiquitination, and glycosylation, although only a handful of these modifications have previously been described in detail. Our data indicate that phosphorylation of the yeast splicing factor Cus2 is important for its function in splicing. Specifically, we find that phosphorylation within a RNA binding domain (RRM) of Cus2 regulates its ability to associate with and mediate folding of its known substrate, the U2 snRNA. We discuss the potential implications of this finding, including the possibility that Cus2 is regulated in response to cellular conditions, including the cell cycle, in order to coordinate splicing with the events that occur during cell division. We further this investigation by exploring potential interactions between Cus2, its substrate U2 snRNA, and the Bur kinase complex. The mammalian homologs of these factors have been shown to physically interact in vitro to positively regulate transcription elongation. We investigated the possibility that a similar relationship exists between these factors in yeast. We were unable to find any evidence for a similar set of interactions in vivo or that the splicing factors have an obvious effect on transcription. Taken together, our data elucidate the role of posttranslational modifications in regulating Cus2’s role in splicing and provide further insight into the connection between splicing and the transcription machinery.
Chapter 1. Introduction

Pre-mRNA splicing

The central dogma first proposed by Sir Francis Crick in the 1950s states that genetic information flows by a residue-by-residue transfer of sequential information from DNA to RNA to protein. (Crick, 1970) Since the 1950s, many of the details of how this flow of information is achieved have been revealed, including the discovery of non-coding sequences within the DNA called introns (Berget et al., 1977; Chow et al., 1977). These non-coding sequences interrupt coding sequences, called exons, and must be removed in a process called pre-mRNA splicing before a functional, mature RNA-based message can properly encode for a protein. Understanding the details of pre-mRNA splicing is fundamental to understanding how the information encoded in genetic material is deciphered.

The removal of introns from pre-mRNA is catalyzed by a large, dynamic ribonucleoprotein complex called the spliceosome. Splice site recognition, spliceosome assembly, and catalysis of the two transesterification steps are achieved by the coordinated activities of five spliceosomal snRNAs (U1, U2, U4, U5, and U6) and their associated proteins (Matlin and Moore, 2007; Valadkhan, 2007; Wahl et al., 2009). The spliceosome is assembled in a step-wise manner (Figure 2.1). The assembling spliceosome intermediates can be isolated biochemically and their components have been well-characterized (Hamm and Lamond, 1998; Jurica et al., 2002; Konarska and
Query, 2005; Matlin and Moore, 2007; Rappsilber et al., 2002; Staley and Guthrie, 1998; Stevens et al., 2002; Valadkhan, 2007; Warkocki et al., 2009; Zhou et al., 2002). Briefly, the branchpoint of a pre-mRNA is marked for assembly by the branchpoint-binding protein BBP (SF1) and Mud2 (U2AF) which bind the polypyrimidine tract and 3’ splice site (ss). The U1 snRNP recognizes the 5’ss and binds in an ATP-independent step to form the CC/Commitment complex. Next, the U2 snRNP stably binds the branchpoint to form the prespliceosome (A complex) in the first ATP-dependent step of assembly. During A complex formation there is a concomitant release of the BBP and Mud2. The tri-snRNP consisting of the U5/U4/U6 trimer binds to form the complete spliceosome which undergoes further rearrangement. These rearrangements position the U4 snRNP for release to form the catalytically active spliceosome which is poised to complete the two transesterifications of the splicing reaction and formation of the intron lariat. Once the reaction is complete, the spliceosome undergoes more rearrangements to facilitate its own disassembly, release of the RNA, and the debranching of the lariat. This pathway accounts for more than 99% of splicing. However, there is also a separate splicing pathway catalyzed by the “minor spliceosome” which recognizes noncanonical splicing substrates. Additionally, some organisms undergo splicing through alternative mechanisms including self-splicing, commonly used in prokaryotes, and trans-splicing, which involves the ligation of two separate RNA molecules. (Di Segni et al., 2008; Fedorova and Zingler, 2007)
The splicing reaction consists of two transesterification reactions resulting in the removal of a branched lariat intron and ligation of the two exons (Figure 1.1). Although the actual chemistry is simple, the process regulating this reaction is complex. The spliceosome is a multi-megaDalton ribonucleoprotein particle whose components undergo dynamic yet tightly regulated rearrangements. There are over 100 different known protein factors that make up the spliceosome, however, this number does not take into account the factors that associate transiently or auxiliary factors required only under certain conditions that are therefore underrepresented in the global analyses used to determine the spliceosome composition thus far.

Figure 1.1. The splicing reaction. The splicing reaction consists of two transesterification reactions, resulting in the ligation of the two exons and release of the intron lariat.
Cis elements within the pre-mRNA define the intronic region and direct intron removal by the spliceosome (Fu, 2004; Wang and Burge, 2008; Wang et al., 2004). The essential cis elements are the 5' splice site GU at the 5' end of the intron, the 3' splice site AG sequence, and the branch point adenine nucleotide. These nucleotides are almost invariant but exist within larger sequences that impart unique consequences for each transcript. In addition to the basic cis elements required of each intron, there are other auxiliary cis elements which have enhancer or silencer effects on splice site usage. These elements have both exonic and intronic varieties denoted ESE, ESS, ISE, and ISS, respectively. These elements have been best characterized for mammalian introns, although there are suggestions that similar sequences exist in yeast (Konarska, personal communications). These cis elements are often employed during a process called alternative splicing.

Alternative splicing is the mechanism whereby multiple splice sites within a transcript are variably used to generate different transcripts that encode separate protein isoforms (Figure 1.2). In the simplest case, a transcript encoding 3 exons can be alternatively spliced to form 2 unique messages. Alternative splicing greatly increases protein diversity. For example, it contributes to the ability of the relatively small coding regions of the human genome to encode for over a million different proteins. (Black, 2003)
**Figure 1.2. Alternative splicing is the main source of protein diversity.** A transcript containing 3 exons (depicted as different colored bars) can be spliced into two alternative transcripts which encode two protein isoforms.

**Outstanding Questions**

The spliceosome is one of the cell’s most complex and dynamic macromolecular machines, which catalyzes the splicing reaction with an exquisite level of fidelity. Since the discovery of splicing by two independent groups in 1977 (Berget et al., 1977; Chow et al., 1977), much has been learned about the splicing reaction, spliceosome assembly, the spliceosomal factors, and some of the basic regulatory modes governing this reaction. However, the dynamic nature of the spliceosome has presented significant challenges to understanding the mechanisms that govern the splicing reaction. Fundamental questions remain about how this process is regulated. In particular, we are only beginning to appreciate the mechanisms that ensure the single-nucleotide fidelity required of the splicing reaction. Disruptions of the splicing process caused by mutation of the cis elements or spliceosome components are responsible for a variety of human disease including cystic
fibrosis, retinitis pigmentosa, and a several of cancer types (Boon et al., 2007; Gonzalez-Santos et al., 2008; Hagiwara, 2005; Sumanasekera et al., 2008). Understanding splicing is key to understanding the complexity of the cell, the mechanics behind dynamic processes, and how disease states occur.

**Spliceosome dynamics**

During spliceosome assembly and splicing catalysis the spliceosome undergoes a series of protein-protein, RNA-protein, and RNA-RNA rearrangements (reviewed in (Brow, 2002)). These rearrangements are dynamic yet tightly ordered. This order is achieved in part by the use of negative regulators of splicing that act as checkpoints before splicing can proceed. In addition, a two-state kinetic model has been proposed for how the stability of spliceosome conformations guide the first and second steps of splicing. (Query and Konarska, 2006) Here, factors which modulate the stability of these conformations are responsible for promoting splicing catalysis or substrate discard.

DExD/H box proteins are known to be important for guiding the rearrangements which contribute to splicing fidelity. Antagonistic pairs involving DExD/H box proteins have been identified, including Sub2 and Mud2/BBP, (Kistler and Guthrie, 2001; Wang et al., 2008b) Prp5 and Cus2, (Perriman and Ares, 2000; Perriman et al., 2003) Brr2 and the U4/U6 helix, (Brenner and Guthrie, 2005; Kim and Rossi, 1999; Raghunathan and
Guthrie, 1998) and Prp28 and U1C.(Chen et al., 2001) and are thought to act as checkpoints during the splicing cycle. For example, Mud2 and BBP mark a substrate for recognition by the spliceosome, yet their removal by the action of Sub2 is necessary to prompt the next step of splicing. These “checkpoints” occur throughout the splicing cycle. Despite the obvious importance of DExD/H proteins in the splicing reaction, the mechanisms that guide their specificity and activities are not well understood. In Chapter 2 we explore the idea that posttranslational modifications (PTMs) on these DExD/H proteins may trigger their catalytic activities or PTMs on other auxiliary proteins may regulate their interactions with the DExD/H proteins. Understanding how these important factors regulate spliceosomal rearrangements will be crucial to understanding spliceosome dynamics.

Co-transcriptional splicing dynamics

In addition to the contributions that core splicing factors make to spliceosomal dynamics, there is a growing appreciation for the contribution that external factors exert on the splicing reaction. Of particular interest is the coordination and coupling of spliceosome assembly and catalysis with transcription. Recognition of the pre-mRNA can occur as soon as the splicing signals are synthesized in a process called co-transcriptional splicing (for review see (Bentley, 2005; Goldstrohm et al., 2001; Kornblihtt, 2007; Maniatis and Reed, 2002; Misteli et al., 1997; Reed, 2003). Early EM imaging studies
demonstrated the co-transcriptional nature of splicing by showing that hnRNP particles associate with nascent transcripts which are then shortened via the removal of introns before release from the polymerase. (Beyer and Osheim, 1988; Osheim et al., 1985)

In addition to the spatial and temporal coordination of these processes, it is clear that functional coupling can also occur. Here, we define coupling as two two distinct biochemical processes that affect one another. For example, splicing factors interact directly with the phosphorylated heptapeptide repeats of the CTD of RNA polymerase II. (Corden and Patturajan, 1997; McCracken et al., 1997b) Truncation of these repeats of the CTD can result in splicing defects in vivo while, addition of CTD fragments can enhance splicing in vitro. (Hirose et al., 1999; McCracken et al., 1997b) Mutations in RNA polymerase II (RNAPII) that slow it down, deletion of transcription elongation factors, or growth of cells in the presence of drugs that negatively affect transcription elongation have been shown to alter exon inclusion and splice site choice. (Cramer et al., 1997; de la Mata et al., 2003; Kadener et al., 2001; Nogues et al., 2002) In addition to the polymerase CTD coordinating these events, other transcription factors including PCBP1, GFI1, p54(nrb), CA150, and even promoter usage have been shown to affect splicing events. (Heyd et al., 2006; Kameoka et al., 2004; Kornblihtt, 2005; Marko et al., ; Meng et al., 2007; Smith et al., 2004)

Splicing, in turn, can affect transcription. The removal of promoter proximal splice sites or introns results in decreased transcription in both
mammalian and yeast systems. (Furger et al., 2002) In mammals, the core splicing component, U1 snRNA regulates transcriptional initiation through its interaction with the transcription factor, TFIIH. (Damgaard et al., 2008; Kwek et al., 2002) In addition to initiation, transcriptional elongation can also be affected by splicing factors. In mammals the deposition of the splicing factor U2AF65 onto the nascent message allows the polymerase to overcome a pause at the U2AF65 binding site. (Ujvari and Luse, 2004) In yeast, the homologue of U2AF65, called Mud2, exhibits synthetic interactions with the CTD modifying enzymes required for transcriptional elongation (T. Johnson, unpublished), suggesting that a similar interaction may exist in S. cerevisiae. P-TEFb, a kinase complex responsible for promoting elongation by phosphorylating the CTD and other factors, associates with complexes containing transcription elongation and splicing factors. (Bres et al., 2008) One of these associations, with Tat-SF1 and the U snRNPs, was shown to enhance elongation in vitro. (Fong and Zhou, 2001) Here it was demonstrated that immunoprecipitates of P-TEFb contained a Tat-SF1-associated splicing complex that was able to stimulate transcriptional elongation. This finding was largely credited as evidence of a role for Tat-SF1 and the U2 snRNP in coupling transcription and splicing, although the detailed mechanism of this effect is unknown.

This exciting discovery demonstrated the importance of reciprocal stimulation of transcription by splicing. Zhou and Bensaude each followed by independently finding that the small nuclear RNA called 7SK inhibits the
elongation factor P-TEFb which consists of a CDK9/cyclin T1 heterodimer through direct association. (Nguyen et al., 2001; Yang et al., 2001) This was the first report of a non-coding RNA’s involvement in directing transcription. Since then, a flurry of reports have demonstrated the importance of non-coding RNAs in regulation of gene expression. Studies have shown that non-coding RNAs play important regulatory roles in Drosophila and E. coli in addition to mammals. (Allen et al., 2004; Espinoza et al., 2004; Sanchez-Elsner et al., 2006; Storz et al., 2006) The influence of these RNAs in diverse organisms presents the intriguing possibility that such mechanisms also exist in simpler eukaryotes such as yeast.

**Conservation of the spliceosome**

Most of the major splicing factors are conserved from yeast to mammals, a fact that has allowed for important mechanistic insights through the use of yeast as a model system. *S. cerevisiae* provide significant advantages to studies of complex processes as they are genetically and biochemically tractable. The yeast genome deletion project has generated a collection of yeast strains in which every ORF is individually deleted. (Cherry et al., 1998) As a result many of the significant advances in understanding splicing have come through studies in yeast. Furthermore, the biochemical assays available in yeast are well-established and have been shown to enable analyses at the mechanistic level.
There are also notable disadvantages to a unicellular model system. For instance, yeast do not have differentiated tissues and therefore questions about the role of splicing in development are not possible. Furthermore, not all metazoan splicing factors are present in lower eukaryotes. Notable examples include the SR class of proteins and other factors important for regulating alternative splicing. The paucity of multi-intron-containing transcripts and the lack of SR proteins in yeast has limited the ability to which yeast can be used to understand complex processes such as alternative splicing and regulated splicing.

Nonetheless, recent studies have challenged the assumption that simpler eukaryotes lack regulated and alternative splicing. In budding yeast Npl3 exhibits some qualities similar to metazoan SR factors and there is a growing appreciation that some yeast transcripts get alternatively spliced (T. Johnson lab unpublished results). (Juneau et al., 2009; Juneau et al., 2007; Kress et al., 2008; Lund et al., 2008)

**The U2 snRNP plays crucial roles in splicing**

The U2 snRNP plays key roles during splicing dynamics and has been implicated in co-transcriptional splicing. (Marzluff, 2007) The importance of the U2 snRNP in these processes makes it an attractive target for studies aimed at understanding the mechanisms underpinning splicing regulation.

The U2 snRNP consists of the U2 snRNA, the stably associated Lea1 (U2-A') and Msl1 (U2-B") polypeptides, and the heteromeric protein complexes
SF3a (consisting of Prp9, Prp11, and Prp21) and SF3b (consisting of Rse1, Hsh155, Hsh49, Cus1, and Rds3). (Will et al., 2002) There are several other auxiliary factors that specifically associate with U2 snRNP at particular times during the splicing cycle, including the DExD/H protein Prp5 and its antagonistic pair Cus2. The U2 snRNP plays several important roles during the splicing cycle. Addition of the U2 snRNP to form the prespliceosome is the first ATP-dependent step in the splicing cycle and commits the spliceosome to the reaction. The U2 snRNP is associated throughout splicing and is thought to play an important role in the actual catalysis reaction. (Dybkov et al., 2006; Gottschalk et al., 2001; Hilliker et al., 2007; Wu and Manley, 1991)

The U2 snRNA has highly conserved secondary structural features that allow it to adopt two different structures (Figure 1.3). During spliceosome assembly, the SF3a and SF3b complexes are responsible for loading U2 onto the branchpoint region of the pre-mRNA. (Igel et al., 1998; Yan et al., 1998) These proteins recognize the stem-loop IIa element in U2. Recognition of the pre-mRNA branchpoint by U2 is directed by an essential and conserved sequence upstream of stem-loop IIa called the branchpoint interaction region. (Ares and Igel, 1990; Igel et al., 1998; Zavanelli and Ares, 1991; Zavanelli et al., 1994)

Recently it was shown that the U2 snRNA toggles between the mutually the IIa structure and the mutually exclusive IIc structure. These rearrangements of the RNA are important for allowing splicing to progress and disruption of either structure reduces splicing fidelity. Another important
feature of the U2 snRNA is the stem-loop IIb. This area may act to stabilize
stem-loop IIa and/or interact with other U2 snRNPs. The conserved
complementarity region resides downstream of stem-loop IIb and is followed
by stem-loops III and IV (not shown in Figure 1.3). Stem-loop III, called the
fungal domain, is unique to yeast and dispensable for the RNA’s role in
splicing. The function of this large stem-loop has not yet been determined.
Stem IV serves as a binding site for Lea1 and Msl1, whose binding stabilizes
the snRNA.

Finally, Perriman and Ares have identified a stem-loop within the
branch-point binding region called the branch-point stem loop (BSL). (Perriman
and Ares) This stem loop (not shown in Figure 1.3) forms early in the splicing
cycle and facilitates proper association with the intron branchpoint.
Figure 1.3. Alternate U2 Structures. (A) Stem-loop IIa structure which is competent for splicing and (B) Alternate structure in which base-pairing within the stem-loop IIa is disrupted by base-pairing with the phylogenetically conserved sequence of complimentarity, C.C.. The branch-point (BP), G53 residue, and stem-loop IIb are also indicated. The 3' end of U2 is not depicted.
**Cus2 facilitates U2 snRNA rearrangements**

Before the U2 snRNP can be loaded onto the message, the snRNP must be activated, which is achieved, in part, by the disruption of competing U2 conformations to form the stem IIa-containing conformation of U2. Formation of U2-IIa is facilitated by the U2 snRNP protein Cus2. Cus2 has been shown to preferentially bind the U2 stem IIc-containing conformation (U2-IIc) and promote formation of activated U2 snRNPs containing U2-IIa, however, the exact mechanism of how Cus2 promotes U2-IIa formation is unclear. (Yan et al., 1998) Once the activated U2-IIa snRNP has formed the DExD/H box factor Prp5 facilitates the disassociation of Cus2 from U2 snRNA in an ATP-dependant manner. (Perriman and Ares, 2000; Perriman et al., 2003) Once Cus2 is removed, an ATP-independent function of Prp5 facilitates the stable binding of U2 snRNP to the pre-mRNA to form the pre-spliceosome. Together the activities of Cus2 and Prp5 result in the regulated formation of prespliceosomes containing a splicing-competent U2 snRNP stably bound to the pre-mRNA.

As described earlier, Cus2 may function as a "checkpoint" factor as it appears to have both negative and positive regulatory roles. Chapter 3 will detail the role of phosphorylation in regulating Cus2 activity and the implications for splicing dynamics.
Mammalian P-TEFb associates with the U2 snRNP

Fong and Zhou demonstrated in 2001 that the kinase complex called Positive Elongation Factor b (P-TEFb) enhances transcription when it is directly associated with the transcription factor TAT-SF1 and its associated U snRNPs. (Fong and Zhou, 2001) The data from this study suggest that interactions between U snRNPs and CTD kinases help to coordinate transcriptional elongation and splicing. This intriguing finding was one of the first to identify factors that potentially couple these two processes, although the mechanism is not yet understood.

The presence of homologous factors to TAT-SF1 and P-TEFb in yeast raises the possibility that yeast possess a similar mechanism for stimulating transcriptional elongation. Sequence homology suggests that the Bur and Ctk complexes are the likely homologs of P-TEFb. (Zhu et al., 1997) However, neither complex alone exhibits all of the functionality of P-TEFb. It has been proposed that together the Bur and Ctk complexes reconstitute the activity of their mammalian counterpart. (Bres et al., 2008; Wood and Shilatifard, 2006)

The Bur and Ctk complexes appear to facilitate transcriptional elongation at the 5' and 3' ends of genes, respectively, through a variety of mechanisms including CTD phosphorylation and chromatin modification (for review see Wood and Shilatifard, 2006)). The Bur complex consists of the Bur1 kinase and its associated Bur2 cyclin. The Bur1 kinase is required for efficient elongation by the polymerase, phosphorylates the CTD at serine 5 in vitro, and regulates histone H2B monoubiquitination. (Keogh et al., 2003) The
Ctk complex consists of the Ctk1 kinase, its associated Ctk2 cyclin, and a third regulatory protein, Ctk3. The Ctk complex is also required for elongation by the polymerase, phosphorylates the serine 2 in vitro, and regulates histone H3 trimethylation. (Cho et al., 2001; Patturajan et al., 1999; Sterner et al., 1995; Youdell et al., 2008) Both of these complexes have high sequence homology to P-TEFb and are likely candidates for participation in an interaction similar to that of TAT-SF1 and P-TEFb if one exists in yeast. In Chapter 4 we describe a detailed analysis of physical and genetic interactions between the yeast splicing factors Cus2 and the U2 snRNA and the yeast P-TEFb homologous complexes, Bur and Ctk. These studies provide important information about the in vivo relationship of these factors and inform current thinking about their potential roles in coupling transcription and splicing.

The studies detailed in the following chapters address many of the outstanding questions described in this introductory chapter. Each chapter addresses the fundamental question of how splicing dynamics are regulated. Chapter 2 provides an in-depth analysis of genome-wide data to highlight the extent of post-translational modification of splicing factors and explores the implications of these modifications in regulating splicing dynamics. Chapter 3 addresses the role of phosphorylation in regulating the activity of the U2 snRNP factor, Cus2, in directing splicing dynamics. We find that phosphorylation within the RNA binding domain (RRM) of Cus2 affects its role in splicing by affecting its ability to associate with RNA. We explore the larger implications of this finding by describing the phosphorylation of other RNA
binding proteins within their RRM domains. Chapter 4 is a detailed analysis of yeast splicing factors and their potential role in facilitating co-transcriptional splicing. These studies explore the evolutionary conservation of the relationship first described by Fong and Zhou that splicing factors associate with the transcription machinery and stimulate transcription. While this comparison is, on its face, compelling, we find no evidence that the yeast homologues of these factors exhibit similar physical or functional interactions. Finally, the implications of this work will be detailed further in the discussion chapter, along with proposed future studies.
Chapter 2. A bird’s-eye view of post-translational modifications in the spliceosome and their roles in spliceosome dynamics

Introduction

In eukaryotes, removal of introns from pre-mRNA is carried out by a large, dynamic macromolecular machine called the spliceosome. Splice site recognition, spliceosome assembly, and catalysis of the two transesterification steps are achieved by the coordinated activities of five spliceosomal snRNAs (U1, U2, U4, U5, and U6) and their associated proteins. Biochemical studies over the last 30 years have revealed much about the protein components of the spliceosomal snRNPs (small nuclear ribonucleoprotein) and their stepwise assembly to form the catalytically active spliceosome (reviewed in (Matlin and Moore, 2007; Smith et al., 2008; Valadkhan, 2007; Wahl et al., 2009) and illustrated in Fig. 2.1). Despite substantial advances in understanding the stepwise assembly of the spliceosome, the complex and highly dynamic nature of the splicing reaction still presents significant challenges to understanding the mechanistic details of splicing regulation.
Figure 2.1. Dynamic RNP rearrangements occur throughout the spliceosome assembly pathway. The snRNPs are depicted as colored balls. DExD/H proteins are shown at each step, and the proteins with which the DExD/H proteins interact (and which have opposing functions) are shown in italics. The GTPase Snu114, which is a ubiquitin conjugate and coordinates Brr2 activity is indicated in green text. Prp8 is also included to illustrate how post-translational modifications fit into the pathway.
Here we explore recent evidence from proteomics studies revealing extensive post-translational modification of splicing factors. Although the functional significance of many modifications remains to be elucidated, we describe recent examples of post-translational modifications for which specific regulated steps have been identified. These studies provide exciting insights into the likely functions of post-translational modifications in regulating the elegant choreography of the spliceosome.

**Post-translational modifications regulate dynamic processes**

Post-translational modifications (PTMs) such as phosphorylation, ubiquitination, acetylation, and O-GlcNAcylation work to fine tune nearly every cellular process by causing changes in a protein’s activity, cellular localization, and interactions with other factors. PTMs may contribute to the splicing cycle and splicing fidelity by facilitating the physical rearrangements of the spliceosome, controlling the timing of these rearrangements, regulating splicing factor activities, or altering the mRNP composition assembled on a particular transcript.

