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INTEGRATION OF SPLICING REGULATION WITH DEVELOPMENTAL GENE EXPRESSION PROGRAMS

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by

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TABLE OF CONTENTS

List of Figures	iv
List of Tables	vi
Abstract	vii
Dedication	ix
Acknowledgements	x
Chapter 1 - Introduction	1
Chapter 2 - Integration of a splicing regulatory network within the meiotic gene expression program of *Saccharomyces cerevisiae*	35
Chapter 3 - Change in competition between pre-mRNAs for the splicing machinery drives global regulation of splicing	53
Chapter 4 - Conclusion	103
Bibliography	113

LIST OF FIGURES

1-1	RNA processing plays a central role in eukaryotic gene expression	3
1-2	Pre-mRNA splicing reaction	6
1-3	Step-wise assembly of the spliceosome	8
1-4	Alternative splicing of pre-mRNA	12
1-5	Splicing regulation is integrated with gene expression programs	17
2-1	Meiotic gene expression in the absence of the Mer1p splicing factor	38
2-2	Derepression of meiotic genes in vegetative cells reveals splicing factor requirements for meiosis	39
2-3	Accumulation of mRNA for Mer1p-responsive genes is delayed relative to other Ume6p-activated genes	41
2-4	*MER1* regulon expression is required for induction of *NDT80* and Ndt80p-regulated genes	42
2-5	Pachytene checkpoint activation persists in *mer3*Δ and *spo22*Δ and to a lesser degree in the *mer1*Δ strain	42
2-6	The *MER1* regulatory network and the meiotic gene expression program	43
2-S1	The URS1 regulatory sequence is found in the promoters of *MER1*, *MER3*, *SPO22*, and *SPO70*	52
3-1	Splicing efficiency improves globally during mid-meiosis	81
3-2	Splicing of meiotic transcripts is more efficient during meiosis than during vegetative growth	82
3-3	Splicing efficiency increases after treatment with rapamycin	83

3-4	Splicing defects are suppressed by down-regulation of RPG transcription	84
3-5	Competition is imposed at early steps of spliceosome assembly	85
3-6	Trans-competition control of splicing	86
3-S1	Rapamycin-induced improvement in splicing	87
3-S2	Competitive inhibition	89

LIST OF TABLES

1-1	Occurrence of non-canonical introns in yeast	19
1-2	Meiotic introns generally have non-consensus splice signals	21
2-1	The majority of meiotic intron-containing genes are transcriptionally activated by Ume6p	40
2-S1	Candidate Mer1p-responsive genes	49
2-S2	Yeast strains	50
2-S3	RT-PCR and RT-qPCR primers	51
3-S1	Data for heatmap in Figure 1	90
3-S2	Expression of spliceosomal components during meiosis, rapamycin treatment, and *IFH1* down-regulation	94
3-S3	Yeast Strains	97
3-S4	RT-PCR and RT-qPCR primers	98

ABSTRACT

INTEGRATION OF SPLICING REGULATION WITH GENE EXPRESSION PROGRAMS

Elizabeth Melissa Munding

Genes expressed within a cell define its identity and functional state. Gene expression in eukaryotes is highly regulated from transcription, to RNA processing, to translation. Pre-mRNA splicing, or the removal of intervening intronic sequences and ligation of expressed exonic sequences, is an essential step of RNA processing and contributes to regulation of the quantity and coding potential of the mRNA. Splicing can vary in complexity from the simpler ligation of neighboring exons, to more complex patterns of alternative splicing where exons or parts of exons may be differentially included or skipped. In order to understand how splicing regulation contributes to gene expression programs as a whole, splicing must be integrated with transcriptional networks and post-transcriptional networks that work together to drive transitions through cell states.

The studies presented here address how splicing regulation is integrated with the meiotic developmental gene expression program in budding yeast. Meiosis is known to depend on both transcription and splicing regulation. In particular, the splicing activator Mer1 is required for meiosis. The genome-wide studies in Chapter 2 define the entire Mer1 splicing network to consist of Mer1 and four regulated transcripts. The contribution of Mer1 to the meiotic gene expression program is also explored. These studies indicate that transcription of the Mer1 network is activated by the first transcriptional wave of meiosis, which is driven by the transcriptional regulators Ume6 and Ime1. We show that co-induction of Mer1 with its regulated transcripts creates a delay in expression of the Mer1-regulated transcripts that depends on accumulation of Mer1 protein. Subsequently expression of two Mer1-target transcripts is required for induction of the second transcriptional wave in meiosis, activated by the Ndt80 transcription factor, and progression through the meiotic program. Thus, the Mer1

splicing network links the early Ume6-dependent transcriptional wave with the second Ndt80-dependent transcriptional wave in meiosis. This study reveals how splicing networks may be interlaced with transcriptional networks to drive progression through a gene expression program.

Genome-wide analyses of splicing during meiosis presented in Chapter 2 also revealed a general increase in splicing efficiency. The global splicing improvement is coincident with the transcriptional repression of ribosomal protein genes (RPGs), which constitute a majority of intron-containing transcripts in the cell. The studies presented in Chapter 3 identify the molecular mechanism for the splicing improvement during meiosis as a relief in competition between pre-mRNAs for the spliceosome. Although relief in competition between transcripts and improved splicing of non-RPG pre-mRNAs is hardwired into the meiotic gene expression program, vegetative cells where RPGs are transcriptional repressed also display improved splicing of other transcripts. This study is the first to show that global splicing regulation depends on the effective load of pre-mRNAs on the splicing machinery. This regulatory mechanism that we are the first to describe is called trans-competition control.

The studies presented in this thesis contribute to understanding how splicing regulation is coordinately integrated with transcriptional networks to promote progression through a gene expression program.

DEDICATION

My dissertation is dedicated to my mom, Tania, my dad, Paul, and my partner in life and love, Peter. I thank them for the support, encouragement, and love that they have unconditionally given me during my graduate career. Thank you.

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In addition, the text of this dissertation includes a reprint of the following published material: Munding et al. “Integration of a splicing regulatory network within the meiotic gene expression program of *Saccharomyces cerevisiae*.” *Genes Dev 24*, 2693-2704 (December 2010). One of the co-authors listed in this publication directed and supervised the research which forms the basis for the dissertation.

CHAPTER 1

INTRODUCTION

While the genetic information contained in cells within a multicellular organism is generally the same, the particular genes that are expressed can differ greatly owing to the diversity of cell types and complexity of higher eukaryotes like mammals. Gene expression must be a highly regulated process that ensures the correct production of RNA and proteins from the DNA template in a given cell at the correct time in the developmental program. Sequencing of the DNA genomes from various organisms has indicated that humans have far fewer protein coding genes than the 100,000 originally predicted to be required to make up a mammal. In fact, humans have just over 20,000 protein coding genes, which is only about four times that of the baker's yeast *Saccharomyces cerevisiae* and about the same as the worm *Caenorhabditis elegans*. Expansion of the coding potential of the human genome is provided by differences in processing of the RNA indicating that regulation of RNA processing is central to the regulation gene expression.

Central dogma of molecular biology

Described over 50 years ago by Francis Crick, the central dogma of molecular biology states that genetic information flows from nucleic acid to protein and never in reverse (Crick, 1958). More specifically, genetic information, stored as DNA (deoxyribonucleic acid), is transcribed into messenger RNA (ribonucleic acid; mRNA) and the mRNA is then translated in protein. In eukaryotes, DNA is transcribed into precursor mRNA (pre-mRNA) and requires additional processing steps for generation of mRNA (Fig1-1).

Processing of pre-mRNA into mRNA includes the addition of a 7-methylguanosine (m^7G) cap to the 5' end and cleavage of the 3' end followed by polyadenylation (pA). Both of these modifications serve to protect the RNA from degradation as well as to promote export into the cytoplasm and translation. Another essential processing step of eukaryotic gene expression is pre-mRNA splicing, a process in which the intervening, non-coding intronic sequences are removed and coding exonic sequences are ligated or "spliced" together.