A recent report nicely illustrates the ways in which a particular PTM—ubiquitination—regulates the timing of spliceosome dynamics in budding yeast (S. cerevisiae). During spliceosome assembly, the U4/U6•U5 tri-snRNP associates with the pre-mRNA, whereupon the spliceosome is “activated,” in part by the release of the U4 snRNP (Fig. 2.2). A member of the DExD/H box family of proteins, Brr2, facilitates this process by unwinding the U4/U6 RNA
duplex. Like Brr2, each of the eight DExD/H box proteins that have been shown to play a crucial role in splicing hydrolyzes ATP to drive energy-rich RNA-RNA and RNA-protein rearrangements (reviewed in Staley, 1999 #157); hence, their activities are tightly regulated. Bellare et al. discovered that the ability of Brr2 to unwind U4/U6 is suppressed by ubiquitin, most likely when it is conjugated to the highly conserved U5 snRNP protein, Prp8. (Bellare, 2006 #63; Bellare, 2008 #62) It is thought that when Prp8 ubiquitination is disrupted (or when the ubiquitin moiety is occluded), Brr2 activity is no longer suppressed, and this activity leads to tri-snRNP disassembly. (Bellare, 2008 #62) Misregulation of Brr2 causes premature unwinding of U4/U6 and aborts the splicing pathway. Since Brr2 is also involved in a later step in splicing—U2/U6 unwinding—during spliceosome disassembly, (Small, 2006 #240) it is possible that this later step is also regulated by Brr2 ubiquitination. This example highlights the importance of ubiquitination in regulating spliceosome dynamics via its effects on both the physical and the temporal interactions of splicing factors.
Figure 2.2. Ubiquitination regulates Brr2’s role in formation of a catalytically active spliceosome. During the splicing reaction, formation of the catalytic spliceosome is facilitated by rearrangements resulting in tri-snRNP disassembly and U4 snRNP release. Ubiquitin negatively regulates spliceosome activation by suppressing Brr2-mediated U4/U6 unwinding. Once the ubiquitin moiety is removed or occluded, Brr2 activity is no longer suppressed and facilitates U4 snRNP dissociation.
Large-scale proteome analysis enables a better understanding of PTMs in splicing

As this example illustrates, it is crucial to understand how PTMs contribute to splicing regulation, but the splicing reaction presents particular difficulties for identifying PTMs and their effects. The spliceosome is highly dynamic, as are most PTMs. Capturing a splicing factor in a particular modified state is difficult given the transient nature of splicing intermediates and of the modifications themselves. Furthermore, identification of the enzyme responsible for a particular modification is difficult, as its association with the spliceosome is likely to be transient. Lastly, as will be discussed below in more detail, some PTMs may be specific to particular environmental conditions.

The advent of proteomics and systems biology presents an opportunity to address some of the complex issues presented by the splicing reaction and machinery. Large-scale -omics investigations involve unbiased global analyses of cellular responses. Subsequent systems-style integration of these data sets allows for better understanding of the underlying biology. In the field of pre-mRNA splicing, proteomics studies have been used to determine the protein composition of each spliceosomal complex, thus providing valuable information about the structural rearrangements that occur at each step of the splicing cycle. (Behrens and Luhrmann, 1991; Behzadnia et al., 2007; Bessonov et al., 2008; Chen et al., 2007; Herold et al., 2009; Jurica et al.,
These studies have been further informed by large-scale approaches to understanding spliceosome transitional states using drugs that block PTMs and reveal the compositions of intermediate complexes. (Kuhn et al., 2009) The other proteomic studies have used mass spectrometry (MS) analysis to identify PTM substrates, including splicing factors, many of which are catalogued in the online database UniProt. (2007) The results of these studies are presented in table 2.1 and the evidence linking each one to splicing is described below.

**Phosphorylation**

Reversible protein phosphorylation is used as a regulatory mechanism in nearly all cellular processes. Dynamic phosphorylation events are achieved via the interplay of protein kinases and phosphatases that promote phosphate addition and removal, respectively. Phosphorylation of proteins within complexes is of particular importance since it alters physical interactions (by disrupting some ionic interactions and facilitating others), protein stability, and enzymatic activities. (Cohen, 2000)

Phosphorylation regulates many of the dynamic interactions that occur throughout spliceosome assembly, splicing catalysis, and spliceosome disassembly. Early work pointed to a role for cycles of protein phosphorylation and dephosphorylation by demonstrating that splicing cannot proceed *in vitro* in the presence of phosphatases or phosphatase inhibitors. (Mermoud et al.,
Indeed, there are splicing factors representing each step in the splicing pathway that are phosphorylated, suggesting that phosphorylation and dephosphorylation are important throughout the splicing cycle (Table 2.1). Although phosphorylation is sure to play a role in guiding spliceosome dynamics, only a handful of the 100+ non-SR splicing factors have been reported as phosphorylation substrates and even fewer have been described at the mechanistic level. Since there are a number of excellent reviews of SR protein phosphorylation, (Blencowe et al., 1999; Fluhr, 2008; Hagiwara, 2005; Stojdl and Bell, 1999; Tenenbaum and Aguirre-Ghiso, 2005; Valcarcel and Green, 1996) these will not be discussed here.

One clear example of how dynamic phosphorylation and dephosphorylation modulates the activity of a single factor is the U2 snRNP protein SAP155. Phosphorylation of mammalian SAP155 occurs prior to or concomitant with splicing catalysis. (Wang et al., 1998) SAP155 is then dephosphorylated during the second step of splicing by the PP1 or PP2A phosphatase or both. (Shi et al., 2006) Recruitment of PP1 to SAP155 is mediated at least in part by NIPP1 (nuclear inhibitor of protein phosphatase 1), which recognizes and binds to hyperphosphorylated SAP155 and stimulates its dephosphorylation. (Tanuma et al., 2008)

Analysis of proteomic data leads to predictions about how phosphorylation affects discrete steps in splicing, including commitment complex formation and pre-spliceosome formation. For example, during commitment complex formation, the yeast factor BBP and its partner Mud2
(SF1 and U2AF65, respectively, in humans) bind the branchpoint region of the pre-mRNA and form a bridging interaction with the U1 snRNP bound to the 5'SS. (Fleckner et al., 1997; Kistler and Guthrie, 2001) Subsequent pre-spliceosome formation requires the exchange of the U2 snRNP for BBP/SF1 and Mud2/U2AF65 at the branchpoint. (Kistler and Guthrie, 2001) Large-scale analyses have revealed that each of these factors is phosphorylated, a result which raises the possibility that the role of these factors in formation of commitment complexes and pre-spliceosomes might be regulated by their modification. (Albuquerque et al., 2008; Smolka et al., 2007) Indeed, genetic interactions between Mud2 and the PP1 phosphatase suggest that the cycle of phosphorylation and dephosphorylation of one or all of these proteins may play a role in this step. (Hershko and Ciechanover, 1998; Wilmes et al., 2008)

Regulated phosphorylation of splicing factors may also be a mechanism by which the activities of specific proteins are regulated in response to cellular conditions. For instance, in two large-scale studies examining cell-cycle specific phosphorylation events, Cus2, the yeast U2 snRNP factor important for facilitating proper U2 snRNA folding. (Perriman and Ares, 2007) was found to be phosphorylated, a finding that raises the possibility that Cus2 has roles in splicing that are cell-cycle dependent. Mutations in Cus2 that suppress the deleterious effect caused by misfolded U2 snRNA lie in close proximity to a putative CKII site within the protein’s acidic domain. This domain is also extensively phosphorylated in the mammalian homolog of Cus2, Tat-SF1, a fact which further suggests that phosphorylation within this domain is
important for Cus2 activity. (Dephoure et al., 2008; Li et al., 2007; Matsuoka et al., 2007; Olsen et al., 2006; Wang et al., 2008a; Yan et al., 1998) Moreover, since recent studies illustrate that, under different environmental conditions, the splicing of a certain subset of genes requires the activity of different splicing factors, (Pleiss et al., 2007a) it is likely that conditions rendered by progression through the cell-cycle also require specific splicing factor activities (modulated by PTMs) to mediate the appropriate responses.

These studies highlight two outstanding questions about the role of phosphorylation in splicing; namely, How does phosphorylation affect protein activities such as RNA binding and protein-protein interactions? And what mechanisms does the spliceosome employ to ensure that protein phosphorylation occurs at the right time? Clearly, new approaches will need to be employed to determine the global effects of phosphorylation on splicing.

**Ubiquitination**

Ubiquitination involves the covalent attachment of the small, conserved peptide ubiquitin (ub) to a target protein, almost exclusively at a lysine residue. Ubiquitin addition is catalyzed by an enzymatic cascade involving an E1 ub-activating enzyme, followed by the activities of an E2 ub-conjugating enzyme and an E3 ub ligase (reviewed in (Pickart and Eddins, 2004)). This process is reversible as a result of the activities of deubiquitinating enzymes (reviewed in (Amerik and Hochstrasser, 2004; Hershko and Ciechanover, 1998)). Monoubiquitination has emerged as an important signaling mechanism
separate from the well-characterized role of polyubiquitination in proteolysis (reviewed in(Sigismund et al., 2004)). Monoubiquitination of both histone and non-histone protein targets plays a role in regulating a number of important reactions, including transcription, DNA repair, signal transduction, and receptor internalization (reviewed in(Welchman et al., 2005)). Ubiquitination, in general, has been shown to regulate protein-protein interactions and often serves as a signal for subsequent phosphorylation events.(Hunter, 2007)

In addition to recent studies suggesting that ubiquitination of Prp8 regulates tri-snRNP disassembly,(Bellare et al., 2006; Bellare et al., 2008) there are other indications that ubiquitin or ubiquitin-like proteins (such as Sumo and Hub1) play additional roles in splicing. These moieties, and the enzymes responsible for their addition/removal, co-purify with splicing factors.(Makarov et al., 2002; Rappsilber et al., 2002) Interestingly, a number of splicing factors that exhibit genetic and physical interactions with one another contain motifs similar to those found in the ubiquitination machinery. The presence of these motifs suggests that the splicing factors themselves are directly involved in ubiquitination events. The formation of the pre-spliceosome and the biogenesis of snRNPs involved in this step appear to be regulated by ubiquitination and deubiquitination. For example, Prp19, an essential member of the Nineteen Complex (NTC) that, along with the tri-snRNP, associates with the pre-mRNA for formation of the pre-catalytic spliceosome, contains an E3 ligase U-box domain and exhibits ubiquitin ligase activity in vitro.(Hatakeyama et al., 2001) Mutations within this U-box domain
disrupt the NTC complex and produce splicing defects. (Ohi and Gould, 2002; Ohi et al., 2003; Ohi et al., 2005) Additionally, Sad1, an essential factor required for U4/U6 biogenesis and tri-snRNP addition, contains a C-terminal hydrolase (UCH) domain, which is typically associated with ubiquitin cleavage. (Brenner and Guthrie, 2005; Hunter et al., 2009) These findings suggest that splicing factors may directly contribute to cycles of ubiquitination and deubiquitination to facilitate progression of the splicing reaction and that the targets of their activities are likely to be other splicing factors. Consistent with this idea, several splicing factors have been identified as ubiquitin conjugates including Snu114, the U5 snRNP GTPase protein which coordinates Brr2 activity (Table 2.1). (Peng et al., 2003; Tagwerker et al., 2006) Snu114 exhibits genetic interactions with Prp19 and Sad1, (Brenner and Guthrie, 2005) a fact which presents the possibility that the Snu114 ubiquitination state is modulated by the activities of these proteins.

Phosphorylation marks are known to promote or inhibit subsequent ubiquitination events by altering E3 ligase binding sites. (Hunter, 2007) This mechanism of sequential modification may serve to calibrate ubiquitination events by directing E3 ligase binding, which normally occurs with little preference for the primary sequence. The highly ordered process of spliceosomal rearrangement is well-suited to such a tightly coordinated series of modifications and leads to the prediction that splicing factors are multiply modified. Indeed, the yeast factors Prp19, Prp43, Snu114, Sad1, Prp8, Cbp80, and Hsh155 have been shown to be both phosphorylated and
ubiquitinated (Table 2.1), and there are likely to be other examples. It is unclear if the addition of these modifications is coordinated or if they serve as completely independent marks, but it is easy to imagine the implications that this type of coordinated modification may have. Prp8 regulation may be an instance of coordinated modification since Prp8 contains many of these modifications, acts throughout splicing, and participates in a large number of sequential and coordinated RNA-RNA and RNA-protein rearrangements. (Grainger and Beggs, 2005) Further studies are needed to address this model directly.

**Acetylation**

Protein acetylation at lysine residues is a dynamic modification whose function in transcription and chromatin regulation has long been of interest. Lysine acetylation and deacetylation are catalyzed by a group of enzymes known as lysine acetyltransferases (KATs) and lysine deacetylases (KDACs). Acetylation of non-histone targets is known to affect protein stability, protein-DNA interactions, sub-cellular localization, and transcriptional activity (reviewed in (Kouzarides, 2000)). Recent proteomics studies have identified acetyl marks on a plethora of proteins involved in diverse cellular processes, including factors involved in splicing. (Basu et al., 2009; Choudhary et al., 2009; Kim et al., 2006; Lin et al., 2009)

In addition to large-scale studies identifying the targets of acetylation, biochemical studies in mammalian cells have shown that splicing factors that
are the targets of acetylation also associate with acetylation/deacetylation machinery. For example, the U2 snRNP factor SF3b130 associates with the KAT complex STAGA (Martinez et al., 2001) and is the target of acetylation. (Choudhary et al., 2009) Likewise, the RNA helicase p68, which facilitates spliceosome formation by destabilizing the U1/5' SS interaction, associates with KDAC1 (Liu, 2002) and is acetylated. (Choudhary et al., 2009) It is not known, however, how acetylation of these proteins affects their functions in splicing. Furthermore, it remains to be determined whether these splicing factors are the actual targets of the enzymes with which they associate.

A more direct role for acetylation in regulating splicing was suggested by Kuhn et al., who demonstrated that both KAT and KDAC inhibitors can affect splicing in vitro. (Kuhn et al., 2009) This study used three KAT and three KDAC small molecule inhibitors to block splicing at distinct steps. The composition of the stalled spliceosomes was then analyzed by mass spectrometry. These studies identified unique splicing complexes that accumulated only in the absence of proper acetylation or deacetylation. For instance, the KAT inhibitor anacardic acid blocked splicing at a complex lacking several U1 snRNP, SR, and NTC proteins, a result which suggests that inhibiting acetylation prevents the stable association of these proteins. Even though in the absence of KAT inhibition these studies could not confirm acetylation of the missing proteins using anti-acetyl antibodies or radioactively labeled acetyl coenzyme A, the majority of the proteins absent from these
stalled complexes have been identified as acetylation substrates in a large-scale study of proteins acetylated in vivo including FBP11, S164, SPF31, hPRP5, SSRp40, SRp55, SC35, SRm160, SRm300, Prp19, and Cdc5. (Choudhary et al., 2009) Further characterization of specific acetylation events using mutational analysis of acetylation substrates will be important for elucidating direct roles for acetylation in splicing.

The well-characterized role for KATs and KDACs in regulating chromatin dynamics presents the exciting possibility that lysine acetylation in vivo functions to coordinate transcription and splicing. Indeed, Gunderson and Johnson have shown that the acetyltransferase activity of the yeast KAT Gcn5 regulates co-transcriptional association of the U2-snRNP with the nascent transcript. (Gunderson and Johnson, 2009) The physical proximity of the KATs and the splicing factors during co-transcriptional splicing allows that acetylation of histones or splicing factors or both could affect their co-transcriptional spliceosome assembly and the activities of specific splicing factors.

Glycosylation

Protein glycosylation—the addition of saccharides to proteins—has been shown to regulate a variety of cellular processes including protein folding, cell signaling, cell adhesion, and protein stability (reviewed in (Hart et al., 2007; Wells and Hart, 2003)). The addition of O-linked N-acetylglucosamine (O-GlcNAc) to serine and threonine residues has been
shown to occur on numerous cytosolic and nuclear proteins. Like other PTMs, this modification is dynamic, with the level of the O-GlcNAc modulated by the opposing activities of O-GlcNAc transferases and β-N-acetylglucosaminidases. (Hart et al., 2007; Hurtado-Guerrero et al., 2008) This reversible modification affects protein-protein and protein-DNA interactions, protein stability and activity, and cell signaling cascades. (Nandi et al., 2006) Given the diverse functional regulatory roles of O-GlcNAc modification, it is possible that this modification also plays a role in splicing dynamics.

Large-scale analyses have identified several splicing factors as modified by O-GlcNAc. SF3b3 was identified in a large-scale screen for O-GlcNAc-modified proteins from HeLa cells. (Nandi et al., 2006) Several yeast splicing factors were identified in a large-scale study using lectins that specifically bind GlcNAc, including Slu7, Prp19, Npl3, and Isy1. (Gelperin et al., 2005; Kung et al., 2009) It remains to be determined whether O-GlycNAcylation of these mammalian and yeast splicing factors is important for their activities in splicing. Interestingly, O-GlcNAc modifies residues that can also be phosphorylated, and O-GlcNAc transferase stably associates with the phosphatase PP1, associations which suggest that this modification may directly compete with phosphorylation. (Hart et al., 2007; Wells and Hart, 2003) Indeed, several factors are both phosphorylated and O-GlycNAcylated on the same residues (Table 2.1), a fact which opens the possibility that there may be a regulated exchange of phosphates for O-GlcNAc on these proteins.
Determining the specific role of O-GlcNAc transferases in splicing will yield valuable insights into the roles of this modification in spliceosome dynamics.

**General roles for post-translational modifications of splicing factors**

In the next two sections we consider how PTMs may contribute to two important aspects of splicing. First we describe how modification of DExD/H box proteins may regulate the timing and fidelity of dynamic rearrangements that occur during splicing. Second, we describe how post-translational modifications of splicing factors may allow splicing to be responsive to changes in the environment.

**DExD/H proteins that guide splicing dynamics and fidelity are post-translationally modified.**

As described above, there are a number of examples illustrating that PTM of splicing factors can regulate the dynamic interactions that occur throughout the splicing pathway. Of particular interest are the eight conserved DExD/H-box proteins that drive many spliceosomal rearrangements and enforce splicing fidelity (Fig. 2.1). This class of proteins is characterized by the presence of a DExD/H motif along with six or seven other conserved motifs that determine their NTP-binding, NTP-hydrolysis, RNA-binding, and unwinding activities (reviewed in [Linder et al., 2001]). This key group of spliceosomal proteins hydrolyzes ATP to catalyze a number of both RNA-RNA and RNA-protein rearrangements that take place throughout the splicing
cycle. (Hamm and Lamond, 1998; Staley and Guthrie, 1998) The functional consequence of these activities is to progress, pause, or abort the splicing pathway. (Konarska and Query, 2005) making DExD/H box proteins major splicing “decision-makers.” A recent report indicates that post-translational modification of splicing factors is at least one mode of regulating this class of proteins.

Mammalian PRP28, a likely homolog of the yeast U5 snRNP DExD/H protein required for exchanging U6 snRNA for U1 snRNA at the 5’SS, (Staley and Guthrie, 1999) must be phosphorylated by SRPK2 for its stable association with the tri-snRNP and formation of the pre-catalytic complex. (Mathew et al., 2008) Although it is not known precisely which protein-protein or protein-RNA interactions are altered by PRP28 phosphorylation, PRP28 phosphorylation appears to promote physical interactions that allow for stable association of the tri-snRNP and pre-spliceosome. Furthermore, proteomics studies show that PRP28 is underrepresented in catalytic spliceosomes, (Bessonov et al., 2008) suggesting that PRP28 dissociates from the spliceosome before catalysis, perhaps as the authors suggest, mediated by its dephosphorylation.

The revelation that PRP28 must be phosphorylated to stably associate with the tri-snRNP, raises the question of whether PTM is a general mechanism for regulating the activity of DExD/H box proteins, and their influence on splicing fidelity. A compelling model of splicing fidelity posits that each transition along the splicing pathway represents two kinetically
competing conformations that must be stabilized or destabilized to advance or abort the splicing pathway. (Query and Konarska, 2004) To this end, the dynamics of splicing often involve the pairing of opposing factors that interact to ensure proper timing and fidelity of the splicing reaction. The DExD/H box proteins play a crucial role in these rearrangements. For instance, Prp16 activity is crucial for the transition between the first and second steps of splicing. (Schwer and Guthrie, 1991, 1992) The activity of Prp16 is opposed by Isy1, a member of the Nineteen Complex (NTC), which acts to stabilize the first step conformation of the spliceosome. (Villa and Guthrie, 2005) Perturbation of either Prp16 or Isy1 decreases splicing fidelity, a result which suggests that the interaction of these proteins regulates the kinetics of the first to second step transition. Similar antagonistic pairs involving DExD/H box proteins have been identified, including Sub2 and Mud2/BBP, (Kistler and Guthrie, 2001; Wang et al., 2008b) Prp5 and Cus2, (Perriman and Ares, 2000; Perriman et al., 2003) Brr2 and the U4/U6 helix (Kim and Rossi, 1999; Raghunathan and Guthrie, 1998; van Nues and Beggs, 2001), and Prp28 and U1C, (Chen et al., 2001) where the interactions mediate substrate rearrangements that are necessary for splicing to progress (Fig. 2.1). It is likely that the other DExD/H proteins (Prp2, Prp22, and Prp43) employ similar mechanisms to ensure proper timing and fidelity of splicing. (Perriman and Ares, 2007; Rutz and Seraphin, 1999)

Despite the obvious importance of DExD/H proteins in the splicing reaction, the mechanisms that guide their specificity and activities are not well
understood. We envision a model of spliceosome assembly whereby the DExD/H proteins associate with RNP subcomplexes and are poised to catalyze rearrangements when the correct conformations are achieved. PTMs on these DExD/H proteins may trigger their catalytic activities or PTMs on other auxiliary proteins may regulate their interactions with the DExD/H proteins. Indeed, post-translational modifications have been identified for all of the spliceosomal DExD/H box proteins and their partner proteins (Table 2.1). (Roy et al., 1995; Tanaka et al., 2007; Tsai et al., 2005; Tsai et al., 2007)

**A possible mechanism for mediating the rapid splicing changes that occur in response to extracellular conditions**

Competitive fitness and cell survival depend on a cell’s ability to swiftly respond to changes in the environment. Post-translational modification of spliceosomal proteins provides an efficient method for making splicing responsive to cellular conditions. Indeed, in metazoans, cell signaling affects alternative splicing by producing changes in SR protein phosphorylation in response to environmental cues. (Shin et al., 2004; Shin and Manley, 2004) The question remains whether general splicing factors are also modified in response to environmental changes.

Two recent studies in budding yeast suggest global splicing patterns are uniquely sensitive to changes in specific splicing factors and changes in environmental conditions. Splicing-sensitive microarrays were used to demonstrate that mutation of core splicing factors produces different splicing
profiles, a demonstration which suggests that splicing of each transcript is sensitive to the activities of specific proteins. (Pleiss et al., 2007b) Moreover, yeast cells exposed to changes in environmental conditions undergo rapid (within 2 minutes) changes in their genome-wide splicing profiles that are unique to the stimuli. (Pleiss et al., 2007a) Taken together, these studies produce an intriguing model that, in response to environmental changes, rapid PTMs alter the activities of specific splicing factors to modulate their roles in removing specific introns.

For example, not only is Prp8 ubiquitinated (as described above), but it also contains a JAB/MNP-like domain, which is implicated in binding ubiquitin. Upon amino acid starvation, two different alleles of Prp8 with mutations that flank the protein’s JAB/MNP-like domain produce different splicing profiles. (Pleiss et al., 2007b) Mutations of the JAB/MNP-like domain reduce both ubiquitin binding and U4/U6-U5 tri-snRNP levels, reductions that demonstrate that ubiquitin contributes to Prp8’s role in splicing. (Bellare et al., 2006; Bellare et al., 2008) These data raise the possibility that modification within this portion of the protein contributes to substrate specificity and implicates ubiquitin binding by Prp8 as important in splicing responses to the environment. While this hypothesis remains to be tested, it will be interesting to determine whether the same stress conditions used in this study produce changes in the levels of ubiquitin-bound Prp8 and U4/U6-U5 tri-snRNP. Furthermore, it will be interesting to determine if ubiquitin-mediated intramolecular actions in Prp8 contribute to its activity.
**Future directions**

Post-translational modifications have proven to be an important mechanism for regulating the highly dynamic interactions that guide the splicing cycle and ensure splicing fidelity. While proteomic studies have provided valuable information about PTM substrates, the next important steps toward understanding the role of PTMs in splicing must involve elucidating the functional consequences of these modifications. Outstanding questions to be addressed include determining which PTM events are required for each step of the splicing cycle, how combinations of modifications affect splicing factor activity, and under what conditions specific modifications are required.

Although current detection technologies and certain biochemical aspects of PTMs present specific challenges in achieving these three goals, integrating the current data sets with new proteomics data that become available will be key to advancing our understanding of global splicing dynamics.

It is also important to note the likelihood that some PTMs identified in proteomics studies are not important for the target’s role in splicing. In addition to regulating a protein’s function, PTMs may mark a protein for proper folding, degradation, or trafficking. Additionally, in the case of proteins that play multiple roles in gene expression, PTMs may be important for other, non-splicing functions of the proteins. It is also possible that some PTMs are nonspecific. For example, recent studies of the evolution of protein phosphorylation sites indicate that some phosphorylation events occur
nonspecifically on disordered protein surfaces. (Holt et al., 2009) Because many kinases are promiscuous, nonspecific modifications may occur, particularly on proteins that are complexed with a true target protein (i.e., are in close proximity with the kinase). These sites probably arise from random mutations and are not conserved until they acquire a function. As discussed below, specific experimental design considerations will facilitate determining which PTMs are important for regulating the splicing cycle.

**Challenge 1: Revealing PTM dynamics during splicing**

Although proteomic studies have greatly increased the number of identified spliceosomal proteins that are post-translationally modified by improving techniques for both enriching modified peptides and detecting them in mixed preparations, this number is still likely to be an underestimate. Furthermore, current MS methods are limited in the reproducibility of the data produced. However, new technologies like multiple reaction monitoring MS can be used to facilitate reliable, quantitative results with greater coverage. (Cox et al., 2005; Gocke et al., 2005; Mollah et al., 2007; Unwin et al., 2009) In addition to the technical challenges of various detection methods, the biology of PTMs presents challenges for identifying and characterizing PTMs during highly dynamic processes like splicing. Modifications are transient and modified peptides are likely to be in low abundance compared to their unmodified counterparts.
To study the timing of PTMs during the splicing cycle on a global scale, it will be necessary to combine proteomic analyses with methods that enrich for proteins specific to each step. For example, conditional alleles of individual splicing factors enrich for particular complexes under non-permissive temperatures. Because splicing is blocked, some PTMs may be stabilized and will provide information about the timing of particular splicing factor modifications. Furthermore, small molecules that inhibit specific modifications can be used to block spliceosomal rearrangements at specific intermediates, and mass spectrometry can complement these in vitro studies by identifying which proteins are modified at each step or within each complex. Improved methods for stabilizing PTMs, enrichment methods, and improvements in detection technologies will all likely contribute to an expanded set of PTMs and their role in splicing.

It is equally important to identify the enzymes responsible for catalyzing PTM addition and removal. However, these enzymes may associate only transiently with their substrates, thus decreasing the likelihood of their detection. Employing techniques such as in vivo crosslinking may enable the capture of such transient interactions in future studies.

**Challenge 2: Elucidating the PTM code**

In addition to the dynamic changes in PTMs during the splicing cycle, it is apparent that some splicing factors are multiply modified, a fact which raises the question of whether these modifications are coordinated. It is known that
such spatial and temporal crosstalk between PTMs is an important aspect of regulation. Roles for processive phosphorylation, crosstalk between multiple modifications within a single protein, and regulated stepwise-protein modification have already been established as important for protein function.

Recognition of particular splice sites is guided by an “mRNP code,” where the concerted activities of multiple splicing factors dictate splice site utilization. (Stamm, 2008) Similarly, splicing may be additionally regulated by a “PTM code”—where crosstalk between modifications within a single protein or multiple proteins modulates spliceosomal rearrangements and stepwise progression of the splicing cycle. Within a single protein there are typically several potential modification sites, some of which can be the target of more than one type of modification. Furthermore, crosstalk can exist between phosphorylation, ubiquitination, acetylation, and O-GlcNAcylation. For instance, phosphorylation can promote ubiquitination by creating a recognition signal for E3 ligase binding (Hunter, 2007; Olsen et al., 2006); ubiquitination can, in turn, stimulate lysine acetylation, while acetylation can inhibit ubiquitination. (Hunter, 2007) Likewise, O-GlcNAcylation occurs at the same serine/threonine residues available for the phosphorylation and can, in fact, inhibit phosphorylation of surrounding residues. (Hunter, 2007) Regulation of the activity of a specific splicing factor may involve multiple modifications that guide its involvement in sequential steps in splicing. Similarly, proteins containing PTM-recognition domains that bind different types of modifications can act as dual recognition systems, binding only when its interacting partners
are properly modified. Prp19, for example, is ubiquitinated, O-GlcNAcylated, and acetylated.

(Albuquerque et al., 2008; Choudhary et al., 2009; Gelperin et al., 2005; Lin et al., 2009; Ohi et al., 2003) It will be interesting to determine whether the addition of these modifications is coordinated and how each modification, whether independently or in combination, defines Prp19 activity.

Challenge 3: From ‘omics to mechanism: determining the functional consequences of PTMs on splicing

The abundance of proteomics and genomics data has added breadth to our current knowledge of splicing factor modifications and how splicing changes under different conditions. However, the differences in the ways in which these data sets were prepared—different growth conditions, sample preparations, mutations, and detection methodologies—have made it difficult to integrate the data. Addressing the functional consequences of splicing factor PTMs requires a systems approach combining proteomics and genomics with analysis of individual factors.

As suggested by genome-wide splicing changes in S. cerevisiae in response to environmental changes, it is possible that certain PTMs occur only under a narrow set of conditions that trigger the activity of specific splicing factors or the splicing of specific transcripts. (Pleiss et al., 2007a) Likewise, particular transcripts may require a modified splicing factor to facilitate their splicing, while others may not. The splicing-specific microarray has proven to be a powerful tool for identifying splicing changes under specific conditions.
Such arrays can be used in combination with PTM inhibitors, mutations in the PTM machinery, or mutations at splicing factor modification sites to identify how changes in PTM profiles produce concomitant changes in splicing. Additionally, the resulting data sets can be compared to identify conditions and mutations that produce overlapping effects on splicing and may reveal factors that act in concert under such conditions.

**Conclusion**

The spliceosome is an exquisitely coordinated macromolecular machine. The revelation that many splicing factors are post-translationally modified strongly suggests that many of the dynamic rearrangements that occur during the splicing cycle are regulated, in part, by the addition and removal of these PTMs. Understanding how the network of PTMs functions in regulating splicing will be the next exciting step toward understanding the coordinated mechanisms of spliceosome dynamics.

**Acknowledgements**

We would like to thank Dr. Stephen Rader, Dr. Mary Fae McKay, Julia Claggett, and Felizza Gunderson for critical reading of the manuscript, Patricia Tu for her work on table 2.1, and members of the Johnson lab for useful comments and suggestions. This work was supported by an NSF CAREER award to T.L.J. (MCB-0448010) and an NSF predoctoral fellowship to S.L.M.

Table 2.1. Comparison of post-translationally modified splicing factors across species.
Dashed lines separate homologous factors which are grouped according to their associated complex. Hu=Human, Mu=Murine, Sc=S. cerevisiae, Sp=S. pombe. Modifications are indicated by P=Phosphorylation, Ac=Acetylation, Ub=Ubiquitination, O-Glc=O-Glycosylation, Su=Sumo. Superscript numbers indicate the reference.

<table>
<thead>
<tr>
<th>Commitment Complex</th>
<th>SF1</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hu</td>
<td>Mu</td>
<td>Sc</td>
<td>Ac(Choudhary et al., 2009)</td>
<td>Jb(Peng and Cheng, 2005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BBP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cbp80</td>
<td>Hu</td>
<td>Mu</td>
<td>Sc</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ac(Choudhary et al., 2009)</td>
<td></td>
</tr>
<tr>
<td>Cbp20</td>
<td>Hu</td>
<td>Mu</td>
<td>Sc</td>
<td>Ac(Choudhary et al., 2009)</td>
<td></td>
</tr>
</tbody>
</table>

P(Beausoleil et al., 2004; Beausoleil et al., 2006; Dephoure et al., 2008; Giorgianni et al., 2007; Molina et al., 2007; Olsen et al., 2006)

P(Shu et al., 2004; Smith et al., 2007; Sweet et al., 2009; Trost et al., 2009; Villen et al., 2007; Zanivan et al., 2008)

P(Albuquerque et al., 2008; Smolka et al., 2007)

P(Dephoure et al., 2008; Wilson et al., 2000)

P(Villen et al., 2007)
Table 2.1. Comparison of post-translationally modified splicing factors across species (con’t).

<table>
<thead>
<tr>
<th>U1 snRNP</th>
<th>Rank</th>
<th>Species</th>
<th>Expression</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>U2AF65</td>
<td>Hu</td>
<td>Sc</td>
<td>Ac(Choudary et al., 2009)</td>
<td>P(Cantin et al., 2008; Dephoure et al., 2008; Gevaert et al., 2005; Imami et al., 2008; Molina et al., 2007; Olsen et al., 2006; Yu et al., 2007) P(Chi et al., 2007)</td>
</tr>
<tr>
<td>Mud2</td>
<td>Sc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U2AF35</td>
<td>Hu</td>
<td></td>
<td></td>
<td>P(Matsuoka et al., 2007; Olsen et al., 2006)</td>
</tr>
<tr>
<td>U1 70k</td>
<td>Hu</td>
<td>Sc, Mu</td>
<td>Ac(Choudary et al., 2009)</td>
<td>P(Beausoleil et al., 2006; Brill et al., 2004; Daub et al., 2008; Dephoure et al., 2008; Gauci et al., 2009; Mayya et al., 2009; Olsen et al., 2006; Rikova et al., 2007; Shin et al., 2004; Yu et al., 2007)</td>
</tr>
<tr>
<td>FBP11</td>
<td>Hu</td>
<td>Sc, Mu</td>
<td>Ac(Choudary et al., 2009)</td>
<td>P(Beausoleil et al., 2004; Cantin et al., 2008; Dephoure et al., 2008; Imami et al., 2008; Olsen et al., 2006) P(Albuquerque et al., 2008) P(Smith et al., 2007; Trost et al., 2009; Villen et al., 2007; Zanivan et al., 2008)</td>
</tr>
<tr>
<td>Prp40</td>
<td>Sc</td>
<td>Sp</td>
<td></td>
<td>P(Wilson-Grady et al., 2008)</td>
</tr>
<tr>
<td>Usa1</td>
<td>Sc</td>
<td></td>
<td>Js(Peng and Cheng, 2005)</td>
<td>P(Albuquerque et al., 2008; Li et al., 2007) P(Soufi et al., 2009)</td>
</tr>
<tr>
<td>Prp39</td>
<td>Hu</td>
<td>Sc</td>
<td>Ac(Choudary et al., 2009)</td>
<td>Jb(Peng and Cheng, 2005)</td>
</tr>
<tr>
<td>U1-A</td>
<td>Hu</td>
<td></td>
<td>Ac(Choudary et al., 2009)</td>
<td>P(Dephoure et al., 2008)</td>
</tr>
<tr>
<td>Snu71</td>
<td>Sc</td>
<td></td>
<td></td>
<td>P(Albuquerque et al., 2008; Li et al., 2007)</td>
</tr>
<tr>
<td>Nam8</td>
<td>Sc</td>
<td></td>
<td></td>
<td>P(Albuquerque et al., 2008)</td>
</tr>
</tbody>
</table>
Table 2.1. Comparison of post-translationally modified splicing factors across species (con’t).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Species</th>
<th>Modification</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>U1-C</td>
<td>Hu</td>
<td>Ac(Choudhary et al., 2009)</td>
<td>(Dephoure et al., 2008; Rush et al., 2005)</td>
</tr>
<tr>
<td>p68</td>
<td>Hu</td>
<td>Ac(Choudhary et al., 2009)</td>
<td>O-Glc(Nandi et al., 2006)</td>
</tr>
<tr>
<td>UAP56</td>
<td>Hu</td>
<td>Ac(Choudhary et al., 2009)</td>
<td>O-Glc(Nandi et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>Sc</td>
<td>Jb(Peng and Cheng, 2005; Tagwerker et al., 2006)</td>
<td>(Albuquerque et al., 2008; Chi et al., 2007; Li et al., 2007; Smolka et al., 2007)</td>
</tr>
<tr>
<td>Sub2</td>
<td>Hu</td>
<td>Ac(Choudhary et al., 2009)</td>
<td>O-Glc(Nandi et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>Sc</td>
<td>Jb(Tagwerker et al., 2006)</td>
<td>(Yu et al., 2007)</td>
</tr>
<tr>
<td>U2AFM</td>
<td>Hu</td>
<td>Ac(Choudhary et al., 2009)</td>
<td></td>
</tr>
<tr>
<td>DHX15</td>
<td>Hu</td>
<td>Ac(Choudhary et al., 2009)</td>
<td></td>
</tr>
<tr>
<td>Prp43</td>
<td>Hu</td>
<td>Ac(Choudhary et al., 2009)</td>
<td></td>
</tr>
<tr>
<td>SPF30</td>
<td>Hu</td>
<td>Ac(Choudhary et al., 2009)</td>
<td></td>
</tr>
<tr>
<td>SPF31</td>
<td>Hu</td>
<td>Ac(Choudhary et al., 2009)</td>
<td></td>
</tr>
<tr>
<td>PUF60</td>
<td>Hu</td>
<td>Ac(Choudhary et al., 2009)</td>
<td></td>
</tr>
<tr>
<td>SPF45</td>
<td>Hu</td>
<td>Ac(Choudhary et al., 2009)</td>
<td></td>
</tr>
<tr>
<td>SNRPA1</td>
<td>Hu</td>
<td>Ac(Choudhary et al., 2009)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mu</td>
<td>Ac(Choudhary et al., 2009)</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.1. Comparison of post-translationally modified splicing factors across species (con’t).

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>AC(Choudhary et al., 2009)</td>
<td></td>
</tr>
<tr>
<td>SNRPB2</td>
<td>Hu</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mu</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prp21</td>
<td>Sc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SF3A1</td>
<td>Hu</td>
<td></td>
<td>AC(Choudhary et al., 2009)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mu</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SF3A2</td>
<td>Hu</td>
<td></td>
<td>AC(Choudhary et al., 2009)</td>
<td>SuVerheggen et al., 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prp11</td>
<td>Sp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SF3A3</td>
<td>Hu</td>
<td></td>
<td>AC(Choudhary et al., 2009)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sap61</td>
<td>Sc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SF3B1</td>
<td>Hu</td>
<td></td>
<td>AC(Choudhary et al., 2009)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mu</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hsh155</td>
<td>Mu</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SF3B2</td>
<td>Hu</td>
<td></td>
<td>AC(Choudhary et al., 2009)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cus1</td>
<td>Sc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SF3B3</td>
<td>Hu</td>
<td></td>
<td>AC(Choudhary et al., 2009)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sap130</td>
<td>Sc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

References:
- Beausoleil et al., 2004
- Beausoleil et al., 2006
- Boudrez et al., 2002
- Cantin et al., 2008
- Daub et al., 2008
- Dephoure et al., 2008
- Han et al., 2008
- Imami et al., 2008
- Matsuoka et al., 2007
- Molina et al., 2007
- Olsen et al., 2006
- Peng and Cheng, 2005
- Nandi et al., 2006
- Olsen et al., 2006
- Rush et al., 2005
- Shu et al., 2004
- Villen et al., 2007
- Zanivan et al., 2008
- Albuquerque et al., 2008
- Wilson-Grady et al., 2008
- Chen et al., 2009
- Vertegaal et al., 2004
Table 2.1. Comparison of post-translationally modified splicing factors across species (con’t).

<table>
<thead>
<tr>
<th>SF3B4</th>
<th>Hu</th>
<th>Ac(Choudhary et al., 2009)</th>
<th></th>
<th>P(Rikova et al., 2007; Rush et al., 2005)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF3B5</td>
<td>Hu</td>
<td>Ac(Choudhary et al., 2009)</td>
<td></td>
<td>P(Matsuoka et al., 2007)</td>
</tr>
<tr>
<td>SF3B14</td>
<td>Hu</td>
<td>Ac(Choudhary et al., 2009)</td>
<td></td>
<td>P(Rush et al., 2005)</td>
</tr>
<tr>
<td>SF3B14b</td>
<td>Hu</td>
<td>Ac(Choudhary et al., 2009)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SF3B125</td>
<td>Hu</td>
<td>Ac(Choudhary et al., 2009)</td>
<td></td>
<td>P(Dephoure et al., 2008; Olsen et al., 2006)</td>
</tr>
<tr>
<td>Tat-Sf1</td>
<td>Mu</td>
<td></td>
<td></td>
<td>P(Bailiff et al., 2004; Smith et al., 2007; Trost et al., 2009; Villen et al., 2007; Zanivan et al., 2008)</td>
</tr>
<tr>
<td>Cus2</td>
<td>Sc</td>
<td></td>
<td></td>
<td>P(Dephoure et al., 2008; Han et al., 2008; Imami et al., 2008; Matsuoka et al., 2007; Molina et al., 2007; Nousiainen et al., 2006; Olsen et al., 2006; Wang et al., 2008a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P(Holt et al., 2009; Li et al., 2007; Ptacek et al., 2005)</td>
</tr>
<tr>
<td>DDX46</td>
<td>Hu</td>
<td>Ac(Choudhary et al., 2009)</td>
<td></td>
<td>P(Beausoleil et al., 2004; Dephoure et al., 2008; Olsen et al., 2006)</td>
</tr>
<tr>
<td>Prp5</td>
<td>Mu</td>
<td></td>
<td></td>
<td>P(Trost et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Sc</td>
<td></td>
<td></td>
<td>P(Ptacek et al., 2005)</td>
</tr>
<tr>
<td>Sad1</td>
<td>Hu</td>
<td>Ac(Choudhary et al., 2009)</td>
<td></td>
<td>Jb(Peng and Cheng, 2005)</td>
</tr>
<tr>
<td></td>
<td>Sc</td>
<td></td>
<td></td>
<td>P(Lizcano et al., 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P(Chi et al., 2007)</td>
</tr>
</tbody>
</table>
Table 2.1. Comparison of post-translationally modified splicing factors across species (con’t).

<table>
<thead>
<tr>
<th>SART1</th>
<th>Hu</th>
<th>Snu66</th>
<th>Sc</th>
<th>Su(Vertegaal et al., 2004)</th>
<th>Prp4</th>
<th>SART3</th>
<th>Hu</th>
<th>Sc</th>
<th>Ac(Choudhary et al., 2009)</th>
<th>Prp3</th>
<th>p54</th>
<th>Hu</th>
<th>Ac(Choudhary et al., 2009)</th>
<th>Prp38a</th>
<th>Prp38b</th>
<th>Hu</th>
<th>Ac(Choudhary et al., 2009)</th>
<th>Prp38</th>
<th>Sc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snu66</td>
<td>Sc</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prp4</td>
<td>Hu</td>
<td>Sc</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ac(Choudhary et al., 2009)</td>
<td></td>
<td></td>
<td></td>
<td>P(Beausoleil et al., 2008)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SART3</td>
<td>Hu</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P(Matsuoka et al., 2007; Olsen et al., 2006)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prp24</td>
<td>Sc</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P(Albuquerque et al., 2008; Smolka et al., 2007)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prp3</td>
<td>Hu</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P(Dephoure et al., 2008; Han et al., 2008; Matsuoka et al., 2007; Molina et al., 2007; Nousiainen et al., 2006; Olsen et al., 2006; Wang et al., 2008a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mu</td>
<td>Sc</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P(Dai et al., 2007; Zanivan et al., 2008)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p54</td>
<td>Hu</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ac(Choudhary et al., 2009)</td>
<td></td>
<td></td>
<td></td>
<td>P(Beausoleil et al., 2006; Cantin et al., 2008; Daub et al., 2008; Dephoure et al., 2008; Molina et al., 2007; Olsen et al., 2006; Tang et al., 2007; Yu et al., 2007)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prp38a</td>
<td>Hu</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ac(Choudhary et al., 2009)</td>
<td></td>
<td></td>
<td></td>
<td>P(Daub et al., 2008; Dephoure et al., 2008; Olsen et al., 2006; Yu et al., 2007)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prp38b</td>
<td>Hu</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ac(Choudhary et al., 2009)</td>
<td></td>
<td></td>
<td></td>
<td>P(Beausoleil et al., 2006; Dephoure et al., 2008; Gauci et al., 2009; Kim et al., 2005; Matsuoka et al., 2007; Mayya et al., 2009; Olsen et al., 2006; Olsen et al., 2006; Yu et al., 2007)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prp38</td>
<td>Sc</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P(Albuquerque et al., 2008)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.1. Comparison of post-translationally modified splicing factors across species (con’t).

| Prp8 | Hu  | Mu | Sc | Ac (Choudhary et al., 2009) | Jb (Bellar et al., 2006) | Su (Vertegaal et al., 2004) | P (Cantin et al., 2008; Dephoure et al., 2008; Matsuoka et al., 2007) | P (Matsuoka et al., 2007; Shu et al., 2004) | P (Albuquerque et al., 2008) |
| Prp6 | Prp1 | Hu  | Sc | Sp | Ac (Choudhary et al., 2009) | Su (Vertegaal et al., 2004) | P (Dephoure et al., 2008) | P (Ptacek et al., 2005) | P (Wilson-Grady et al., 2008) |
| Brr2 | Hu  | Sc | Ac (Choudhary et al., 2009) | Su (Vertegaal et al., 2004) | P (Beausoleil et al., 2004; Carrascal et al., 2008; Daub et al., 2008; Dephoure et al., 2008; Han et al., 2008; Nousiainen et al., 2006; Olsen et al., 2006; Wang et al., 2008a; Wang et al., 2006) | P (Albuquerque et al., 2008; Smolka et al., 2007) |
| SNRP116 | Snu114 | Hu  | Sc | Ac (Choudhary et al., 2009) | Su (Vertegaal et al., 2004) | P (Beausoleil et al., 2004; Matsuoka et al., 2007; Olsen et al., 2006) | P (Albuquerque et al., 2008; Gruhler et al., 2005; Li et al., 2007) |
| Prp28 | Hu  | Sc | Ac (Choudhary et al., 2009) | Su (Vertegaal et al., 2004) | P (Dephoure et al., 2008; Imami et al., 2008; Molina et al., 2007; Olsen et al., 2006; Rush et al., 2005) | P (Smolka et al., 2007) |
| Prp2 | Hu  | Sc | Ac (Choudhary et al., 2009) | Su (Vertegaal et al., 2004) | P (Dephoure et al., 2008; Imami et al., 2008; Molina et al., 2007; Olsen et al., 2006; Rush et al., 2005) | P (Smolka et al., 2007) |
| Prp31 | Hu  | Su (Vertegaal et al., 2004) | P (Dephoure et al., 2008) |
| Lsm2 | Hu  | Su (Vertegaal et al., 2004) | P (Matsuoka et al., 2007; Rush et al., 2005) |
Table 2.1. Comparison of post-translationally modified splicing factors across species (con’t).

<table>
<thead>
<tr>
<th>Nineteen Complex</th>
<th>U6 snRNP</th>
<th>Sp/Sc</th>
<th>Ac/0-Glc</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lsm3</td>
<td>Sp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lsm4</td>
<td>Sc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lsm6</td>
<td>Hu</td>
<td></td>
<td>O-Glc</td>
<td>Wilson-Grady et al., 2008</td>
</tr>
<tr>
<td>Cwc15</td>
<td>Hu/Sc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ecm2</td>
<td>Mu</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Syf1</td>
<td>Hu</td>
<td></td>
<td>Ac(Choudhary et al., 2009)</td>
<td>Dephoure et al., 2008</td>
</tr>
<tr>
<td>Syf2</td>
<td>Sc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spp381</td>
<td>Sc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isy1</td>
<td>Hu/Sc</td>
<td></td>
<td>Ac(Choudhary et al., 2009)</td>
<td>O-Glc(Gelperin et al., 2005)</td>
</tr>
<tr>
<td>Prp19</td>
<td>Hu/Sc</td>
<td></td>
<td>Ac(Choudhary et al., 2009)</td>
<td>O-Glc(Gelperin et al., 2005)</td>
</tr>
<tr>
<td>Cwc2</td>
<td>Sc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SKIP</td>
<td>Hu</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prp46</td>
<td>Sc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ntc20</td>
<td>Sc</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Reference:
- Prp19: Daub et al., 2008; Dephoure et al., 2008; Olsen et al., 2006
- Prp46: Zanivan et al., 2008
- Prp46: Albuquerque et al., 2008
- Ntc20: Albuquerque et al., 2008
- Isy1: Matsuoka et al., 2007
- Prp19: Dephoure et al., 2008; Albuquerque et al., 2008
Table 2.1. Comparison of post-translationally modified splicing factors across species (con’t).

<table>
<thead>
<tr>
<th>Ntr2</th>
<th>Sc</th>
<th>P(Albuquerque et al., 2008)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prp16</td>
<td>Hu</td>
<td>Ac(Choudhary et al., 2009)</td>
</tr>
<tr>
<td>Prp18</td>
<td>Mu Sc</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDC5</td>
<td>Hu</td>
<td>Ac(Choudhary et al., 2009)</td>
</tr>
<tr>
<td>DHX8</td>
<td>Hu</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sc</td>
<td></td>
</tr>
<tr>
<td>SRRM2</td>
<td>Hu</td>
<td>Ac(Choudhary et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Sp</td>
<td></td>
</tr>
<tr>
<td>Cwc21</td>
<td>Sp</td>
<td></td>
</tr>
<tr>
<td>Cwc22</td>
<td>Mu Sc</td>
<td></td>
</tr>
<tr>
<td>Cwc24</td>
<td>Sc</td>
<td></td>
</tr>
<tr>
<td>Cwc25</td>
<td>Sp</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.1. Comparison of post-translationally modified splicing factors across species (con’t).

<table>
<thead>
<tr>
<th>Auxiliary Factors</th>
<th>Species</th>
<th>Description</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slu7</td>
<td>Hu</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sc</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glc(Kung et al., 2009)</td>
<td></td>
<td>P(Albuquerque et al., 2006; Carrascal et al., 2008; Olsen et al., 2006; Wang et al., 2008a; Yu et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>P(Albuquerque et al., 2008; Li et al., 2007; Smolka et al., 2007)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spp2</td>
<td>Sc</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sp</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P(Albuquerque et al., 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P(Wilson-Grady et al., 2008)</td>
</tr>
<tr>
<td>PRP17</td>
<td>Hu</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mu</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P(Olsen et al., 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P(Sweet et al., 2009)</td>
</tr>
<tr>
<td>Lsm1</td>
<td>Sc</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P(Ptacek et al., 2005)</td>
</tr>
<tr>
<td>DBR1</td>
<td>Hu</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prp26</td>
<td>Mu</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sc</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ac(Choudhary et al., 2009)</td>
<td>P(Dephoure et al., 2008; Mayya et al., 2009; Olsen et al., 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P(Trost et al., 2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P(Li et al., 2007)</td>
</tr>
<tr>
<td>Smd2</td>
<td>Sc</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P(Soufi et al., 2009)</td>
</tr>
<tr>
<td>Npl3</td>
<td>Sc</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P(Albuquerque et al., 2008; Ficarro et al., 2002; Gruhler et al., 2005; Li et al., 2007; Smolka et al., 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P(Wilson-Grady et al., 2008)</td>
</tr>
<tr>
<td>Srp2</td>
<td>Sp</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P(Albuquerque et al., 2008; Li et al., 2007; Smolka et al., 2007)</td>
</tr>
<tr>
<td>Spp41</td>
<td>Sc</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Su(Zhou et al., 2004)</td>
</tr>
<tr>
<td>WBP11</td>
<td>Hu</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mu</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ac(Choudhary et al., 2009)</td>
<td></td>
<td>P(Albuquerque et al., 2008; Li et al., 2007; Smolka et al., 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P(Dai et al., 2007; Shu et al., 2004; Villen et al., 2007)</td>
</tr>
<tr>
<td>Urm1</td>
<td>Sc</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P(Albuquerque et al., 2008)</td>
</tr>
<tr>
<td>Aar2</td>
<td>Sc</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sp</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P(Albuquerque et al., 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P(Wilson-Grady et al., 2008)</td>
</tr>
</tbody>
</table>
Table 2.1. Comparison of post-translationally modified splicing factors across species (con’t).

<table>
<thead>
<tr>
<th>Factor</th>
<th>Species</th>
<th>Post-translationally Modified Splicing Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>KHSRP</td>
<td>Hu</td>
<td>Ac (Choudhary et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Mu</td>
<td>O-Glc (Nandi et al., 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P (Daub et al., 2008; Dephoure et al., 2008; Gauci et al., 2009; Matsuoka et al., 2007; Mayya et al., 2009; Molina et al., 2007)</td>
</tr>
<tr>
<td>TDP43</td>
<td>Hu</td>
<td>Glc (Nandi et al., 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P (Olsen et al., 2006)</td>
</tr>
<tr>
<td>KIAA1966</td>
<td>Hu</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mu</td>
<td></td>
</tr>
<tr>
<td>ZRAB2</td>
<td>Hu</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mu</td>
<td></td>
</tr>
<tr>
<td>MAGOH B</td>
<td>Hu</td>
<td>Ac (Choudhary et al., 2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GEMIN5</td>
<td>Hu</td>
<td>Ac (Choudhary et al., 2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Su (Vertegaal et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>Mu</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTB</td>
<td>Hu</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.1. Comparison of post-translationally modified splicing factors across species (con't).

<table>
<thead>
<tr>
<th>hnRNP</th>
<th>Hu</th>
<th>Mu</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td></td>
<td></td>
<td>P(Cantin et al., 2008; Daub et al., 2008; Dephoure et al., 2008; Gauci et al., 2009; Heibeck et al., 2009; Imami et al., 2008; Molina et al., 2007; Olsen et al., 2006; Rikova et al., 2007; Rush et al., 2005; Yu et al., 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P(Shu et al., 2004; Sweet et al., 2009; Trinidad et al., 2006; Villen et al., 2007; Zanivan et al., 2008; Zhou et al., 2008)</td>
</tr>
<tr>
<td>A3</td>
<td></td>
<td></td>
<td>P(Cantin et al., 2008; Daub et al., 2008; Dephoure et al., 2008; Gauci et al., 2009; Imami et al., 2008; Mayya et al., 2009; Molina et al., 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Su(Vertegaal et al., 2004)</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td>P(Beranova-Giorgianni et al., 2006; Carrascal et al., 2008; Daub et al., 2008; Dephoure et al., 2008; Giorgianni et al., 2007; Han et al., 2008; Molina et al., 2007; Olsen et al., 2006; Tang et al., 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P(Albuquerque et al., 2008; Shu et al., 2004; Trost et al., 2009; Villen et al., 2007; Zanivan et al., 2008)</td>
</tr>
<tr>
<td>D</td>
<td></td>
<td></td>
<td>P(Beausoleil et al., 2004; Carrascal et al., 2008; Daub et al., 2008; Dephoure et al., 2008; Han et al., 2008; Molina et al., 2007; Olsen et al., 2006; Tao et al., 2005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P(Zanivan et al., 2008)</td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
<td>P(Beausoleil et al., 2004; Daub et al., 2008; Dephoure et al., 2008; Matsuoka et al., 2007; Olsen et al., 2006; Rush et al., 2005)</td>
</tr>
</tbody>
</table>
Table 2.1. Comparison of post-translationally modified splicing factors across species (con’t).

<table>
<thead>
<tr>
<th>hnRNP</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>Hu</td>
<td>Mu</td>
<td>P(Beausoleil et al., 2004; Daub et al., 2008; Dephoure et al., 2008; Imami et al., 2008; Olsen et al., 2006; Rush et al., 2005; Yu et al., 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P(Shu et al., 2004; Sweet et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Ac(Choudhary et al., 2009)</td>
<td>Su(Vertegaal et al., 2004)</td>
<td>P(Dephoure et al., 2008)</td>
</tr>
<tr>
<td>hnRNP L</td>
<td>Hu</td>
<td>Mu</td>
<td>P(Beausoleil et al., 2006; Daub et al., 2008; Ficarro et al., 2002; Olsen et al., 2006; Wang et al., 2008a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P(Ballif et al., 2004; Shu et al., 2004)</td>
</tr>
<tr>
<td>hnRNP M</td>
<td>Hu</td>
<td>Ac(Choudhary et al., 2009)</td>
<td>Su(Vertegaal et al., 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mu</td>
<td></td>
</tr>
<tr>
<td>hnRNP U</td>
<td>Hu</td>
<td>Ac(Choudhary et al., 2009)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mu</td>
<td></td>
</tr>
<tr>
<td>RBM22</td>
<td>Hu</td>
<td>Ac(Choudhary et al., 2009)</td>
<td></td>
</tr>
<tr>
<td>Raly</td>
<td>Hu</td>
<td>Ac(Choudhary et al., 2009)</td>
<td>P(Beranova-Giorgianni et al., 2006; Carrascal et al., 2008; Daub et al., 2008; Dephoure et al., 2008; Giorgianni et al., 2007; Matsuoka et al., 2007; Olsen et al., 2006; Wang et al., 2008a; Yu et al., 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mu</td>
<td>P(Sweet et al., 2009; Trost et al., 2009; Villen et al., 2007; Zanivan et al., 2008; Zhou et al., 2008)</td>
</tr>
</tbody>
</table>
Chapter 3. Phosphorylation within an RRM domain regulates Cus2 activity by affecting RNA binding

Introduction

Pre-mRNA splicing—the removal of noncoding intron sequences that interrupt the coding region of eukaryotic genes—is a key target of regulation in eukaryotic cells. The pre-mRNA splicing reaction is catalyzed by the spliceosome, a large ribonucleoprotein complex made up of five small nuclear RNAs (snRNAs) that associate with over a hundred proteins to form small nuclear ribonucleo-protein complexes (snRNPs). These snRNPs assemble onto the pre-mRNA to form the catalytically active spliceosome. Both the snRNA and protein components of the spliceosome are highly conserved, and in vitro studies confirm that there is strong functional conservation of the spliceosome from yeast to metazoans. (Jurica and Moore, 2003; Ruby and Abelson, 1991; Sharp, 1994; Staley and Guthrie, 1998)

The spliceosome undergoes intricate and dynamic rearrangement of its components, which results in splicing of the pre-mRNA. First the U1 snRNP binds to the 5’ splice site of the pre-mRNA, while other non-snRNP proteins associate in the branchpoint region. The U2 snRNP binds to the branchpoint to form the pre-spliceosome. Next the U4/U6•U5 triple snRNP binds to the pre-spliceosome and forms the pre-catalytic spliceosome. Extensive snRNA base pairing rearrangements lead to the removal of the U1 and U4 snRNPs and formation of the catalytic spliceosome. (Staley and Guthrie, 1998; Wahl et
Detailed biochemical and genetic analysis has revealed that the DExH/D-box RNA helicases, an evolutionarily conserved class of splicing factors, drive the conformational changes of the spliceosome that ensure precise timing of these dynamic molecular interactions and exquisite fidelity of the splicing reaction. There are important implications to these highly coordinated, carefully timed events. Mutations in the proteins that affect the coordination and timing of the rearrangements lead to defects in splicing and a decrease in splicing fidelity. Although the dynamic nature of these spliceosome rearrangements has been a key focus of studies of splicing mechanisms, it is still unclear how they are coordinated.

Attempts to understand the regulation of snRNA rearrangements by their associated splicing factors has led to an interest in one regulatory mechanism that is likely to play an important role in directing the activity of splicing factors: post-translational protein modifications. Post-translational modifications are thought to contribute to the splicing cycle and splicing fidelity by facilitating or disrupting protein-protein and protein-RNA interactions that restrict the timing of specific rearrangements or alter the mRNP composition that is assembled on a particular transcript.

An extensive survey of the available genome-wide analyses of post-translational modifications reveals evidence of splicing factor acetylation, ubiquitination, glycosylation, etc., all of which have been implicated in splicing (Chapter 2). The protein modification that is likely to be one of the most widespread and important for regulation of the activity of the spliceosome is
phosphorylation. Splicing extracts derived from human cell lines reveal evidence of protein kinases that phosphorylate splicing factors to directly regulate splicing events. For example, phosphorylation of hPrp28 by the kinase SRPK2 (Mathew et al., 2008) is necessary for the U4/U6•U5 triple snRNP addition to the pre-spliceosome to form the pre-catalytic spliceosome. Additionally, the mammalian protein Sap155 (yeast Hsh155) is phosphorylated prior to the first catalytic step, and must be dephosphorylated prior to the second catalytic step (Wang et al., 1998; Zhou and Yik, 2006). In addition to core spliceosome components, in mammals the SR proteins are regulated by cycles of phosphorylation and dephosphorylation (Reviewed in (Graveley, 2000; Manley and Tacke, 1996)). Importantly splicing is blocked in vitro by the PP1 phosphatase, and phosphatase inhibitors block splicing before the first catalytic step, two facts that indicate that dynamic cycles of phosphorylation are important (Mermoud et al., 1994).

Far less is known about the role of phosphorylation in regulating spliceosomal rearrangements in yeast, although—as in mammals— it is likely to be important. In fission yeast, the kinase Prp4, which is essential for splicing, affects phosphorylation of the splicing protein Prp1 in vivo, although the precise role of this phosphorylation is unclear (Schwelnus et al., 2001). In S. cerevisiae, the SR-like protein Npl3 is phosphorylated by the Sky1 kinase and is required for splicing of a subset of genes (Kress et al., 2008), although the specific role of Npl3 phosphorylation in splicing remains to be elucidated. Our own observations of the large number of splicing proteins that undergo
phosphorylation suggest that this may be an important, albeit poorly characterized feature of spliceosome activity.

One of the proteins reported to be phosphorylated is Cus2 (Holt et al., 2009; Li et al., 2007), which is involved in modulating one of the most dynamic steps in spliceosome assembly, U2 snRNP recognition of the branchpoint. The U2 snRNP recognizes the pre-mRNA branchpoint sequence in the first ATP-dependent step of splicing and remains associated with the spliceosome throughout splicing. Previous studies have demonstrated that the U2 snRNA toggles between two (or more) secondary structures during both the first and second steps of splicing and that these rearrangements are essential for both splice site recognition and splicing fidelity. (Hilliker et al., 2007; Perriman and Ares, 2007) Cus2 is intricately involved in guiding U2 snRNA rearrangements. Cus2 mutants suppress growth and splicing defects caused by misfolded U2 snRNA. Furthermore, Cus2 preferentially binds the U2-IIc form of U2 snRNA and facilitates its rearrangement to the “activated” U2-IIa form. (Perriman and Ares, 2007) Cus2 then must be removed before the activated snRNP can be loaded onto the pre-mRNA. The DExH/D protein Prp5, and particularly its ATPase activity, plays a critical role in Cus2 removal and U2 snRNP activation. In fact, the ATPase activity of Prp5 can be bypassed by deletion of CUS2 (Perriman et al., 2003). Despite the fact that deleting CUS2 eliminates the requirement for the ATPase activity of Prp5, conferring ATP-independence leads to a decrease in splicing fidelity (Perriman and Ares, 2007). These findings suggest that Cus2 associates with U2
snRNA to facilitate its association with the spliceosome, and Cus2 remains bound to the snRNP to prevent inappropriate interactions until the correct U2 snRNP rearrangements are established for accurate splice site recognition. When the correct spliceosomal conformation is established, Cus2 is removed by Prp5 via its ATPase activity. However, since U2 rearrangements also occur at later steps in splicing, it is possible that Cus2 also affects these rearrangements as well.

Cus2’s positive and negative regulatory roles in U2 snRNA rearrangements underscore the importance of Cus2 in regulating the timing and fidelity of splicing. Since Cus2 undergoes post-translational modification, we have carried out a mutational analysis at the phosphorylation sites identified in vivo to address the question of whether these modifications affect its role in splicing. Cus2, like a number of other splicing factors, is phosphorylated at a conserved tyrosine residue within the protein’s RNA Recognition Motifs. Here we show that mutation of this tyrosine leads to synthetic lethality when combined with misfolded U2 snRNA. Surprisingly, mutations that would be expected to either prevent phosphorylation or mimic phosphorylation have a similar effect. Consistent with this, both of these mutants abrogate Cus2 binding to U2 and bypass the requirement for the ATPase activity of Prp5. These data demonstrate that this tyrosine residue is crucial for the activity of the RRM and suggest that dynamic phosphorylation at this site may be required for the tyrosine’s role in U2 snRNP rearrangements. Surprisingly, mutation of a nearby serine that has been shown to be
phosphorylated in vivo also exhibits synthetic interactions with the U2 snRNA, particularly under conditions that hyperstabilize an improperly folded RNA. The inability of this residue to undergo phosphorylation leads to an increase in RNA binding, suggesting that removal of this phosphorylated residue contributes to release of U2 snRNA interactions with Cus2. Taken together these results suggest that Cus2 phosphorylation within the conserved RRM contributes to its role in pre-mRNA splicing. We discuss the implications of phosphorylation within RRM factors as a general mechanism for regulating splicing factor activities.

Results

Tyrosine Phosphorylation within RNPs is a common feature of RNA splicing factors

Reversible phosphorylation is a common regulatory mechanism used in nearly every cellular process. However, relatively little is understood about how this modification directs the events that take place during pre-mRNA splicing. To begin to understand the role of phosphorylation in regulating splicing we performed a directed search of the wealth of data produced in several large-scale analyses of global phosphorylation in yeast and metazoans. This search identified many factors involved in messenger RNA splicing. Importantly, many of these factors were phosphorylated within key functional motifs including RNA-binding motifs (RRMs). We hypothesized that
phosphorylation events important for the function of these factors would occur at conserved residues within these domains. Indeed this search identified many phosphorylated residues within key functional motifs and at conserved residues. Notably, several splicing factors are phosphorylated at conserved residues within the signature RRM sequence motifs called ribonucleoprotein 1 and 2 (RNP1 and RNP2) (Figure 3-1).

Structure comparisons of RRMs from a variety of proteins have shown that the residues within the RNP motifs form stacking interactions with single stranded RNAs. Specifically, RNP1 positions 1, 3, and 5 and RNP2 position 2 have been implicated in directly interacting with the nucleic acid. Our search identified several factors as phosphorylated at tyrosine residues at RNP2 position 2 (Figure 3.1). The fact that several splicing factors are phosphorylated at this conserved tyrosine residue thought to be important for directing interactions with RNA, suggests that this modification affects the RNA binding activity of these factors. Pre-mRNA splicing has been shown to be rapidly and tightly regulated by a variety of cellular conditions including the cell cycle and environmental stress. It is possible that tyrosine phosphorylation is a general mechanism for regulating RNA binding of these splicing factors or, perhaps, in response to particular conditions.
Figure 3.1. Splicing factors phosphorylated at conserved tyrosine residues within RRM-RNP motifs. The phosphorylated residues are indicated (*).

The yeast splicing factor Cus2 is phosphorylated *in vivo* and *in vitro*

To investigate the role of phosphorylation at this conserved tyrosine residues, we characterized this phosphorylation at this tyrosine within the yeast splicing factor Cus2. Cus2 consists of 3 primary domains—2 RRM and a C-terminal acidic domain (Figure 3-2). Global phosphorylation analyses have shown Cus2 to be phosphorylated *in vivo* at residue S163 within the...
linker domain (Figure 3-2). (Li et al., 2007) Our own mass spectrometry analysis has confirmed the S163 phosphorylation. A more recent global analysis by Holt et al, (2009) identified two residues, Y48 and S50 within Cus2’s first RRM, as being phosphorylated. (Holt et al., 2009) Our initial mass spectrometry analysis did not identify these sites however, due to the technical challenges of this type of analysis it is not uncommon for different experiments to display different levels of coverage. Therefore, it is difficult to make a conclusion from this negative result. We predict that improvements in the technical aspects of our mass spectrometry in identifying phosphorylated amino acids will allow better coverage of the Cus2 protein and will identify these (and perhaps other) phosphorylated amino acids in the protein.
Figure 3.2. Cus2 and U2 snRNA structures. A. Diagram of Cus2 showing phosphorylation sites identified by mass spectrometry (green arrows). B. Schematic of the U2 snRNA showing the mutually exclusive U2-IIa and U2-IIc forms. The position of the G53A substitution is shaded red. The sequences that base-pair to form the IIc stem-loop are shaded in blue. The region of U2 important for binding the branchpoint of pre-mRNA is shaded in green.
In addition to the *in vivo* studies, both recombinant and immunoprecipitated Cus2 can be readily phosphorylated *in vitro*. Recombinant protein is phosphorylated in splicing extract (personal communication, M. Ares) and immunoprecipitated Cus2 protein is phosphorylated by a variety of kinases in an *in vitro* kinase assay (Supplemental Figure 3-1A). We attempted to identify the residues phosphorylated *in vitro* by mutating predicted phosphorylation sites identified by the online program YeastNetPhos 1.0 and subjecting these mutant proteins to an *in vitro* kinase assay to look for reduced signal compared to the wild-type protein. However, these mutants still gave a phosphorylation signal, a fact that suggests that Cus2 can be phosphorylated at several sites *in vitro* (Supplemental Figure 3-1B). Initial experiments to detect phosphorylated Cus2 in immunoprecipitates (e.g., immunoblotting using commercially available antibodies against phosphorylated serine side chains) were not successful. However, this is not surprising as the amount of phosphorylated Cus2 protein is likely to be low in relation to the total protein amount and these general antibodies may not be sensitive enough for accurate and specific detection. Additional experiments, beyond the scope of this study, will be required to identify the additional site(s) of phosphorylation and thus provide detailed confirmation of the part played by it in the splicing cycle.

To determine the functional relevance of phosphorylation events identified *in vivo* each of the residues identified in the mass spectrometry
analyses were individually mutated. Specifically, mutants were generated with alanine (A) substitutions and either glutamic acid (E) or aspartic acid (D) in order to mimic the effects of an unphosphorylated or phosphorylated residue, respectively. Previous studies have demonstrated the utility of such substitutions in characterizing phosphorylation events. (Arguello et al., 1996; Corbin-Lickfett et al., ; Dulhanty et al., 1995; Hao et al., 1996; Iglesias et al., 1998; Potel and Elliott, 2005; Potter and Hunter, 1999; Russo et al., 2001; Schneider and Fanning, 1988; Suzuki et al., 2002; Yan and Templeton, 1994)

Cus2 has been shown to be important for facilitating proper folding of the U2 snRNA during prespliceosome formation. To address the possibility that phosphorylation regulates the ability of Cus2 to facilitate U2 snRNA folding we performed a genetic analysis of our mutants and a mutant U2 snRNA, depicted in Figure 3-2B. If the in vivo phosphorylation events are important for either Cus2’s ability to bind U2 or facilitate its rearrangements, then we expect Cus2 phospho-mutants to exhibit altered growth in combination with U2 mutants. We analyzed these mutants to determine their effect on U2-G53A growth and cold sensitivity (Figure 3-3A). Previously it was shown that deletion of CUS2 exacerbates the growth defect conferred by mutant U2 snRNA alleles, while mutations in CUS2 suppress the cold sensitivity of this allele. Both of these phenotypes are confirmed here. Substitutions at S163 do not exhibit a genetic interaction with the U2-G53A allele and these cells grow like wild-type in all of the conditions tested. Similarly, neither S50A nor S50D exhibit growth defects with U2-G53A at
30°C. However, S50A exacerbates the cold-sensitive phenotype of U2-G53A, a result that suggests this mutant has decreased RNA folding capability. Strikingly, both the alanine and aspartic acid substitutions at residue Y48 render these cells synthetically lethal in combination with the G53A U2 mutant snRNA in a manner similar to CUS2 deletion. To confirm that these mutations do not lead to protein destabilization, the protein levels of each of these mutants was analyzed (Figure 3-3B).
Figure 3.3. Cus2 RRM1 phospho-mutants exhibit genetic interactions with U2-IIc (G53A). Strains harboring Cus2 phospho-mutants were grown to the same OD<sub>600</sub> and four ten-fold serial dilutions were spotted and grown at the indicated temperature for 3-6 days. Cus2-9 is a known suppressor of U2-IIc sensitivity. B. Cus2 phospho-mutants are expressed at wild-type levels. Whole cell protein extracts from strains harboring HA-tagged Cus2 mutants were separated by SDS-PAGE, transfered to nitrocellulose, and immunoblotted against the HA epitope for Cus2 and against histone H3 as a loading control.
To determine the effect of the mutants on splicing efficiency, we performed an *in vivo* splicing analysis using strains harboring wild-type or the U2-G53A allele. Here, the levels of unspliced U3A and U3B RNA are assessed by primer extension using an oligo that recognizes both the unspliced and mature U3 RNAs. As was previously reported, U2-G53A causes a splicing defect that is exacerbated at cold temperatures and deletion of *CUS2* exacerbates this splicing defect (Figure 3–4). Neither S163 mutant exhibits altered splicing efficiency when compared to wild-type Cus2 (lanes 11 & 12 compared to lane 5) in agreement with the genetic analysis results. Similarly, the cold-sensitivity conferred by the S50A mutation leads to decreased splicing efficiency (lane 9). Although both the Y48A and Y48D mutants exhibit similar genetic phenotypes with U2-G53A, the effect of these mutants on splicing efficiency at 18°C differs. The splicing defect of Y48A is more pronounced compared to that of the Y48D mutant.

If Y48 phosphorylation is important for activating Cus2’s role in facilitating RNA folding, then we would expect to reveal differential effects of the unphosphorylatable and phospho-mimetic mutants. The similar genetic interaction of Y48A and Y48D with U2-G53A were therefore surprising and suggested that phosphorylation at Y48 may affect an activity of Cus2 that takes place upstream of its role in mediating U2 snRNA folding.
Figure 3.4. Cus2 RRM1 alanine mutants exacerbate the splicing defect caused by the U2-G53A allele. Yeast strains carrying the indicated alleles of U2 and CUS2 were grown at 30°C and (in B) shifted to 18°C for 4 h before extraction of RNA and primer extension with a labeled oligonucleotide complementary to U3 snRNA. Samples were normalized to contain the same amount of spliced U3 so that the level of unspliced U3 indicates splicing inhibition.
To address the possibility that phosphorylation regulates Cus2’s ability to bind the U2 snRNA *in vivo*, an RNA immunoprecipitation analysis was performed. Here, strains harboring epitope-tagged Cus2 mutant proteins were subjected to crosslinking and immunoprecipitated. The immunoprecipitates were extensively washed, the crosslinks reversed, and primer extension was performed to probe for the co-immunoprecipitation of the U2 snRNA. Similar to previously reported *in vitro* binding results, the Y48D mutation severely abrogates RNA binding relative to wild-type. Strikingly, the Y48A mutant has a similar effect on binding (Figure 3-5). These data suggest that mutations at this conserved tyrosine residue interfere with the protein’s ability to efficiently bind RNA and are consistent with the effects conferred by these mutants on growth.

In contrast, the S50A mutant exhibits enhanced U2 snRNA binding whereas the S50D mutant does not. Taken together with the cold-sensitive phenotypes of this mutant, these data suggest that the S50A mutant exhibits inappropriate RNA binding that affects the efficiency of splicing and cell viability. This result is consistent with a role for phosphorylation at this residue in regulating Cus2’s ability to bind RNA. These data also favor the hypothesis that the effect of these mutants represents a requirement for phosphorylation at S50 (and perhaps Y48) for proper Cus2 activity.
Figure 3.5. Cus2 RRM1 mutants show altered binding of the U2 snRNA. Formaldehyde crosslinked HA-tagged Cus2-RNA complexes were immunoprecipitated using an anti-HA antibody conjugated to Protein G sepharose beads. Complexes were extensively washed, treated with Proteinase K, and incubated at 65°C to reverse the crosslinks. RNA from the precipitate was extracted with phenol and used as a template in a primer extension reaction with U1, U2, and U4 snRNA-specific primers. Lea1 is a U2 snRNP protein and used as a positive control. Input is 1/20th.
**Cus2 phospho-mutants bypass the need for Prp5 ATPase activity**

CUS2 deletion rescues the lethal prp5-GNT ATPase mutant suggesting that the ATPase function of Prp5 is important for Cus2 removal. To determine whether Cus2 phosphorylation signals Cus2 removal we tested the ability of Cus2 phospho-mutants to suppress the lethality caused by mutating the NTP-binding domain of Prp5 (prp5-GNT). Similar to CUS2 deletion, Y48A and Y48D suppress a normally lethal prp5-GNT allele (Figure 3-6). Conversely, neither the S50A nor S50D mutant is able to rescue. In addition, none of the alleles tested, including CUS2 deletion, is able to suppress the prp5-GNT allele at low temperatures, a fact that suggests that Prp5 ATPase activity is required to facilitate rearrangement of an additional structure, perhaps within the U2 snRNA, that is stabilized at cold temperatures. Taken together with the RNA binding data, these results suggest that phosphorylation at Y48 disrupts Cus2’s interaction with the U2 snRNP via the U2 snRNA which abolishes both the ability of Cus2 to facilitate U2 snRNA folding and to negatively regulate prespliceosome formation.
Figure 3.6. *prp5*-GNT mutation is suppressed by Cus2 RRM1 mutants. The indicated strains were grown in Sc-ura-leu-trp to the same OD$_{600}$ and ten-fold serial dilutions were plated onto plates containing 5-FOA to shuffle out the *PRP5* wild-type plasmid. Plates were incubated at the indicated temperatures for 7 days.

Discussion

There is mounting evidence that many splicing factors undergo extensive posttranslational modifications, although the functional consequence of these phosphorylation events remain largely unexplored (Chapter 2). The data presented here inform the current model of Cus2 activity and suggest phosphorylation as a mechanism for Cus2 regulation. Our data demonstrate
that alteration of Cus2 phosphorylation sites within its RRM abrogates its ability to associate with the U2 snRNA and facilitate prespliceosome assembly whereas the role of a phosphorylation site within the protein’s linker region remains elusive. Because Cus2 has been shown to enforce the ATP-dependence of pre-spliceosome assembly, these results have implications for regulated splicing events.

Previous global phosphorylation studies identified Cus2 as phosphorylated at both Y48 and S50 residues within its RRM. (Holt et al., 2009) To investigate the role of these post-translational modifications in regulating Cus2 activity, we mutated each of these sites to phosphomimetic aspartate or non-phosphorylatable alanine. The tyrosine residue at position 48 lies within the protein’s RRM and is predicted to play a crucial role in directly binding nucleic acids via base stacking with its aromatic side chain. (Clery et al., 2008; Kielkopf et al., 2004; Maris et al., 2005; Shamoo et al., 1995) Cus2 is intolerant of other amino acid substitutions at this position, likely because of the important structural contribution of this residue. Although this intolerance prevents us from drawing direct conclusions from our mutational analysis of this residue, we favor the model in which phosphorylation of this tyrosine residue prevents Cus2’s association with RNA and ultimately blocks its function in splicing. Indeed there are several examples in the literature of tyrosine phosphorylation regulating the RNA-binding functions of proteins from viruses to metazoans. (Derry et al., 2000; Green et al., 1992; Haegebarth et al., 2004; Knirsch and Clerch, 2001; Lisitsky
and Schuster, 1995; Ostrowski et al., 2000; Pype et al., 1994) Furthermore, our directed search revealed that several RNA-binding factors are phosphorylated at this conserved residue within the RNP motif as shown in table 3.1. Although the function of each of these modifications remains to be elucidated, the prevalence and location of this modified residue is highly suggestive of a common function, namely regulating RNA binding.

Mutational analyses of phosphorylation sites often reveal opposing effects of the phospho-mimetic glutamic acid and aspartic acid substitutions and the alanine substitution. These opposing effects are interpreted as being indicative of the role phosphorylation plays at that site. For example, such phospho-mutant substitutions were used to demonstrate the role of DEK phosphorylation in facilitating proper splice site choice by U2AF in mammals. Here, a mutant harboring an alanine substitution at a putative serine phosphorylation site abolished the ability of DEK to enforce discrimination by U2AF between 3' splice site AG and CG dinucleotides, while the phospho-mimetic glutamic acid substitution mutant had the opposite effect. (Soares et al., 2006) In other instances the alanine and phospho-mimetic substitutions do not have opposing effects. This has been interpreted to be indicative of several things including a requirement for dynamic phosphorylation, a change in structure, or an upstream effect of the one being assay. For example, the ability of the viral protein ICP27 to disassociate from splicing speckles was impaired when both alanine and glutamic acid substitutions were made at its putative phosphorylation sites. (Corbin-Lickfett et al.) These effects were
interpreted as a requirement for both phosphorylation an dephosphorylation in regulating proper ICP27 function. Taken together, these studies highlight the value, but also the complexity, of analyses of these phospho-mutants.

It is unclear why both Y48 and S50 phosphorylations occur given the strong effect of changes made at the single tyrosine residue. Multisite phosphorylation can evolve by adding to or augmenting the specificity of the functional interactions regulated by the original phosphorylation site (Holt et al, 2009). Phosphorylation of S50 may work in a similar manner to fine-tune Cus2’s associations with RNA or proteins. The S50A mutant exacerbates the splicing defect of U2-G53A, exhibits a cold-sensitive growth defect, and binds U2 snRNA with a higher affinity than wild-type, but is not lethal like the Y48 substitutions. The S50D mutant is indistinguishable from wild-type and exhibits no phenotypes. The opposing effects of these mutations suggest that dynamic S50 phosphorylation contributes to proper Cus2 regulation. Other examples exist where the function of a primary phosphorylation site is specified by an auxiliary phosphorylation event. For instance, phosphorylation of the RNA-binding protein YB-1 at a residue proximal to its RNP motif does not alter RNA binding but does alter the protein’s interaction with the RNA’s cap.(Bader et al., 2003; Bader and Vogt, 2008) Mutations of either the RNP residues or this phosphorylation site within YB-1 results in reduced activity, a result which suggests that these two parts of the protein cooperate to specify this protein’s activity.
Tyrosine phosphorylation is an important transducer of cell signaling events and regulates a variety of cellular processes including growth, proliferation, and metabolism. Even in yeast, which lack true members of the protein tyrosine kinase family, tyrosine phosphorylation signals a variety of cellular transitions including pheromone signaling and cell cycle regulation. Yeast lack members of the true protein tyrosine kinase family, however, there are several yeast kinases that have tyrosine phosphorylation activity. (Booher et al., 1993; Brewster et al., 1993; Dailey et al., 1990; Errede and Levin, 1993; Gartner et al., 1992; Hoekstra et al., 1994; Irie et al., 1993; Lim et al., 1993; Ma et al., 1995; Sorger and Murray, 1992; Sun et al., 1996; Zheng et al., 1993) The majority of these proteins are involved in cell signaling. One of these kinases with activity directed at tyrosine residues is Swe1, which exhibits genetic, biochemical, or physical interactions with the splicing factors Snu16, Lin1, Yhc1, and Urn1. (Costanzo et al.,; Ptacek et al., 2005; Yu et al., 2008) It will be interesting to determine whether Swe1 is the kinase responsible for phosphorylating Cus2, perhaps during a specific cell cycle event. All three of the phosphorylation sites within Cus2 were identified under cell-cycle related conditions. Previous studies have demonstrated a connection between the events that take place during the cell cycle and pre-mRNA splicing. It is thought that splicing is generally down-regulated during mitosis, although the mechanisms of how this regulation is achieved remain to be elucidated. (Shin and Manley, 2004) There are several examples of splicing factors regulated by phosphorylation during cell-cycle specific events. SAP49
is phosphorylated at the conserved tyrosine residue within RNP2 and has been shown to affect cell cycle progression. (Terada and Yasuda, 2006) The RNA-binding activity of HuR is affected by phosphorylation sites within and proximal to its RRMs, presumed to be directed by the cell-cycle checkpoint kinase Chk2. (Abdelmohsen et al., 2007) The mammalian Sam68 is an RNA-binding protein whose tyrosine phosphorylation prevents its association with RNA. (Wang et al., 1995) In addition to other processes, this phosphorylation event affects pre-mRNA splicing through several mechanisms including inhibiting U2AF association with pre-mRNAs. (Tisserant and Konig, 2008) It will be important to explore other examples of signal-dependent splicing events.

**Why is Cus2 phosphorylated?**

Previous work has demonstrated that phosphatases and phosphatase inhibitors abrogate pre-mRNA splicing *in vitro*, a finding which indicates that rounds of phosphorylation and dephosphorylation are required during the splicing cycle. It is possible that Cus2 is dynamically phosphorylated in this way during its role in splicing. Consistent with this, it is also possible that Cus2 is phosphorylated in a signal-dependent manner to specifically prevent its entry into splicing complexes. In addition, Cus2 is not an essential protein and splicing can occur in its absence, a fact that further suggests that Cus2 phosphorylation is specific for regulating particular splicing events. Conversely, Cus2 may have other yet unidentified RNA substrates whose
binding is the main target of Cus2’s phosphorylation. It will be interesting to determine whether this is the case and whether phosphorylation alters the interactions and under what conditions.

This study has highlighted data from large-scale studies, which has shown that many splicing factors are phosphorylated at residues important for facilitating RNA binding. The work described here characterizing the role of tyrosine phosphorylation in regulating the RNA binding activity of the yeast splicing factor Cus2 suggests that this mechanism is potentially evolutionarily conserved. Future studies will be needed to explore the possibility that tyrosine phosphorylation may be a general, conserved mechanism for rapid and efficient regulation of RNA binding proteins. These studies also indicate a supporting role for phosphorylation at other residues within the RRM in modulating RNA binding. Future studies of this type will provide clues as to phosphorylation events that regulate both general and signal-dependent splicing events.

**Materials and Methods**

**Yeast strains and growth.**

The strains used in this study are listed in table 3.2 and plasmids are listed in table 3.3. All strains were propagated according to standard procedures in the appropriate selective media. Plasmid shuffling was performed on selective 5- fluoroorotic acid (5-FOA) plates. Standard methods for transformations and media preparation were used as described in Methods
in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual. The RP01 strain used for the genetic analyses with U2 snRNA alleles carries a chromosomal GAL-driven U2 gene is described elsewhere and was a generous gift from M. Ares. The wild-type CUS2 TRP1-marked (pRS314CUS2) and HA-HIS-tagged URA3-marked (pTAGnCUS2) plasmids are described elsewhere (Yan et al., 1998) and were also generous gifts from M. Ares. Standard site-directed mutagenesis was used to generate the CUS2 mutant alleles on both pRS314CUS2 and pTAGnCUS2 using oligos listed in table 3.4. CUS2 mutant alleles on pRS315 (centromeric plasmid with LEU2) were generated by subcloning the CUS2 gene from the pRS314 vector backbone into the pRS315 vector.

The CUS2 phospho-mutant genetic analysis in Figure 3.2 was performed by cotransforming the RP01 strain with the appropriate U2 LEU2-marked and CUS2 TRP1-marked plasmids. Transformants were selected on synthetic galactose media lacking uracil, leucine, and tryptophan. Growth was determined by serially diluting equal amounts of cells onto glucose-containing selective media to shut down the GAL-driven chromosomal U2 gene as previously described. (Yan et al., 1998)

**Genetic analysis of **PRP5. **Genetic analysis of the CUS2 mutant alleles with the prp5-GNT allele was performed by cotransforming the DS4D strain carrying the pIP45 plasmid containing wild-type U2 and PRP5 genes with the wild-type U2 gene on**
pRS317 (centromeric plasmid with LYS2) and the appropriate CUS2 allele on pRS315, and PRP5 allele on pRS314. Transformants were selected on synthetic media lacking uracil, lysine, leucine, and tryptophan. Growth of the mutant alleles was determined by shuffling out the pIP45 plasmid on plates containing 5-FOA lacking lysine, leucine, and tryptophan.

**RNA Immunoprecipitation.**

RIP was performed as described in Gilbert & Svejstrup (2006). 150ml of cells were grown in selective media to an O.D.600 0.5–0.7 and then crosslinked for 15 minutes with formaldehyde to a final concentration of 1%. Cells were disrupted with glass beads (0.5 mm) for 30 minutes at 4°C and lysates were cleared by centrifugation. To shear chromatin, lysates were sonicated with a Branson sonicator and 1/8” tip for a total of two minutes at 30% intensity (15 seconds on, 30 seconds off on ice). Input samples were removed and HA-tagged proteins were immunoprecipitated from 200 microliters of lysate with 12CA5 (Roche) antibody. After immunoprecipitation, samples were washed and incubated with Proteinase K (Invitrogen) and then at 65°C to reverse crosslinking. RNA was purified using phaselock tubes (Eppendorf) and analyzed by primer extension.
Yeast whole cell extract/western blot analysis

Whole cell extracts were prepared from BY4743 *cus2Δ* strains harboring the pTAGnCUS2 mutant plasmids as described elsewhere. (Gunderson and Johnson, 2009) Briefly, cells were grown to an O.D.600 of 1.0 and lysed using FA-1 Lysis buffer (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA pH 8.0, 1% Triton-X, 0.1% Deoxycholate, plus protease inhibitors) and 0.5 mm glass beads with 8 minutes of vortexing at 4°C. The supernatant was cleared by centrifugation and protein concentration was determined by Bradford Assay (Bio-Rad). 30 µg of total protein was fractionated by SDS-PAGE electrophoresis and transferred to a nitrocellulose membrane for immunoblotting with 1:2000 dilution of anti-histone H3 (AbCam ab1791) and 1:1000 dilution of anti-HA 12CA5 (Covance), followed by chemiluminescent detection (Pierce).

In vivo splicing analysis

RNA was prepared from strain RPO1 carrying the CUS2-TRP-marked and U2-LEU-marked plasmids. Cells were grown in selective galactose media overnight, washed, and used to inoculate 10mls of selective glucose media. Strains were grown for 12 hours at 30°C and then (if indicated) shifted to 18°C for 4 hours. Total RNA was prepared using phaselock tubes (Eppendorf) and used as a template for primer extension with a labeled U3-specific oligonucleotide.
Table 3.1. List of yeast strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS4D</td>
<td>MATa, prp5::kanMX4, cus2::kanMX4, SNR20::HIS3, trp1, Perriman et al., 2003</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DS4Dura3, leu2, lys2, pIP45 (PRP5 plus SNR20 on URA3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MATa, leu2-3,112, ura3-52, trp1, pep4-3, cus2::HIS3, Yan et al., 1998</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RP01 glucose-repressible SNR20 gene</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2. List of plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Plasmid Number</th>
<th>Description</th>
<th>backbone</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRS314</td>
<td>CUS2 WT</td>
<td>pRS314</td>
<td>pRS314</td>
<td>M. Ares</td>
</tr>
<tr>
<td>pTAGn</td>
<td>CUS2-HA-6XHIS</td>
<td>pYES1.2</td>
<td>pYES1.2</td>
<td>(Yan et al., 1998)</td>
</tr>
<tr>
<td>pSM051</td>
<td>CUS2-Y48A</td>
<td>pRS314</td>
<td>pSM051</td>
<td>This study</td>
</tr>
<tr>
<td>pSM052</td>
<td>CUS2-Y48D</td>
<td>pRS314</td>
<td>pSM052</td>
<td>This study</td>
</tr>
<tr>
<td>pSM053</td>
<td>CUS2-S50A</td>
<td>pRS314</td>
<td>pSM053</td>
<td>This study</td>
</tr>
<tr>
<td>pSM054</td>
<td>CUS2-S50D</td>
<td>pRS314</td>
<td>pSM054</td>
<td>This study</td>
</tr>
<tr>
<td>pSM055</td>
<td>CUS2-S163A</td>
<td>pRS314</td>
<td>pSM055</td>
<td>This study</td>
</tr>
<tr>
<td>pSM056</td>
<td>CUS2-S163E</td>
<td>pRS314</td>
<td>pSM056</td>
<td>This study</td>
</tr>
<tr>
<td>pSM057</td>
<td>CUS2-Y48A</td>
<td>pRS315</td>
<td>pSM057</td>
<td>This study</td>
</tr>
<tr>
<td>pSM058</td>
<td>CUS2-Y48D</td>
<td>pRS315</td>
<td>pSM058</td>
<td>This study</td>
</tr>
<tr>
<td>pSM059</td>
<td>CUS2-S50A</td>
<td>pRS315</td>
<td>pSM059</td>
<td>This study</td>
</tr>
<tr>
<td>pSM060</td>
<td>CUS2-S50D</td>
<td>pRS315</td>
<td>pSM060</td>
<td>This study</td>
</tr>
<tr>
<td>pSM061</td>
<td>CUS2-S163A</td>
<td>pRS315</td>
<td>pSM061</td>
<td>This study</td>
</tr>
</tbody>
</table>
Table 3.2. List of plasmids used in this study (con’t)

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Vector</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSM062</td>
<td>$CUS2-S163E$</td>
<td>pRS315</td>
<td>This study</td>
</tr>
<tr>
<td>pSM063</td>
<td>$CUS2-Y48A$</td>
<td>pRS315</td>
<td>This study</td>
</tr>
<tr>
<td>pSM064</td>
<td>$CUS2-Y48D$</td>
<td>pRS315</td>
<td>This study</td>
</tr>
<tr>
<td>pSM065</td>
<td>$CUS2-S50A$</td>
<td>pRS315</td>
<td>This study</td>
</tr>
<tr>
<td>pSM066</td>
<td>$CUS2-S50D$</td>
<td>pRS315</td>
<td>This study</td>
</tr>
<tr>
<td>pSM067</td>
<td>$CUS2-S163A$</td>
<td>pRS315</td>
<td>This study</td>
</tr>
<tr>
<td>pSM068</td>
<td>$CUS2-S163E$</td>
<td>pRS315</td>
<td>This study</td>
</tr>
<tr>
<td>pSM069</td>
<td>$CUS2-Y48A$</td>
<td>pYES1.2</td>
<td>This study</td>
</tr>
<tr>
<td>pSM070</td>
<td>$CUS2-Y48D$</td>
<td>pYES1.2</td>
<td>This study</td>
</tr>
<tr>
<td>pSM071</td>
<td>$CUS2-S50A$</td>
<td>pYES1.2</td>
<td>This study</td>
</tr>
<tr>
<td>pSM072</td>
<td>$CUS2-S50D$</td>
<td>pYES1.2</td>
<td>This study</td>
</tr>
<tr>
<td>pSM073</td>
<td>$CUS2-S163A$</td>
<td>pYES1.2</td>
<td>This study</td>
</tr>
<tr>
<td>pSM074</td>
<td>$CUS2-S163E$</td>
<td>pYES1.2</td>
<td>This study</td>
</tr>
<tr>
<td>pSM075</td>
<td>$CUS2-Y48A$</td>
<td>pYES1.2</td>
<td>This study</td>
</tr>
<tr>
<td>pSM076</td>
<td>$CUS2-Y48D$</td>
<td>pYES1.2</td>
<td>This study</td>
</tr>
<tr>
<td>pSM077</td>
<td>$CUS2-S50A$</td>
<td>pYES1.2</td>
<td>This study</td>
</tr>
<tr>
<td>pSM078</td>
<td>$CUS2-S50D$</td>
<td>pYES1.2</td>
<td>This study</td>
</tr>
<tr>
<td>pSM079</td>
<td>$CUS2-S163A$</td>
<td>pYES1.2</td>
<td>This study</td>
</tr>
<tr>
<td>pSM080</td>
<td>$CUS2-S163E$</td>
<td>pYES1.2</td>
<td>This study</td>
</tr>
</tbody>
</table>
Table 3.2. List of plasmids used in this study (con’t)

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pIP45</td>
<td>PRP5 plus SNR20</td>
<td>Perriman et al, 2003</td>
</tr>
<tr>
<td>pRS316</td>
<td>PRP5</td>
<td>Perriman et al, 2003</td>
</tr>
<tr>
<td>pRS317U2</td>
<td>WT U2</td>
<td>M. Ares</td>
</tr>
<tr>
<td>Prp5</td>
<td>WT PRP5</td>
<td>Perriman et al, 2003</td>
</tr>
<tr>
<td>PRP5-GNT</td>
<td>PRP5-GNT</td>
<td>Perriman et al, 2003</td>
</tr>
</tbody>
</table>

Table 3.3. Primers used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>S50A F</td>
<td>5’ CAAAATACTTCAATATATATTGCCGTCTTCCACAGAC AAAACAAACG 3’</td>
</tr>
<tr>
<td>S50A R</td>
<td>5’ CGTTGTTCATGCGGGAAGACCGGCAATATATATTGAA GTATTTTTTG 3’</td>
</tr>
<tr>
<td>S50D F</td>
<td>5’ CAAAATACTTCAATATATATTGACGTCTTCCACAGAC AAAACAAC 3’</td>
</tr>
<tr>
<td>S50D R</td>
<td>5’ GGGTTTCTGCGGGAAGACCGTGCAATATATATTGAAGT ATTTTTTG 3’</td>
</tr>
<tr>
<td>Y48A F</td>
<td>5’ CCTCTTAACTTTCTTCAATAGCCATTCTGCTTCCACACA GAC 3’</td>
</tr>
<tr>
<td>Y48A R</td>
<td>5’ GTCTCGGAAAGACCGAATGGCTATTGAAGTATTTTTT GAGG 3’</td>
</tr>
<tr>
<td>Y48D F</td>
<td>5’ GCCTCAAAAAATCTTTCAATAGAC 5’</td>
</tr>
<tr>
<td>U2-430R</td>
<td>5’ CAATAATGTGATTGG</td>
</tr>
<tr>
<td>U1-R</td>
<td>GAATGGAAACGTCAGCAACAC</td>
</tr>
<tr>
<td>U4-R</td>
<td>ACCATGAGGAGACGGTCTGG</td>
</tr>
</tbody>
</table>
Acknowledgements

We are grateful to Manuel Ares, Imre Barta, and their colleagues for providing plasmids and yeast strains. We thank Manuel Ares and Rhonda Perriman for helpful discussions and sharing unpublished data. This work was supported by an NSF CAREER award to T.L.J. (MCB-0448010) and an NSF predoctoral fellowship to S.L.M.

Supplemental Figures

Figure S3.1. Recombinant Cus2 is phosphorylated by several kinases in vitro. Immunoprecipitated Bur, Ctk, and Hsl1 kinase complexes were used to phosphorylate 0.5ug of recombinant Cus2 protein with γP-ATP. Reactions were incubated at room temperature for 30 minutes, resolved on a 12% SDS-PAGE gel, transferred to nitrocellulose, and exposed to X-ray film or phosphoimager plate.
Chapter 4. Genetic dissection of a role for yeast U2 snRNP components in transcription: Analysis of the Cus2:Tat-SF1 homology

In eukaryotes, removal of introns from pre-mRNA is carried out by a large, dynamic macromolecular machine called the spliceosome. Splice site recognition, spliceosome assembly, and catalysis of the two transesterification steps are achieved by the coordinated activities of five spliceosomal snRNAs (U1, U2, U4, U5, and U6) and their associated proteins. Once thought to be a distinct biochemical process, work in the last 10 years has done much to demonstrate that pre-mRNA splicing can occur co-transcriptionally as the pre-mRNA is being transcribed by RNA polymerase II (RNAP II). Recent work has established that molecular and functional interactions take place between the RNAPII elongation complex and the RNA splicing machinery. (Conrad et al., 2000; Corden and Patturajan, 1997; McCracken et al., 1997b) These interactions work to coordinate the two processes with one another in a manner that is thought to ensure efficient production and processing of mRNA.

A variety of studies have demonstrated that RNAPII is important for coordinating pre-mRNA splicing and transcription. The polymerase carboxy-terminal domain (CTD) has been shown to physically interact with splicing factors and to positively regulate splicing in vitro and in vivo. (Hirose et al., 1999; McCracken et al., 1997b) The carboxy-terminal domain of RNAPII (CTD) consists of a series of heptide repeats of YSPTSPS conserved from yeast (with 26 repeats) to humans (with 52 repeats) that undergo cycles of
phosphorylation and dephosphorylation during transcription. These changes that occur on serine-5 and serine-2 of the heptapeptide repeats correlate with transcription at the 5' and 3' ends of the gene, respectively (reviewed in (Palancade and Bensaude, 2003) and (Phatnani and Greenleaf, 2006)). Furthermore, these phosphorylation patterns appear to be important for coordinating the localization of transcription and RNA processing factors to the elongating polymerase complex.(Egloff and Murphy, 2008) Recently, it was discovered that in addition to serine-5, serine-7, of the heptide repeat is phosphorylated early in transcription, but the function of this modification, particularly in coordinating transcription and splicing, is not yet fully understood.(Akhtar et al., 2009; Chapman et al., 2007; Egloff et al., 2007) Post-translational modifications of the polymerase CTD by kinases, phosphatases, and prolyl isomerases have been shown to affect co-transcriptional splicing through multiple mechanisms (for reviews see (Buratowski, 2009; Doonan and Kitsios, 2009; Egloff and Murphy, 2008; Kim et al., 2009; Loyer et al., 2005)). For instance, the physical tethering of pre-mRNA processing factors to the CTD is dependent upon its phosphorylation status and structure. (Xu et al., 2003) Splicing factors shown to interact with the CTD include the capping enzymes (Cho et al., 1997; McCracken et al., 1997a), the SR and SR-like proteins in mammals (reviewed in (Corden and Patturajan, 1997)), and the yeast splicing factor Prp40 (Morris and Greenleaf, 2000). In addition to facilitating physical interactions, phosphorylation of the
CTD can affect the processive of transcription, which has been shown to, in turn, affect splice site choice.

The mammalian kinase complex P-TEFb is an important regulator of CTD phosphorylation and has multiple roles in coordinating transcription and pre-mRNA processing. (Bres et al., 2008) P-TEFb is recruited to the polymerase to stimulate serine-2 phosphorylation, a modification associated with productive elongation. CTD serine-2 phosphorylation in turn recruits SR splicing factors such as SF2/ASF and SC35 to the site of transcription, leading to stimulation of co-transcriptional splicing. (Cramer et al., 1999; Ge et al., 1998; Lin et al., 2008; Misteli et al., 1998) In addition, P-TEFb phosphorylation of other substrates, including Suppressor of Ty Homolog-5 (SPT5) and Negative Elongation Factor-E (NELF-E), has been shown to affect a complex network of chromatin modifications. (Pirngruber et al., 2009) These chromatin modifications, such as histone H3 lysine 36 trimethylation (H3K36me3), in turn affect splicing events including downstream recruitment of the U2 snRNP. (Sims et al., 2007) The splicing factors recruited by serine-2 phosphorylated CTD and these chromatin marks in turn stimulate P-TEFb recruitment and transcription. (Fong and Zhou, 2001; Lin et al., 2008) Taken together, these studies emphasize the reciprocal relationship of P-TEFb and splicing factors.

The role of P-TEFb at the interface between splicing and transcription was highlighted by an important report by Fong & Zhou in 2001. (Fong and Zhou, 2001) Here it was demonstrated that immunoprecipitates of P-TEFb
contained a Tat-SF1-associated splicing complex that stimulated transcriptional elongation. This finding was largely considered to be evidence for a role of Tat-SF1 and the U2 snRNP in stimulating transcription, although the detailed mechanism of this effect and whether this interaction occurs *in vivo* remained unknown.

The yeast homolog of TAT-SF1, *CUS2*, has been characterized in yeast as a U2 snRNP-associated splicing factor. The homology between *CUS2* and the TAT-SF1 has raised the intriguing question of whether Tat-SF1 also has a role in regulating splicing and whether *CUS2* has a role in regulating transcription. Recently it was shown that deletion of *CUS2* reduced influenza RNA synthesis in viral ribonucleoprotein complex (vRNP)-infected yeast cells.\(^{(Naito et al., 2007)}\) Analysis of Tat-SF1 knockdown in influenza-infected mammalian cells demonstrated that this protein facilitates formation of vRNP particles, independent of RNA synthesis or processing. These studies suggest that the observed effect of *CUS2* deletion on viral synthesis was due to a similar role for Cus2 as a chaperone for viral RNP assembly. Furthermore, this study raised the question of whether *CUS2* is capable of exhibiting a stimulatory effect on transcription similar to the effect of Tat-SF1 as reported by Fong and Zhou. Lastly, the involvement of viral components in these studies leaves open the question of whether these effects are specific to viral systems or represent a more general role for these factors in coupling transcription with splicing.
In addition to the homology between CUS2 and TAT-SF1, yeast also possess factors homologous to P-TEFb. The cyclin-dependent kinases (CDKs) Bur and Ctk exhibit equal sequence homology with P-TEFb (Zhu et al., 1997), although neither complex exhibits complete P-TEFb function. It has been proposed that together the Bur and Ctk complexes reconstitute the activity of their mammalian counterpart P-TEFb (Bres et al., 2008; Wood and Shilatifard, 2006). The Bur and Ctk complexes appear to facilitate transcriptional elongation at the 5' and 3' ends of genes, respectively, through a variety of mechanisms including CTD phosphorylation and chromatin modification (for review see (Wood and Shilatifard, 2006)). The Bur complex consists of the Bur1 kinase and its associated Bur2 cyclin. The Bur1 kinase is required for efficient elongation by the polymerase, phosphorylates the CTD at serine-5 in vitro, and regulates histone H2B monoubiquitination (Keogh et al., 2003). The Ctk complex consists of the Ctk1 kinase, its associated Ctk2 cyclin, and a third regulatory protein, Ctk3. The Ctk complex is also required for elongation by the polymerase, phosphorylates the serine-2 in vitro, and regulates histone H3 trimethylation (Youdell et al., 2008). (Cho et al., 2001; Patturajan et al., 1999; Sterner et al., 1995) Both of these complexes are likely candidates for participation in an interaction similar to that of Tat-SF1 and P-TEFb.

In yeast, the TAT-SF1 homolog, CUS2, aids in folding the U2 snRNA into a specific stem-loop structure that renders the RNA competent for its function in splicing. The ability of Cus2 to facilitate U2 snRNA folding is
abrogated by a mutation within one of its RRM{s} that abolishes U2 snRNA binding \textit{in vitro} (Yan et al., 1998) A corresponding mutation in Tat-SF1 has been shown to disrupt the protein-snRNP interaction necessary to enhance elongation. Interestingly, this mutation enhances the binding of Tat-SF1 to the cyclin, CYTC-1, of P-TEFb, sequestering the kinase into a complex that is unable to enhance elongation. This raises the possibility that the ability of Tat-SF1 to associate with the U2 snRNP, perhaps mediated by the snRNA’s secondary structure, is responsible for the effects on elongation described by Fong and Zhou (Fong and Zhou, 2001).

To address the possibility that the interactions between P-TEFb, Tat-SF1, and the U2 snRNP are conserved, we analyzed the relationship of the homologous factors in yeast. The stimulatory effect of Tat-SF1 on transcription is dependent upon its association with P-TEFb and the U2 snRNP. Perturbation of these interactions abrogated the stimulatory effect and inhibited transcription (Fong and Zhou, 2001). We therefore hypothesized that if a similar interaction exists in yeast, disruption of either CUS2 or the U2 snRNA would result in the same transcription-related phenotypes exhibited by perturbation of the CDKs. Furthermore, we predicted that these factors would physically associate in a manner similar to their mammalian counterparts.

Establishing the existence of a similar complex in yeast would allow for a deeper mechanistic analysis, so we analyzed Cus2 and U2 snRNA mutants using well-characterized assays of transcription elongation. These assays have been used previously to assess the P-TEFb homologs’ roles in
transcription. We find that, unlike the P-TEFb homologs, mutations of the U2 snRNP components do not exhibit sensitivity to 6-azauracil, inositol auxotrophy, or the Suppressor of Ty (Spt*) or Bypass UAS Requirement (BUR+) phenotypes. In addition, neither mutation of CUS2 nor the U2 snRNA exhibit genetic interactions with the Bur and Ctk complexes. Finally, we were unable to detect physical interactions between these factors. Taken together, we find a lack of evidence for a functional complex containing the Bur complex and the U2 snRNP.

Results

The TAT-SF1 yeast homolog CUS2 does not exhibit genetic interactions with the yeast homologs of P-TEFb

To address the possibility that Cus2 coordinates splicing and transcriptional elongation in yeast we began by examining genetic interactions between CUS2 and transcription factors, particularly the putative P-TEFb homologs. Synthetic interactions (such as synthetic lethality) are a hallmark behavior of genes involved in a common function.(Guarente, 1993) Not surprisingly, CUS2 deletion exhibits synthetic interactions with genes encoding other splicing factors including the U2 snRNA and several U2 snRNP-associated proteins including Prp5 and Cus1.(Perriman et al., 2003; Wells et al., 1996; Yan et al., 1998) Similarly, the CDK complexes exhibit genetic interactions with factors involved in transcriptional elongation including RNAPII, TFIIS, the Spt4/5 complex, and with one another.(Cho et al., 2001;
We predicted that if the Bur or Ctk complexes shared a functional overlap with CUS2, as is the case with P-TEFb and Tat-SF1, we would observe synthetic mutant phenotypes when mutations in the Bur or Ctk complexes were combined with deletion of CUS2.

To address this possibility, a cus2Δ strain was crossed with both bur2Δ and ctk2Δ strains and the double mutant progeny of these crosses were analyzed (Figure 4.1). BUR2 and CTK2 are both non-essential components of their P-TEFb-like complexes. Deletion of BUR2 or CTK2 eliminates the kinase activity of their respective complexes (Hautbergue and Goguel, 2001; Yao et al., 2000). A cus2Δ mutant grows indistinguishably from wild type cells, whereas a bur2Δ mutant shows a significant growth defect (Figure 4.1A). This slow growth phenotype is unchanged in the cus2Δ bur2Δ strain. Similarly, deletion of CTK2 results in a slow growth phenotype that is unaffected by CUS2 deletion (Figure 4.1B). Unlike other factors that contribute to Bur or Ctk activity and reveal this through synthetic interactions, we find no evidence of such a relationship between these factors and Cus2. Because we have found similar genetic interactions between both the Bur and Ctk complexes and CUS2, we focused only on the BUR interaction.

Factors involved in the same pathways are sometimes able to compensate for one another when overexpressed. (Forsburg, 2001) So, we considered the possibility that the CDK complexes and CUS2 acted in the same pathway such that deletion of one gene masked the phenotype
associated with deletion of the other. To test this, a genetic analysis was performed using strains in which BUR2 has been deleted and CUS2 is overexpressed (Figure 4.1C). Here, expression from a plasmid encoding a HA-tagged Cus2 protein under the control of the GAL1 promoter is induced by growing the cells on galactose media. GAL-induced Cus2 protein expression from this plasmid has been confirmed by western blot analysis using antibodies directed against the epitope (data not shown). Overexpression of CUS2 produces no effect in either WT or bur2Δ cells. These data suggest that CUS2 overexpression is not sufficient to bypass the need for BUR2.
Figure 4.1. Cus2 does not exhibit genetic interactions with p-TEFb-associated cyclin homologs. A. Analysis of \textit{bur2Δcus2Δ} double mutant. The indicated strains were grown in YPD and four ten-fold serial dilutions were spotted onto YPD plates and grown at 30°C for 3 days. B. Analysis of \textit{ctk2Δcus2Δ} double mutant. The indicated strains were grown in YPD and four ten-fold serial dilutions were spotted onto YPD plates and grown at 30°C for 3 days. C. \textit{CUS2} deletion does not exhibit genetic interactions with \textit{RPB1} truncation mutants. The indicated strains were grown in YPD and four ten-fold serial dilutions were spotted onto YPD plates and grown at 30°C for 3 days. (C. Chung) D. Cus2 overexpression does not complement \textit{bur2Δ} growth defect. The indicated strains carrying URA3-marked plasmids (vector or \textit{CUS2} under the GAL1 promoter) were grown in SC-ura and four ten-fold serial dilutions were spotted onto SC-ura plates containing glucose or galactose and grown at 30°C for 4 days and 6 days respectively.
CUS2 does not exhibit general transcription-related phenotypes

To determine whether CUS2 alone exhibits global defects in transcription, we performed a genetic analysis of CUS2 deletion combined with mutations in the most critical part of the transcription machinery, RNAP II itself. In yeast, the C-terminal domain of RNA polymerase II (CTD) is comprised of 26-52 heptapeptide “YSPTSPS” repeats and is phosphorylated at the transition from initiation to elongation. The number of heptapeptide repeats is important for facilitating proper elongation by the polymerase: reducing the number of repeats has been shown to cause slowed growth, temperature- and cold-sensitivity, and an ability to activate transcription of some genes in vitro and in vivo. (Meisels et al., 1995)

A common phenotype for mutation of transcription elongation factors is that cells become dependent on a fully functional, full-length CTD. In fact, mutations in BUR1 and CTK1 exhibit strong synthetic growth defects in combination with CTD truncation mutants. (Lindstrom and Hartzog, 2001; Murray et al., 2001) If CUS2 affects transcriptional elongation in general, and especially if it affects the activities of these CTD kinase complexes, then we expect to reveal this function through genetic interactions of CUS2 and the CTD truncation mutants. To analyze CUS2 interactions with the CTD, strains harboring deletion of this gene was crossed with an rpb1Δ strain harboring RPB1 on a plasmid. The double mutants were transformed with CTD truncation mutants on plasmids and the wild-type plasmid was shuffled out on
5-FOA to look at the effect of progressive truncations in the \( \text{cus2}\Delta \) background.

As the CTD is increasingly truncated, growth is slowed, but the yeast strains remain viable with a CTD truncated to eleven heptad repeats. These truncations confer increasing temperature and cold sensitivity. Additionally, mutation of either \( \text{BUR1} \) or \( \text{CTK1} \) results in an enhanced requirement for the CTD repeats and renders cells inviable in combination with CTD truncations less than 13 repeats. (Lindstrom and Hartzog, 2001; Murray et al., 2001)

\( \text{CUS2} \) deletion in combination with these CTD truncation mutants exhibits no synthetic growth defects at any temperature (Figure 4.1D for 30°C and data not shown). As with the genetic analysis of \( \text{cus2}\Delta \text{bur2}\Delta \) and \( \text{cus2}\Delta \text{ctk2}\Delta \), these data strongly suggest that \( \text{CUS2} \) does not exhibit functional overlap with the polymerase.

**\( \text{CUS2} \) does not suppress the transcription-related phenotypes of the Bur complex**

Deletion of \( \text{BUR2} \) results in several phenotypes that are classic indicators of defects in transcription including sensitivity to the drug 6-AU, inositol auxotrophy, Spt- and BUR- phenotypes. (Prelich and Winston, 1993; Yao et al., 2000) These phenotypes are commonly used to identify factors with roles in transcription, including factors that exhibit functional overlap with the CDK complexes, although not all transcription factors exhibit each of these phenotypes. To further investigate whether \( \text{CUS2} \) has functional overlap with
the Bur complex, we performed several genetic tests to determine if CUS2 deletion or overexpression affects these known phenotypes.

Hence, mutations in several components of the transcription elongation machinery, including the Ctk complex, render cells sensitive to the drug 6-azauracil. (Riles et al., 2004) Treatment of cells with 6-AU results in nucleotide starvation and an enhanced requirement for a fully functioning transcription apparatus for efficient transcription. To determine whether CUS2 deletion confers 6-AU sensitivity or affects the 6-AU sensitivity of bur2Δ, we analyzed growth of cus2Δ and cus2Δ bur2Δ on media containing 6-AU. Deletion of BUR2 alone results in strong sensitivity to 6-AU (Figure 4.2a). However, deletion of CUS2 exhibits no 6-AU sensitive phenotype alone nor does this mutation exacerbate the 6-AU sensitivity relative to bur2Δ alone.

Next, we tested the ability of CUS2 overexpression to suppress the 6-AU sensitivity of BUR2 deletion. CUS2 overexpression does not confer 6-AU sensitivity in cus2Δ cells nor does overexpression suppress the 6-AU sensitivity of bur2Δ cells (Figure 4.2b).
Figure 4.2. Cus2 does not suppress bur2Δ 6-AU sensitivity. A. CUS2 deletion does not affect the 6-AU sensitivity of bur2Δ. Strains were grown overnight in SC-ura, harvested, normalized by OD_{600}, and spotted onto plates in a series of four ten-fold dilutions. Dilutions were spotted onto solid media in the presence of 6-AU (100 μg/ml) or medium lacking uracil and incubated at 30°C for 3 days and 2 days, respectively. B. CUS2 overexpression does not affect 6-AU sensitivity of bur2Δ. The indicated strains carrying URA3-marked plasmids (vector or CUS2 under the GAL1 promoter) were grown in SC-ura and four ten-fold serial dilutions were spotted onto SC-ura galactose plates in the presence of 6-AU (100 μg/ml) or medium lacking uracil and grown at 30°C for 4 days and 6 days respectively. Dilutions from figure A and B were grown on the same plate.

CUS2 does not exhibit the Spt- phenotype

The mammalian factors P-TEFb, Tat-SF1, and Spt5 associate in a large complex that binds both Tat and RNAP II. These factors act in concert and are each required for Tat-dependent transcription. (Kim et al., 2003; Parada and Roeder, 1999) In addition to phosphorylating the polymerase CTD, P-TEFb phosphorylates Spt5 within these complexes to switch the Spt4/5 complex NELF from one that inhibits elongation to one that promotes elongation. (Kim et al., 2003) Therefore, the kinase activity of P-TEFb activates transcriptional elongation in two distinct, but complementary, ways.
A parallel interaction occurs in yeast. *BUR1* interacts genetically with *SPT4* and its partner *SPT5* and has been shown to phosphorylate the CTD of Spt5 to positively regulate transcriptional elongation. (Liu et al., 2009) If the stimulatory effect of P-TEFb and Tat-SF1 on transcriptional elongation are mediated, in part, by Spt5 phosphorylation, then we predict that perturbation of the Spt4/5 complex would abrogate this effect.

The Suppressor of Ty (SPT) genes were originally identified by their ability to genetically suppress the transcriptional defects conferred by Ty element insertion. (Winston et al., 1984) Strains harboring a δ Ty element in the *LYS2* gene promoter are Lys- but in the presence of *spt* mutations become Lys+. The *spt* mutants suppress these transcriptional defects through several different mechanisms, all involving alteration of transcription. Mutations in *SPT4*, *SPT5*, *BUR1* and *BUR2* confer Spt- phenotypes indicative of their roles in regulating transcription events. (Malone et al., 1993; Winston et al., 1984) Overexpression of the *bur1-3* allele exhibits a dominant negative Spt- phenotype. If *CUS2* exhibits functional overlap with the Bur complex, then overexpression of *CUS2* mutants may also confer the Spt- phenotype.
Figure 4.3. *CUS2* overexpression does not complement the *Spt* phenotype of an *spt4Δ* strain. Four ten-fold serial dilutions were spotted onto SC-ura galactose plates in the presence or absence of lysine and grown at 30°C for 3 days. All strains harbor the *lys2-128δ* locus. (Bottom) Diagram of the *lys2-128δ* locus, which contains a Tyδ element (triangle), and the predicted position of the RNA transcripts from *Spt+* and *Spt−* strains.

In order to determine the effect of *CUS2* on the *Spt−* phenotypes, *CUS2* was overexpressed in SPT+ (*lys2-128δ*) and SPT− (*spt4Δ lys2-128δ*) strains grown on media lacking lysine. General growth of the transformants was assessed on control media lacking uracil for plasmid selection (Figure 4.3). Growth in the presence of overexpressed *CUS2* is similar in both the *SPT4* and *spt4Δ* strains, suggesting that, unlike the Bur complex, *CUS2* does not interact genetically with *SPT4* deletion. The effect of *CUS2* overexpression on
the SPT- phenotype of \textit{spt4}\Delta was assessed on galactose plates lacking lysine. As previously reported, the SPT\textsuperscript{−} strain (\textit{spt4}\Delta \textit{lys2-128}\delta + empty vector) strain allows for growth on these plates. \textit{CUS2} overexpression does not reverse this phenotype or affect the lysine auxotrophy of the SPT\textsuperscript{+} strain. These studies suggest that \textit{CUS2} does not confer an Spt\textsuperscript{−} phenotype or affect the Spt\textsuperscript{−} phenotypes of factors known to interact with the Bur complex.

**U2 snRNA does not exhibit genetic interactions with the yeast homologs of P-TEFb.**

Mutations in TAT-SF1 that prevent its association with snRNPs are also unable to stimulate transcription.(Fong and Zhou, 2001) This effect was attributed to a role for the U2 snRNP, as depletion of the U2 snRNA from extracts also resulted in decreased \textit{in vitro} transcription. The requirement for the U2 snRNA in mediating Tat-SF1’s stimulatory effect on transcription suggests that U2 may have functional overlap with the P-TEFb homologs. The U2 snRNA has highly conserved secondary structural features that allow it to adopt two mutually exclusive structures (Figure 4.4A). One of these structures contains the stem-loop IIa essential for the RNA’s function in splicing.(Zavanelli and Ares, 1991) A sequence of conserved-complementarity to this loop facilitates formation of an alternate structure, called the U2-IIc conformation.(Ares and Igel, 1990; Zavanelli and Ares, 1991; Zavanelli et al., 1994) During splicing, the U2 snRNA toggles between the U2-IIa and U2-IIc structures. These rearrangements of the RNA are essential for
U2’s role in splicing and are mediated, in part, by Cus2. Cus2 has been shown to preferentially bind the IIC form of U2 snRNA. (Perriman and Ares, 2007) U2 snRNA mutations that destabilize stem IIa are synthetically lethal when combined with CUS2 deletion. Taken together, these data present the possibility that the U2 snRNA, specifically in the IIC conformation, associates with Cus2 to stimulate transcription. Notably, in mammals the U1 snRNA has been shown to stimulate transcription initiation by TFIIH, suggesting a precedent for spliceosomal snRNAs in affecting transcriptional events. (Kwek et al., 2002)

To address the possibility that the U2 snRNA affects transcriptional elongation we began by examining genetic interactions between U2 snRNA and transcription factors, particularly the putative P-TEFb homologs. We generated mutant strains in which the genomic U2 (SNR20) and BUR2 or CTK2 genes were deleted. The double mutants harbored a plasmid encoding a wild-type U2 gene on a pRS316 (URA3, CEN/ARS) backbone. This U2 snRNA plasmid (U2Δ2) lacks the nonessential fungal domain, an extended stem-loop found only in some yeast strains. Previous studies have shown this allele to grow and function similar to wild-type U2 snRNA. (Igel and Ares, 1988) Strains bearing mutant U2 alleles on LEU2 plasmids were transformed into these strains. To determine if a recessive phenotype was conferred by these mutants, we plated onto media containing the drug 5-FOA, which selects for cells that have lost the U2Δ2 plasmid, leaving only the mutant gene.
Figure 4.4. U2 snRNA does not exhibit genetic interactions with P-TEFb-associated cyclin homologs. A. Diagram of the 5' portion of yeast U2 snRNA mutually exclusive U2-IIa and U2-IIc conformations. The U2 snRNA mutants used in this study promote either U2-IIa or U2-IIc as indicated. The G53 position is shaded red. The sequences that base pair to form the U2-IIc stem are shaded in blue. B. The indicated strains were grown in YPD and four ten-fold serial dilutions were spotted onto YPD plates and grown at 30°C for the indicated number of days.
As previously reported, in a wild-type background, a G53 to A mutation preferentially forms the U2-IIc conformation and confers a slow growth phenotype (Figure 4.4B) and a splicing defect (data not shown) especially at lowered or elevated temperatures. However, mutations that stabilize stem IIa by deletion of the region of phylogenetically conserved complementarity to stem-loop IIa combined with conversion of the AU stem-pairs to more thermodynamically stable GC pairs confers no growth defect (Figure 4.4B) or splicing defect (data not shown).

We then tested the effect of these mutations in a strain in which the nonessential cyclin subunit of the Bur complex, \textit{BUR2}, was deleted. Deletion of \textit{BUR2} results in slow growth and cold-sensitivity. Expression of the mutant U2-IIa or -IIc snRNA alleles does not alter the general growth pattern of \textit{bur2}Δ, nor is the cold sensitivity suppressed (data not shown). Similarly, the slow growth phenotype conferred by \textit{CTK2} deletion is not altered by the U2 structural mutants (Figure 4.4B). These data suggest that alterations in U2 snRNA conformation do not display functional overlap with the Bur or Ctk complexes.

\textbf{U2 snRNA does not suppress 6-AU sensitivity of the Bur complex}

Although neither \textit{CUS2} deletion nor its overexpression affected the 6-AU sensitivity conferred by \textit{BUR2} deletion, it is possible that U2 snRNA secondary structure may, independent of Cus2, be required for suppression of this Bur-related phenotype. To determine the requirement of particular U2 snRNA conformations in mediating the \textit{bur2}Δ 6-AU sensitivity, the double
mutant strains were grown in the presence and absence of the drug (Figure 4.5). In the WT background, the U2 snRNA alleles exhibit no growth defect in the presence of 6-AU. Cells in which \textit{BUR2} was deleted were unable to grow in the presence of 6-AU, as previously reported. The addition of either U2-IIa or U2-IIc snRNA alleles does not suppress this sensitivity. Deletion of \textit{CTK2} confers sensitivity to 6-AU but is significantly less sensitive to the drug than \textit{bur2}\textsuperscript{Δ} cells. As is the case with \textit{BUR2} deletion, the U2 snRNA alleles do not affect the 6-AU sensitivity of \textit{ctk2}\textsuperscript{Δ} cells. These data indicate that U2 snRNA is not able to compensate for loss of Bur2 or Ctk2 in the presence of the transcription defects caused by 6-AU addition.
Figure 4.5. U2 snRNA does not exhibit genetic interactions with transcription elongation factor mutants or alter their transcription-related phenotypes. A. Strains with indicated genotypes carrying LEU2-marked U2 snRNA plasmids were grown in SC-ura-leu to log phase. Four 10-fold dilutions were spotted on solid SC-ura media lacking or containing 6-AU (100 µg/ml) allowed to grow at 30°C for the indicated number of days. All strains carry URA3-marked plasmids to support growth on plates lacking uracil.
**U2 snRNA does not exhibit genetic interactions with other general transcription factors**

It is possible that U2 snRNA may affect transcription, but that *in vivo* this activity may only be revealed or required under certain conditions. To address whether U2 snRNA participates in Bur-mediated regulation of transcription, a genetic analysis was performed of U2 snRNA folding mutants and factors known to interact genetically with the Bur complex. Here, we looked for synthetic interactions between the elongation factor, *TFIIS (DST1)* which is synthetically lethal in combination with *bur2Δ* (Laribee et al., 2005), *SPT4* whose activity is regulated by the Bur complex (Lindstrom and Hartzog, 2001), and members of the PAF complex (Laribee et al., 2005; Squazzo et al., 2002) responsible for regulating histone modifications that promote active transcription. Components of the PAF complex have genetic interactions with the Bur complex (Mulder et al., 2007). Furthermore, both the PAF complex and U2 snRNP-associated SF3a complexes are detected in immunoprecipitates of histone H3 that is trimethylated at lysine 4, suggesting that the association of these factors is coordinated by this chromatin mark (Sims et al., 2007). Therefore, we looked for genetic interactions between the U2 snRNA alleles and a non-essential component of the PAF complex, *LEO1*.

Double mutants harboring U2 snRNA *LEU2* plasmid alleles and deletions of *DST1*, *LEO1*, or *SPT4* were tested for changes in growth (Figure 4.5). The U2-IIa allele grows at a rate comparable to that of WT U2 snRNA in
each strain tested. Similarly, the U2-IIc slow growth phenotype is not significantly altered in strains in which any of the transcription factors are deleted.

Deletion of *DST1, LEO1*, and *SPT4* renders cells sensitive to 6-AU, indicative of their roles in regulating transcription. (Malagon et al., 2006; Malone et al., 1993; Squazzo et al., 2002) To determine whether U2 snRNA alleles affect the 6-AU sensitivity of these strains we tested the growth of these double mutants on medium containing 6-AU. Alterations in the U2 snRNA secondary structure failed to produce significant changes in the 6-AU sensitivity of these strains. Taken together, these genetic analyses suggest that U2 snRNA conformation mutants do not exhibit functional overlap with transcription factors that interact with the Bur complex.

**The U2 snRNA does not affect the inositol auxotrophy conferred by BUR2 deletion**

Deletion of *BUR2* leads to severe defects in transcription of a number of Pol II transcripts. For example, *BUR2* deletion abrogates expression of *INO1*, the gene encoding inositol-1-phosphate synthase which converts glucose-6-phosphate into inositol. *INO1* is an intronless gene whose expression is highly sensitive to perturbations in the transcription machinery. For example, *bur2* mutant strains are unable to grow in the absence of inositol. (Yao et al., 2000) To determine if the U2 snRNA could affect expression of *INO1*, cells harboring the U2-IIa and U2-IIc alleles were grown in the absence of inositol
These strains do not exhibit inositol auxotrophy which suggests that the U2 snRNA alone has no effect on \textit{INO1} expression. To determine whether changes in U2 snRNA secondary structure are capable of suppressing the inositol auxotrophy associated with \textit{BUR2} deletion, we tested the growth of \textit{bur2Δ} cells harboring wild type, hyperstabilized or destabilized U2 snRNA stem-loop IIa mutants. As previously reported, cells in which \textit{BUR2} was deleted were unable to grow on plates lacking inositol. The addition of U2-IIa or U2-IIc alleles did not suppress the \textit{INO1} auxotrophy, suggesting that U2 mutants do not bypass the need for Bur-dependent expression of the \textit{INO1} gene.

\textbf{Figure 4.6. U2 snRNA does not suppress the inositol auxotrophy of \textit{bur2Δ}.} The indicated strains were spotted onto solid media containing (+INO) or lacking (-INO) 100 uM inositol and grown for 2-3 days (WT) or 4 days (\textit{bur2Δ}).

Because the inositol auxotrophy of transcription factor mutants is associated with an inability to properly transcribe the \textit{INO1} gene, we wanted to determine whether the U2 snRNA alleles were affecting \textit{INO1} transcript levels.
We performed a Northern blot analysis to examine *INO1* steady state mRNA levels. *INO1* transcription is stimulated during inositol starvation, so cells were grown in media containing inositol were spun down, washed, and switched to medium lacking inositol. Cells were then harvested at 0 and 12 hours followed by RNA extraction and Northern blot analysis (Supplemental Figure 4.1A). All forms of U2 were able to support elevation of *INO1* transcript levels in wild-type cells. After 12 hours in the absence of inositol, wild-type cells exhibited high levels of the *INO1* transcript in the presence of all of the U2 mutants. However, cells in which *BUR2* was deleted were unable to induce *INO1* transcription even when the IIa or Iic form of U2 was present. These data demonstrate that these U2 snRNA conformations do not affect levels of *INO1* mRNA transcription.

**U2 snRNA does not exhibit overlapping phenotypes with the Bur complex**

Deletion of *BUR2* has pleiotropic effects, and the resulting phenotypes associated with this deletion are generally severe. These effects may mask any subtle effects on transcription conferred by U2 snRNA. If U2 snRNA is involved in regulating transcription, then mutations which hyperstabilize the mutually exclusive U2-IIa or U2-Iic forms of the RNA may exhibit transcription-related phenotypes. We hypothesized that, similar to *BUR2* deletion, mutations in the U2 snRNA may also result in some of these classic
transcription phenotypes. Here, we tested both the IIa and IIc forms of U2 for Bur- and Spt- phenotypes.

*BUR1* and *BUR2* were initially identified in a screen for mutants that exhibited increased transcription of the *SUC2* gene in the absence of its upstream activating sequence (UAS). (Winston et al., 1984) Deletion of the *SUC2* upstream activating sequence (UAS) (*suc2Δuas*) abolishes transcription of this gene, causing an inability to grow on media containing sucrose as the carbon source. Mutations which Bypass the UAS Requirement exhibit the Bur phenotype and increase transcription from *suc2Δuas*. (Prelich and Winston, 1993) To determine whether U2 snRNA alleles also exhibit the Bur phenotype, strains harboring the *suc2Δuas* mutations were crossed with a *SNR20::HIS3* strain harboring the U2 alleles on plasmids. The resulting strains were tested for their ability to grow on sucrose plates. Deletion of *SPT4* was previously been shown to be Bur- and serves as a positive control for growth (Figure 4.7A). Both U2-IIa and U2-IIc snRNA alleles fail to grow on sucrose plates suggesting that these alleles are unable to affect transcription in the absence of the UAS.
Figure 4.7. U2 snRNA does not exhibit transcription-related phenotypes. A. U2 snRNA mutants do not exhibit the BUR- phenotype. *suc2Δuas* strains carrying LEU U2 snRNA mutant plasmids were struck on minimal media with sucrose as the carbon source (+1 µg/ml antimysin A). *spt4Δ* is BUR- allowing it to grow on sucrose plates. B. U2 snRNA mutants do not exhibit the Spt- phenotype. Four ten-fold serial dilutions were spotted onto YPD or media lacking lysine and grown at 30°C for 3 days. All strains harbor the *lys2-128δ* locus.
To determine whether U2 snRNA alleles confer a SPT- phenotype, we crossed the U2::HIS strain with a strain containing the lys2-128δ mutation similar to the experiments shown in Figure 4.7B. The resulting double mutant strain was tested for restored growth on media lacking lysine. The SPT-spt4Δ/lys2-128δ strain serves as a positive control for suppression. (Swanson and Winston, 1992) Both U2-IIa and U2-IIc alleles fail to suppress the Ty element insertion and do not support growth on media lacking lysine.

**Neither Cus2 nor U2 snRNA exhibit physical interactions with the Bur complex**

Fong and Zhou demonstrated that Tat-SF1 and accompanying U2 snRNP associate with P-TEFb via interactions with the cyclin subunit T1. (Fong and Zhou, 2001) If a parallel interaction exists in yeast, then we would expect to detect physical interactions between the cyclin Bur2 and both Cus2 and U2 snRNA. Previously it was demonstrated that both Bur1 and Bur2 co-immunoprecipitate indicating that the Bur2 pulldown used in these studies represents pulldown of both factors.

To determine whether Cus2 physically interacts with Bur2 we performed a co-immunoprecipitation experiment of a Bur2TAP tagged strain harboring a 6XHIS tagged CUS2 gene under the control of the GAL1 promoter. Anti-TAP immunoprecipitations were performed on whole cell
lysates made the Bur2-TAP tagged strains in which *CUS2* was overexpressed by galactose induction. *CUS2* expression can be seen in the input sample (Figure 4.8A). However, the Cus2 protein is not detected in the Bur2TAP immunoprecipitate. These data indicate that any physical interactions between Cus2 and the Bur complex are undetectable under these conditions.

To determine whether the U2 snRNA physically associates in a complex containing Bur2 (either directly or indirectly) we performed a modified RNA immunoprecipitation assay. Here, TAP-tagged Bur2 was precipitated using IgG sepharose beads in the presence of a plasmid-borne wild-type U2 snRNA. The beads were then washed with increasing concentrations of salt to reduce non-specific interactions and probed for the presence of U2 via primer extension using a U2-specific oligomer. We detect U2 in both the mock and Bur2-TAP precipitate indicating that the RNA associates with the IgG beads in a non-specific manner (Figure 4.8B). Although RNA is detectable in each sample tested, we failed to detect an enhanced interaction that is specific to the Bur pulldown. The inability of the Bur2 pulldown to enrich for U2 snRNA suggests that any interaction between the Bur complex and U2 snRNA is not detectable under these conditions.
Figure 4.8. The Bur complex does not exhibit physical interactions with Cus2 or U2 snRNA. A. Cus2 does not co-immunoprecipitate the Bur complex. Anti-HIS and anti-TAP immunoprecipitations were performed on whole cell lysates made from Bur2-TAP tagged strains carrying a vector or a HIS tagged GAL-CUS2 plasmid grown in galactose media. Immunoprecipitations were separated by SDS-PAGE, transferred to nitrocellulose, and blotted with anti-TAP or anti-HIS as indicated. WB, western blot. IP, immunoprecipitate. B. Bur2 immunoprecipitation does not enrich for U2 snRNA. Anti-TAP immunoprecipitations were performed on whole cell lysates made from Bur2-TAP or untagged strains. Precipitates were washed with increasing concentrations of salt to reduce non-specific interactions (50-250 mM NaCl₂). Total RNA was isolated from the protein precipitates and probed for the presence of U2 snRNA by primer extension from a DNA oligo complementary to U2 snRNA.
Discussion

Previous studies have suggested that transcription and splicing are functionally coupled. Studies in mammalian cells have directly implicated the transcriptional elongation factors P-TEFb and Tat-SF1 in this coupling. Specifically, work by Fong & Zhou implicated a role for Tat-SF1 in coordinating transcription with splicing via its interactions with factors involved in both processes. Subsequent studies demonstrated that the yeast homolog of Tat-SF1, CUS2, could affect synthesis of viral RNAs in vivo. (Naito et al., 2007) These studies led us to hypothesize that Cus2, like Tat-SF1, is capable of regulating transcription in addition to its known role in splicing.

The experiments described here tested whether the yeast homologs of P-TEFb and components of the U2 snRNP display properties of a functionally coupled complex. We find that the U2 snRNP components do not affect transcription in a detectable way. Furthermore, we were unable to detect physical interactions between the U2 snRNP component and the Bur complex. Taken together, we find a lack of evidence for a functional complex containing the Bur complex and the U2 snRNP. However, advances in both whole genome analyses and single gene/cell technologies have demonstrated that factors thought to have general roles in transcription actually have specific effects on the expression of particular genes under specific conditions. We cannot rule out the possibility that the U2 snRNP may affect transcription of specific genes and under specific conditions. Future studies will be needed to specifically address this possibility.
How do P-TEFb and Tat-SF1 couple transcription and splicing *in vivo*?

The interpretation of our results is confounded somewhat by the lack of a clear understanding about the functional relationship of the mammalian factors. Several studies have demonstrated that P-TEFb, Tat-SF1, and splicing factors can be detected within the same complexes in extracts, although the functional consequence of these interactions remains to be elucidated. (Fong and Zhou, 2000; Li and Green, 1998; Zhou et al., 2000; Zhou et al., 1998; Zhou and Sharp, 1996) Tat-SF1 has been implicated in general transcriptional elongation (Chen et al., 1999; Chen and Zhou, 1999; Li and Green, 1998; Parada and Roeder, 1999) and in Tat-transactivation of TAR-dependent HIV-1 transcription (Zhou and Sharp, 1996). However, no clear role for Tat-SF1 in regulating splicing has been established yet. Conversely, P-TEFb has been shown to indirectly affect splicing of a reporter minigene via its phosphorylation of the polymerase CTD. (Barboric et al., 2009) It remains to be determined whether P-TEFb and Tat-SF1 work together to coordinate transcription and splicing *in vivo*, independent of Tat, on non-viral templates.

Is the ability of Tat-SF1 to stimulate viral transcripts evolutionarily conserved?

It is possible that through evolution P-TEFb and Tat-SF1 have acquired additional functions that allow them to associate in a novel way not seen in yeast. Although Tat-SF1 and CUS2 are closely related and contain two highly similar RRM s, the extensive C-terminal acidic domain of Tat-SF1 is greatly
reduced in Cus2. It is possible that the lengthening of this acidic domain through evolution has allowed Tat-SF1 to acquire a new function absent in its yeast counterpart. The acidic domain of Tat-SF1 is required to facilitate the protein’s association with P-TEFb and for efficient transactivation of a TAR-containing reporter. In yeast, this portion of the Cus2 protein is important for its function in U2 snRNA folding as deletion of this region results in reduced function toward misfolded U2 snRNA. (Yan et al., 1998) The extended acidic domain of Tat-SF1 is therefore an attractive candidate for the region of the protein that coordinates splicing and transcription.

Contrary to this hypothesis, a recent report identified Cus2 as an important factor for influenza virus RNA synthesis in infected yeast cells. (Naito et al., 2007) Here, yeast cells supporting the replication and transcription of the influenza genome demonstrated lowered viral RNA expression in the absence of CUS2. This effect was recapitulated with siRNA-mediated knockdown of Tat-SF1 in influenza infected HeLa cells. The effect was shown to be due to a direct role for Tat-SF1 in facilitating assembly of viral RNPs that are essential for downstream viral RNA synthesis. These data suggest that viral machineries are able to utilize Cus2 and Tat-SF1 in a similar manner through recognition of homologous portions of these proteins rather than the extended C-terminal acidic domain. It is clear that at least in some cases, viruses utilize host factors in ways unique to viral infection. It will be interesting to determine whether yeast cells transfected with HIV-1
components will reveal a role for CUS2 on transcriptional elongation in a manner similar to that of Tat-SF1.

The fact that a relationship exists between transcription and subsequent RNA processing is well documented. Examples of the tight coordination between splicing and transcription exist in yeast and metazoans, a fact that suggests that this coordination evolved a long time ago. Continued effort toward understanding the mechanisms of this coordination is crucial for a more complete understanding of gene expression.

Materials and Methods

Yeast strains and growth

The strains used in this study are listed in table 2.1 and are in the BY4743 strain background with the exception of the SPT- BUR- strains, provided by Karen Arndt. Strains containing multiple disruptions with the same auxotrophic marker were obtained from genetic crosses and confirmed by PCR. All strains were propagated according to standard procedures in the appropriate selective media. Plasmid shuffling was performed on selective 5-fluoroorotic acid (5-FOA) plates. Sucrose plates prepared as described elsewhere (Costa and Arndt, 2000) and supplemented with 1 µg/ml antimycin A. Inositol media were prepared from a yeast nitrogen base containing ammonium sulfate but lacking inositol (Q BIOgene) and supplemented with the appropriate nutrients; inositol was added to 100 µM where indicated. Standard methods for transformations and media preparation were used as
described in Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual. The plasmids used in this study are listed in table 2.2.

**Viability assay/dilution series**

For growth analysis, strains were grown overnight in the appropriate selective media at 30°C. Cells were diluted and incubated at 30°C until all strains reached the same O.D.600 (between 0.3-0.5). A ten-fold serial dilution of each strain was spotted onto the proper selective plates and incubated for the indicated number of days.

**6-azauracil plate assay**

The yeast strains used in this assay were transformed with the URA3<sup>+</sup> CEN plasmid pRS316, and selected on synthetic complete media lacking uracil (SC-URA) plate. The transformed colonies were grown in SC-URA liquid media up to O.D.<sub>600</sub> of approximately 0.5, serially diluted 10-fold onto SC-URA plates with or without 100µg/ml 6-azauracil. Plates were incubated at 30°C for the indicated number of days.

**Co-immunoprecipitation**

Co-immunoprecipitation (co-IP) experiments were carried out according to the general procedure described in (Cardenas et al., 1994) with the following modifications. Yeast strains were grown in 50 mL of SC-URA to an O.D.<sub>600</sub> of approximately 0.8. The cells were harvested by centrifugation, washed, and lysed by vortexing with glass beads in cold lysis buffer (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA pH 8.0, 1% Triton-X, 0.1% Deoxycholate; 1mM PMSF; 1µg/mL pepstatin; 2mM benzamidine; 2.5µg/mL
leupeptin; 5µg/mL aprotinin). Whole cell extracts were cleared by centrifugation at top speed for 15 minutes at 4ºC. Cleared whole cell extracts were then incubated for 4 hours at 4ºC with IgG Sepharose 6 Fast Flow beads (GE Healthcare) or NiNTA beads (Qiagen) as appropriate. Beads were washed three times with cold lysis buffer before eluting bound protein by boiling the beads for 10 minutes in 20µL of SDS-PAGE sample buffer. Samples were fractionated by SDS-PAGE electrophoresis and transferred to a nitrocellulose membrane for immunoblotting with 1:3000 dilution of anti-TAP (Upstate 12-342) and 1:5000 dilution of anti-HIS (Santa Cruz Biotechnology 804), followed by chemiluminescent detection (Pierce).

**RNA co-immunoprecipitation**

RNA co-immunoprecipitation experiments were carried as above with the following modifications. Yeast strains were grown in 50 mL of YPD to an O.D.₆₀₀ of approximately 0.8. The cells were harvested by centrifugation, washed, and lysed by vortexing with glass beads in cold lysis buffer (50 mM HEPES-KOH pH 7.5, 50 mM NaCl, 1 mM EDTA pH 8.0, 1% Triton-X, 0.1% Deoxycholate; 1mM PMSF; 1µg/mL pepstatin; 2mM benzamidine; 2.5µg/mL leupeptin; 5µg/mL aprotinin, 40U RNasin (Promega)). Whole cell extracts were cleared by centrifugation at top speed for 15 minutes at 4ºC. Whole cell extracts were then pre-cleared for 1 hour with CL4B Sepharose beads (Sigma). The precleared samples were then used for immunoprecipitation with IgG Sepharose 6 Fast Flow beads for 4 hours with rotation at 4ºC. Beads were washed three times with cold lysis buffer before eluting bound protein
with TEV protease (Invitrogen) at 18°C for 1 hour. RNA in eluted protein sample was extracted using acid phenol and ethanol precipitated. The pool of precipitated RNAs was probed for the presence of U2 by primer extension using the U2-430R oligo: 5' CAAAAATGTGTATTG.

**In vivo splicing analysis**

Cells were grown in selective galactose media overnight, washed, and used to inoculate 10mls of selective glucose media. Strains were grown for 12 hours at 30°C and then (if indicated) shifted to 18°C for 4 hours. Total RNA was prepared using phaselock tubes (Eppendorf) and used as a template for primer extension with a labeled U3-specific oligonucleotide.

**RNA isolation**

Cells grown at 30° were harvested by centrifugation, washed with water, and frozen. RNA isolation, Northern analysis, and Northern blot probe preparation have been described. (Hossain et al., 2009) The Northern probe was generated by PCR with the following primer pair to amplify INO1: 5'-INO1-F 5'-GGTCTAAGGAGATTTCAAAAAGTTC.  INO1-R 5' TTACAACATCTCCTCTCGAATCTTAG. The gel-purified PCR product was labeled with [a-32P] dCTP using the Rediprime random labeling system (GE Healthcare).
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>MATα ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 MATα ura3Δ0 leu2Δ0 his3Δ1 met15Δ0</td>
<td>This study</td>
</tr>
<tr>
<td>bur2Δ</td>
<td>bur2::kanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>cus2Δ</td>
<td>cus2::kanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>ctk2Δ</td>
<td>ctk2::kanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>bur2Δ cus2Δ</td>
<td>bur2::kanMX4 cus2::kanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>ctk2Δ cus2Δ</td>
<td>ctk2::kanMX4 cus2::kanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>rpb1Δ+pRP112</td>
<td>rpb1::URA3 [pRP112]</td>
<td>This study</td>
</tr>
<tr>
<td>cus2Δ</td>
<td>MATα ura3Δ0 leu2Δ0 met15Δ0 his3Δ1</td>
<td>This study</td>
</tr>
<tr>
<td>rpb1Δ+pRP112</td>
<td>cus2::kanMX4 rpbl::URA3 [pRP112]</td>
<td>This study</td>
</tr>
<tr>
<td>dst1Δ</td>
<td>dst1::kanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>KY616</td>
<td>MATα his4-912Δ lys2-128Δ leu2Δ1 ura3-52 SUC2ΔUAS(-1900/-390)</td>
<td>This study (2000)</td>
</tr>
<tr>
<td>KY616 spt4Δ</td>
<td>MATα his4-912Δ lys2-128Δ leu2Δ1 ura3-52 SUC2ΔUAS(-1900/-390) spt4::kanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>KY616</td>
<td>MATα his4-912Δ lys2-128Δ leu2Δ1 ura3-52 SUC2ΔUAS(-1900/-390) U2::HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>U2Δ+pAB146</td>
<td>U2::HIS3 [pAB146]</td>
<td>This study</td>
</tr>
<tr>
<td>bur2Δ</td>
<td>MATα ura3Δ0 leu2Δ0 his3Δ1 met15Δ0</td>
<td>This study</td>
</tr>
<tr>
<td>U2Δ+pAB146</td>
<td>bur2::kanMX4 U2::HIS3 [pAB146]</td>
<td>This study</td>
</tr>
<tr>
<td>ctk2Δ</td>
<td>MATα ura3Δ0 leu2Δ0 his3Δ1 met15Δ0</td>
<td>This study</td>
</tr>
<tr>
<td>U2Δ+pAB146</td>
<td>ctk2::kanMX4 U2::HIS3 [pAB146]</td>
<td>This study</td>
</tr>
<tr>
<td>leo1Δ</td>
<td>MATα ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0</td>
<td>This study</td>
</tr>
<tr>
<td>U2Δ+pAB146</td>
<td>leo1::kanMX4 U2::HIS3 [AB146]</td>
<td>This study</td>
</tr>
<tr>
<td>spt4Δ</td>
<td>MATα ura3Δ0 leu2Δ0 his3Δ1 met15Δ0</td>
<td>This study</td>
</tr>
<tr>
<td>U2Δ+pAB146</td>
<td>spt4::kanMX4 U2::HIS3 [pAB146]</td>
<td>This study</td>
</tr>
<tr>
<td>dst1Δ</td>
<td>MATα ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0</td>
<td>This study</td>
</tr>
<tr>
<td>U2Δ+pAB146</td>
<td>dst1::kanMX4 U2::HIS3 [pAB146]</td>
<td>This study</td>
</tr>
<tr>
<td>Bur2TAP</td>
<td>MATα BUR2-TAP:HIS3 ura3Δ0 leu2Δ0 his3Δ1 met15Δ0</td>
<td>OpenBiosystems</td>
</tr>
</tbody>
</table>
### Table 4.2 List of plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid Number</th>
<th>Plasmid Description</th>
<th>Plasmid backbone</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTAGCus2</td>
<td>Cus2-HA-6XHIS</td>
<td>pTAG</td>
<td>(Yan et al., 1998)</td>
</tr>
<tr>
<td>PTAG</td>
<td>HA-6XHIS</td>
<td>pYES 1.2</td>
<td>(Yan et al., 1998)</td>
</tr>
<tr>
<td>pAB146</td>
<td>WT U2</td>
<td>pRS316</td>
<td>A. B.</td>
</tr>
<tr>
<td>pAB131</td>
<td>WT U2</td>
<td>PRS315</td>
<td>A. B.</td>
</tr>
<tr>
<td>pAB138</td>
<td>U2-IlA ΔCC/GC stem</td>
<td>PRS315</td>
<td>A. B.</td>
</tr>
<tr>
<td>pAB143</td>
<td>U2-IlC G53A</td>
<td>pRS315</td>
<td>A. B.</td>
</tr>
<tr>
<td>pRP112</td>
<td>WT RPB1, Z26, N247</td>
<td>YCp50</td>
<td>R. Young</td>
</tr>
<tr>
<td>pRP1-101</td>
<td>rpb1Δ101, C1, N259,</td>
<td>pRP114</td>
<td>R. Young</td>
</tr>
<tr>
<td>pRP1-103</td>
<td>rpb1Δ103, C3, N261</td>
<td>pRP114</td>
<td>R. Young</td>
</tr>
<tr>
<td>pRP1-110</td>
<td>rpb1Δ110, V5, N327</td>
<td>pRP114</td>
<td>R. Young</td>
</tr>
</tbody>
</table>

### Acknowledgements

We are grateful to Manuel Ares, Karen Arndt, Imre Barta, Richard Young and their colleagues for providing plasmids and yeast strains. We thank Manuel Ares and Rhonda Perriman for helpful discussions and ideas. Christina Chung contributed Figure 4.1D. This work was supported by an NSF CAREER award to T.L.J. (MCB-0448010) and an NSF predoctoral fellowship to S.L.M.
Supplemental Figure 4.1. A. U2 snRNA does not affect INO1 transcription. The indicated strains were grown in liquid media in the presence 100 µg/ml 6-AU at 30°C for the indicated times, in hours, prior to RNA isolation. Each lane contained 10 µg of total RNA. Nitrocellulose blots were probed with a Redi-prime PCR product corresponding to an INO1 sequence. B. U2 snRNA does not suppress the bur2Δ defect in H3 K4 trimethylation. Wild-type or bur2Δ whole cell extracts were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti H3 K4 trimethylation antibody.
Chapter 5. Discussion

The work detailed in this dissertation has addressed two of the major outstanding questions in the splicing field: How are splicing dynamics regulated? and How is splicing regulated in relation with other cellular processes? Using U2 snRNP factors as a model, we have investigated the mechanisms that regulate splicing dynamics and the roles these factors play in coupling splicing and transcription. In this section I highlight several key questions that follow from this work. These questions include: How can Systems Biology contribute to our understanding of splicing regulation? What is the importance of co-transcriptional splicing? Is Cus2 a dual function protein?

How can Systems Biology contribute to our understanding of splicing regulation?

Chapter 2 highlights the contribution of global analyses of post-translational modifications to our wealth of understanding about the role of these modifications in regulating splicing. There is an additional need for integration of these data sets to increase the depth of understanding. There is a striking disconnect between the proteomics analyses and the functional analyses. For example, several analyses have been done to illustrate the wealth of posttranslational modifications triggered by cell cycle events.(Amerik
and Hochstrasser, 2004; Choudhary et al., 2009; Daub et al., 2008; Dephoure et al., 2008; Graub et al., 2008; Gruhler et al., 2005; Li et al., 2005; Shin et al., 2004; Shin and Manley, 2002, 2004; Smolka et al., 2007; Tripathi and Parnaik, 2008) However, the functional consequence of these modifications remains largely unanswered. A parallel analysis of transcription and splicing under the same conditions used in identifying the PTMs would provide important information about how these modifications translate into changes in gene expression.

Given the automated nature of many of these global analyses, the use of gene and protein chips, and the significant advances in both sequencing and proteomics technology, it seems clear that such integrated studies are currently possible. In the proposed scenario, protein and RNA samples collected from a single experiment can be put through a series of relatively automated and unbiased analyses. For example, several proteins, including Cus2, are found to be phosphorylated after arresting the cell-cycle by alpha factor treatment. (Li et al., 2007) To identify the functional consequence of these phosphorylation events during alpha factor arrest, these same conditions could be used to prepare samples for expression and splicing-sensitive microarrays. In the case of Cus2 and other splicing proteins, a parallel analysis of the phosphorylation events with the changes in pre-mRNA splicing would allow for identification of the protein factors mediating these changes and their potential substrates. These types of analyses would allow for rapid, unbiased identification of groups of factors mediating specific cellular
responses and could be followed with analyses of the mechanisms involved. Furthermore, these analyses would allow for clustering of factors that appear to be regulated in a common way, perhaps allowing for the identification of unique and unexpected coordinated activities.

Systems Biology is an inter-disciplinary field of study that aims to integrate data to generate models of biological systems. Systems Biology uses powerful new computational and mathematical approaches to create a comprehensive molecular description of complex biological processes. (Deisboeck et al., 2009; Geschwind and Konopka, 2009; Grigoriev, 2004; Hecker et al., 2009; Ma’ayan, 2008; Ng et al., 2006; Peng et al., 2009; Srinivasan et al., 2007; Thomas and Ganji, 2006) By examining multiple components simultaneously, this type of quantitative analysis can reveal unexpected trends and properties of biological systems. For example, data from proteomics, genomics, and metabolomics can be integrated to explore questions of cause and effect that analysis of the single data sets alone cannot answer. Studies of this type will also further our understanding of how cells monitor and adapt to their changing environment. Computational approaches to the integration of global analyses will no doubt further our understanding of cellular responses during a variety of conditions including cell divisions, apoptosis, infection, and stress. These computational methods can be used on the numerous data sets already available, but it will be important to design future experiments involving parallel analyses to enable more meaningful integration. Only Systems Biology approaches will be adequate for
understanding how specific splicing events are regulated under different conditions.

**What is the importance of co-transcriptional splicing?**

The results reported by Fong & Zhou (2001) of Tat-SF1’s role in stimulating HIV-1 transcription have been commonly extrapolated to mean that P-TEFb and Tat-SF1 functionally couple transcription and splicing in mammals. This assumption has been made despite the fact that the analyses were done in extracts using viral templates and in the presence of a viral protein, Tat. A clear role for Tat-SF1 in coupling transcription and splicing in vivo remains to be established for both endogenous and viral transcripts.

The work presented in this study presented several testable predictions including:

- The functional coupling of P-TEFb, Tat-SF1, and the U2 snRNP should be recapitulated in vivo.
- Because the U2 snRNP stimulated transcription of both intronless and intron-containing genes, these factors should generally influence transcription of endogenous genes.
- The U2 snRNP should associate with the transcription machinery in vivo at intronless genes.

Our work described in Chapter 3 is the first to address some of these questions through genetic and biochemical analyses in yeast. We found no evidence for the in vivo coupling of the yeast homologs of P-TEFb, Tat-SF1,
and the U2 snRNP. Our findings have raised questions about potential mechanisms guiding the stimulatory effects of Tat-SF1 and the U2 snRNP on transcription. Specifically it is important to consider how the use of viral components and the in vitro nature of these experiments influence the results.

Viruses have been shown to hijack host factors to facilitate replication of the viral genome. (Amara and Littman, 2003; Cavrois et al., 2008; Dickerson et al., ; Lilley et al., 2007; May and Machesky, 2001; McLean et al., 2008; Mulhern et al., 2009; Plattner and Soldati-Favre, 2008; Santoro et al., 2003) How viruses utilize host factors is a fascinating question. It is clear that at least in some cases, the viruses utilize host factors in ways unique to viral infection. Fong & Zhou nicely demonstrate a clear effect on HIV transcription which is dependent on precipitates containing P-TEFb, Tat-SF1, and the U2 snRNA, although the specific contributions of these factors remains unclear. Furthermore, it remains to be seen whether similar stimulatory effects occur for endogenous eukaryotic genes in the absence of viral proteins such as Tat.

There are several hints in the literature that both P-TEFb and Tat-SF1 have unique interactions with viral components. For instance, Tat-SF1 was shown to promote viral genome synthesis. (Naito et al., 2007) This effect, however, was shown to be due to increased vRNP assembly and therefore not likely to be indicative of a direct effect on transcription elongation or splicing. It was shown that a similar chaperone activity exists for the splicing factor UAP56 to the detriment of splicing suggesting that UAP56 is hijacked by the virus to perform a novel, viral-specific role. (Momose et al., 2001) These
studies underscore the ability of viral systems to utilize host factors in unique ways not seen in uninfected hosts.

It is also important to consider questions of whether the effects of Tat-SF1-containing complexes on transcription and splicing represent the coupling of these processes by these factors, as proposed in the authors' model, or whether these effects represent independent effects on concurrent processes. Here, we define coupled processes as those whose activities influence one another, while concurrent processes occur at the same time and place, but do not affect the other’s activities. Fong and Zhou report that transcripts containing splice sites are transcribed more efficiently than transcripts with mutated splice sites. They conclude that the enhanced transcript levels are due to coupling of transcription and splicing, perhaps by recruitment of snRNPs to the splice signals. Other studies have demonstrated that the relative stability of splice site-containing transcripts can vary and therefore confound these types of analyses. (Hicks et al., 2006; Lazarev and Manley, 2007) Determining the relative stabilities of these reporter transcripts, either by examining their transcription in an in vitro reaction using purified components, or using a T7 promoter, will be important for gaining a more complete understanding of these results. In addition, it is curious that mutation of the branchpoint—where the U2 snRNP binds—is the only mutant transcript that does not display reduced transcript levels. These data are not consistent with the model proposed in which U2 snRNP recruitment to the nascent transcript results in enhanced transcription. Chromatin immunoprecipitation
(ChIP) experiments of U2 snRNP recruitment would be a nice in vivo way to
further explore their model. Lastly, immunoprecipitates of Tat-SF1 are
capable of reconstituting splicing in a micrococcal nuclease-treated extract
and it was concluded that these precipitates are therefore capable of coupling
transcription and splicing. However, it is important to note that coupled
processes are distinct from concurrent ones. Further analyses will be needed
to determine whether the Tat-SF1-containing complexes specifically alter
splicing in a transcription-dependent manner, or whether these complexes
contain multiple activities that promote each process independently.

Data from our lab have demonstrated a role for the Bur complex in
regulating splicing, independent of transcription. Deletion of BUR2 confers an
in vivo splicing defect, which is suggestive of a role for this kinase complex in
regulating splicing. Since we have demonstrated that the Bur complex is
capable of phosphorylating Cus2 in vitro, we hypothesized that this splicing
defect may represent a role for the Bur complex in mediating U2 snRNA
folding via regulation of Cus2. For example, if the Bur complex promotes
formation of the U2-IIa form of U2, then we would predict that a
hyperstabilized U2-IIa mutant may bypass the need for BUR2 and suppress
the bur2Δ splicing defect. To address this possibility, we performed a bur2Δ in
vivo splicing assay in the presence of different forms of U2 snRNA. However,
we found that neither the U2-IIa nor U2-IIc forms were able to suppress the
splicing defect (Figure 5-1A). Because the U2 snRNA is a RNAP II transcript,
we performed a control experiment using RNA prepared from bur2Δ cells
strains to confirm that the splicing defect conferred by BUR2 deletion was not due to lowered U2 snRNA levels in the absence of BUR2 (Figure 5-1B).

These data suggest that the role of the Bur complex in splicing is not likely to be in mediating U2 snRNA folding. Ongoing experiments in our lab, independent of this thesis work, are working to further characterize this novel role of the Bur complex.

![Figure 5.1. bur2Δ has an in vivo splicing defect that cannot be repaired by alternate forms of U2 snRNA.](image)

RNA prepared from WT or bur2Δ cells carrying LEU2-marked U2 snRNA plasmids were used as template for primer extension with a labeled oligonucleotide complementary to U3 snRNA. Samples were normalized to contain the same amount of U6 snRNA.
Co-transcriptional versus post-transcriptional splicing

Many important strides have been made in understanding the phenomenon of co-transcriptional splicing. Additional questions need to be addressed to further our understanding the functional significance of co-transcriptional splicing. The clearest consequence of co-transcriptional splicing has been demonstrated for alternative splicing, although the mechanisms underlying this effect remain in debate. (Goldstrohm et al., 2001; Laurencikiene et al., 2006; Lin et al., 2008; Lynch, 2006; Pandya-Jones and Black, 2009)

Although co-transcriptional splicing can occur, it is important to note that post-transcriptional splicing also occurs and may have significant functional consequences. Global chromatin immunoprecipitation studies done in yeast have suggested that the majority of splicing occurs post-transcriptionally. (Tardiff et al., 2006) Just as co-transcriptional splicing is thought to enhance the efficiency of mRNA synthesis, mechanisms that dictate that a transcript is post-transcriptional spliced are likely to impart some advantages that are not yet fully understood. For example, one could imagine that pre-synthesized transcripts can be stored by the cell until the proper stimulus triggers their splicing. The ability to rapidly generate mature messages ready for translation would impart a selective advantage to generating each transcript de novo. Indeed, unspliced transcripts accumulate in nuclear speckles and others have been shown to be rapidly spliced upon certain cellular signals. (Mintz and Spector, 2000; Sacco-Bubulya and Spector,
It is likely that both co- and post-transcriptional splicing contribute to the ability of cells to rapidly respond to environmental stimuli. It will be important to establish why both mechanisms exist and what the relationship is between them.

To better understand the adaptive consequences of co- and post-transcriptional splicing, one could design a series of experiments to uncouple splicing and transcription in vivo. Treatment of mammalian cells with 5,6-dichloro-1-β-D-ribobenzimidazole (DRB) blocks transcription in a reversible manner. RNA collected during a timecourse from cells treated with DRB can then be analyzed on a splicing-sensitive microarray. Here, the particular transcripts whose splicing is altered by DRB treatment can be identified. Unspliced transcripts that accumulate after DRB treatment are likely to be more dependent on co-transcriptional splicing than those whose splicing appears unaffected. One could then do a screen to identify splicing factors whose mutation abrogates the effect of DRB treatment on splicing and are therefore likely candidates for mediating the co-transcriptional splicing. Because DRB is reversible, the adaptive contribution of co-transcriptional splicing can be assessed in cells after DRB treatment. Here, one could subject DRB-treated cells to a number of known stressors, such as heat shock, to determine the effect of DRB treatment on subsequent viability. It would be interesting to determine whether one could correlate the accumulation of unspliced transcripts with a reduced adaptive response to these stress conditions. Although yeast cells appear to be resistant to DRB,
another chemical compound, called H8, appears to have similar inhibitory effects on yeast transcription. (Cho et al., 1997) It would be interesting to perform parallel analyses to those proposed above in yeast cells after H8 treatment.

**What factors regulate Cus2 activity?**

In Chapter 4 we demonstrate that phosphorylation regulates Cus2 function. Preliminary work has shown that Cus2 can be phosphorylated by several kinases *in vitro*. Furthermore, Cus2 in which all of the predicted phosphorylation sites within the acidic domain (thought to be the main target for phosphorylation) have been mutated is still a substrate for phosphorylation (Figure 5-2). It will be important to determine what enzymes are responsible for this Cus2 modification and under what conditions they occur. To begin to identify the enzymes that modify Cus2, one could do a directed genetic screen using mutants of the yeast kinases capable of tyrosine phosphorylation (there are 6). Candidate enzymes can then be tested for their *in vitro* activity toward Cus2. *In vivo* analyses will be important, but since we were unable to find conditions that enrich for Cus2 phosphorylation, the *in vivo* analyses will require some optimization.

It will also be important to take an unbiased approach to understanding the function of Cus2 phosphorylation. Microarray analysis of both splicing and mRNA expression with the phosphorylation mutants will provide information
about how these mutants affect gene expression. It will be interesting to
determine whether the Y48 mutants exhibit genomic changes similar to CUS2
deletion or whether having an intact protein present makes a difference. The
S50A mutant may be particularly interesting given its enhanced binding and
conditional phenotypes.

The Y48 and S50 Cus2 phosphorylations were identified under
conditions in which the major cell-cycle regulator CDK1 was inhibited.(Holt et
al., 2009) It will also be informative to determine how splicing is affected under
these conditions. These data will provide information about how splicing
events are affected by changes in the cell cycle and can be compared with the
Cus2 phospho-mutant array data to determine which transcripts are likely to
be specifically regulated by Cus2.

Lastly, I have identified genetic interactions between Cus2 and the later
acting splicing factors Prp19 and Prp2 (Figure 5-3). These data suggest that
Cus2 plays an additional role in splicing. One could do a genetic analysis
between these factors and the suite of Cus2 phospho-mutants available to
determine whether Cus2 phosphorylation regulates this later activity.
Figure 5.2. **CUS2 deletion suppresses phenotypes of Prp19 and Prp2 alleles.** Strains were grown in YPD to the same OD$_{600}$ and four ten-fold serial dilutions were spotted and grown at the indicated temperature for 3-6 days. $prp2^{-1}$ is temperature sensitive and exhibits slow growth.

**Is Cus2 a dual function protein?**

Through our investigations of the Cus2 function it became clear that Cus2 has interesting connections to processes other than splicing. For instance, Cus2 has a number of genetic, biochemical, and physical interactions with factors involved in cell-cycle regulation. (Collins et al., 2007; Ptacek et al., 2005; Wilmes et al., 2008) Furthermore, although Cus2 has been characterized by the GFP screen as a nuclear protein, Cus2 has several interesting interactions with cytoplasmic factors. (Collins et al., 2007; Ptacek et al., 2005; Wilmes et al., 2008)

Cus2 was identified as a phospho-protein under two cell-cycle-related conditions. In the first, Cus2 was shown to be phosphorylated at S163 after alpha factor arrest in G1 phase. (Li et al., 2007) In the second, Cus2 phosphorylation at Y48 and S50 was detected after the CDK inhibition as
described above. However, phosphorylated Cus2 was not detected in the untreated sample. The fact that Cus2 is found to be phosphorylated under these conditions suggests that Cus2 is regulated with the cell cycle.

Splicing has been shown to be regulated with the cell cycle. (Li et al., 2005; Shin et al., 2004; Shin and Manley, 2002, 2004; Tripathi and Parnaik, 2008) For example, splicing has been shown to be generally down-regulated during mitosis, particularly of ribosomal protein genes (RPGs). These findings raise the possibility that phosphorylation of Cus2 helps to mediate these cell cycle-specific effects on splicing.

In addition, there are hints that Cus2 activity may be regulated in response to other cellular events. Previously it was demonstrated that yeast undergo rapid changes in splicing in response to 3-Amino-1,2,4-triazole (3-AT) treatment which induces amino acid starvation. (Pleiss et al., 2007a) Here, unspliced transcripts of RPGs were specifically accumulated. The kinetics of this response suggested a rapid mechanism such as posttranslational modification of splicing factors in mediating this effect. Unpublished results from the Guthrie lab have shown that CUS2 deletion reverses this effect, and the responsiveness of RPG splicing is reduced. These data suggest that Cus2 mediates the cellular responses to amino acid deprivation and may function to inhibit splicing. Our model of Cus2 phosphorylation predicts that Cus2 phosphorylation results in an inability to associate with RNA. The effect of Cus2 during 3-AT treatment informs this model and suggests that Cus2 is phosphorylated in response to cellular stress and results in splicing inhibition.
Lastly, *CUS2* has genetic interactions with factors involved in translation including *RIA1, TAD2, KRS1, BRX1, RPA34, NOP15, RSA1, RSA3, and REX3.* (Wilmes et al., 2008) It is possible that these genetic interactions represent the role of *CUS2* in regulating the splicing of ribosomal genes, particularly in response to the cell cycle or during stress.

Several other splicing factors have connections to the ribosome. The splicing factor Prp43 has been shown to have a role in regulating ribosome biogenesis and Lea1, Prp4, Smd3, Hsh49, Sub2, Prp43, Prp8, Brr2, and Prp24 are all predicted to interact with nucleolar factors. (Leeds et al., 2006; Staub et al., 2005) It will be interesting to investigate the role of Cus2 and other splicing factors in regulating ribosome biogenesis.

These interactions present the possibility that Cus2 has RNA substrates other than U2 snRNA. RNA samples from the crosslink RIP experiment in Chapter 4 are still available for analysis by RT-PCR or deep sequencing. Analyses of the other potential RNA-binding partners for Cus2 will potentially expand our understanding about the cellular role(s) of Cus2.

**Acknowledgements**

We are grateful to Manuel Ares, Imre Barta, and their colleagues for providing plasmids and yeast strains. We thank Manuel Ares, Rhonda Perriman, and Christine Guthrie for sharing unpublished data. This work was
supported by an NSF CAREER award to T.L.J. (MCB-0448010) and an NSF predoctoral fellowship to S.L.M.

References
References


complexes expands the catalog of participating factors. Nucleic acids research 35, 3928-3944.


Dickerson, J.E., Pinney, J.W., and Robertson, D.L. The biological context of HIV-1 host interactions reveals subtle insights into a system hijack. BMC systems biology 4, 80.


that hSpt4 and hSpt5 exert their roles in transcriptional elongation as parts of the DSIF complex. Genes Cells 8, 371-378.


Marko, M., Leichter, M., Patrīnou-Georgoula, M., and Guialis, A. hnRNP M interacts with PSF and p54(nrb) and co-localizes within defined nuclear structures. Experimental cell research 316, 390-400.

Martinez, E., Palhan, V.B., Tjernberg, A., Lymar, E.S., Gamper, A.M., Kundu, T.K., Chait, B.T., and Roeder, R.G. (2001). Human STAGA complex is a chromatin-acetylating transcription coactivator that interacts with pre-mRNA
splicing and DNA damage-binding factors in vivo. Molecular and cellular biology 21, 6782-6795.


McCracken, S., Fong, N., Rosonina, E., Yankulov, K., Brothers, G., Siderovski, D., Hessel, A., Foster, S., Shuman, S., and Bentley, D.L. (1997a). 5'-Capping enzymes are targeted to pre-mRNA by binding to the phosphorylated carboxy-terminal domain of RNA polymerase II. Genes & development 11, 3306-3318.


tyrosine phosphorylation in the regulation of the interaction of heterogenous nuclear ribonucleoprotein K protein with its protein and RNA partners. The Journal of biological chemistry 275, 3619-3628.


Potel, C., and Elliott, G. (2005). Phosphorylation of the herpes simplex virus tegument protein VP22 has no effect on incorporation of VP22 into the virus but is involved in optimal expression and virion packaging of ICP0. Journal of virology 79, 14057-14068.


Schneider, J., and Fanning, E. (1988). Mutations in the phosphorylation sites of simian virus 40 (SV40) T antigen alter its origin DNA-binding specificity for
sites I or II and affect SV40 DNA replication activity. Journal of virology 62, 1598-1605.


van Nues, R.W., and Beggs, J.D. (2001). Functional contacts with a range of splicing proteins suggest a central role for Brr2p in the dynamic control of the order of events in spliceosomes of Saccharomyces cerevisiae. Genetics 157, 1451-1467.


cerevisiae that encodes a nuclear serine/threonine/tyrosine kinase. Molecular and cellular biology 13, 5829-5842.