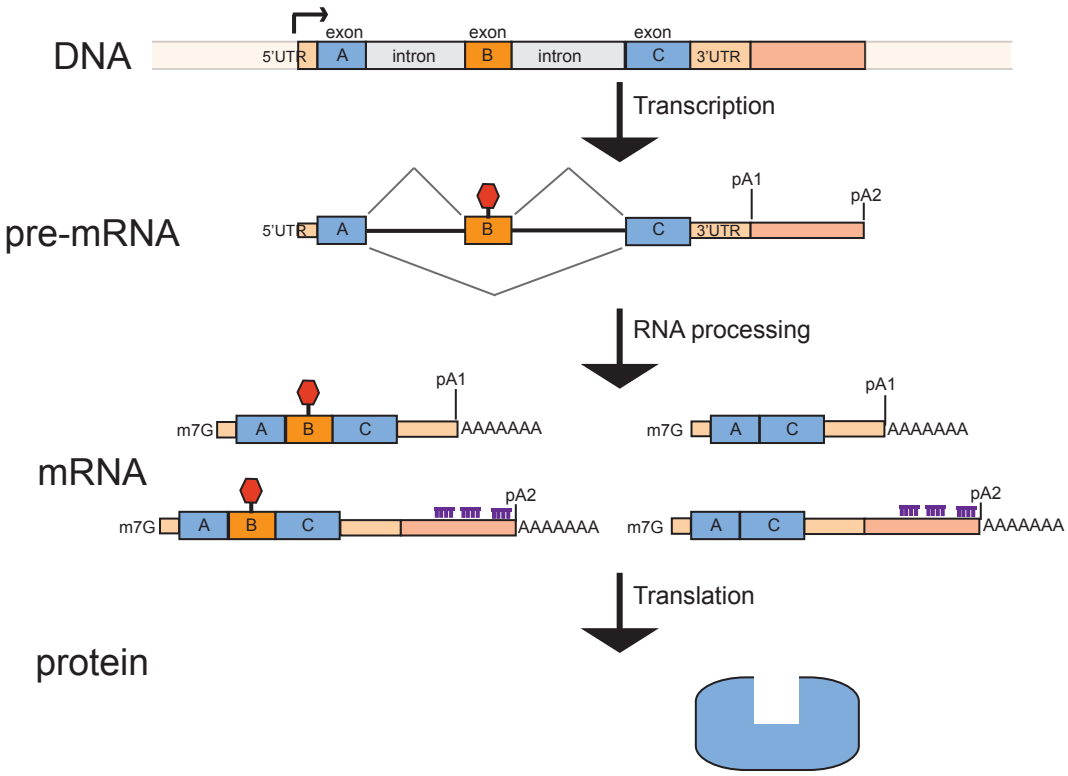

Fig 1-1: RNA processing plays a central role in eukaryotic gene expression.
DNA is transcribed into pre-mRNA which undergoes RNA processing to generate mRNA. RNA processing includes capping of the 5' end, polyadenylation of the 3'end, and pre-mRNA splicing. Following RNA processing and export into the cytoplasm, the mRNA may be translated into protein. In the example above, inclusion of exon B, which contains a premature termination codon, targets the mRNA for degradation through non-sense mediate decay. Only the mRNAs where exon B is skipped are translated into protein.

Splicing can vary in complexity from the simpler intron removal and ligation of neighboring exons to more elaborate patterns of alternative splicing where differential inclusion and skipping of exons can generate many diverse mRNA isoforms from a single pre-mRNA template (discussed below).

An important amendment to the central dogma is that the steps of gene expression do not occur in isolation and are instead coupled and integrated with one another. Pre-mRNA splicing is largely co-transcriptional (reviewed in Carrillo Oesterreich et al., 2011) as are capping and polyadenylation (reviewed in Moore and Proudfoot, 2009). Upon processing, the mRNAs become complexed with proteins that recognize the cap or pA tail as well as other stretches of the mRNA and which in turn promotes export into the cytoplasm and subsequent localization and translation (Moore and Proudfoot, 2009). Therefore gene expression is made up of interconnected processes whereby transcription of a gene is coupled to RNA processing and RNA processing promotes mRNA translation.

Pre-mRNA splicing by the spliceosome

Splicing was first described in an adenoviral mRNA using electron microscopy (Berget et al., 1977; Chow et al., 1977). These studies imaged displaced DNA loops that were created when an mRNA was hybridized to DNA and sequences present in DNA but not in mRNA resulted in the displacement of DNA from the hybrid creating loops. These sequences were introns and were predicted to be removed from the pre-mRNA through a process called splicing.

In vitro splicing systems have been instrumental in defining the requirements of both the splicing substrate and the splicing machinery (reviewed in Green, 1991). In these systems, exogenous, naturally occurring or artificial pre-mRNAs are added to yeast or mammalian cellular extracts in which different components of the splicing machinery may be depleted. The findings from such studies conceptualize a canonical splicing pathway, described below, observed for several model substrates.

Three different sequence features of the substrate are essential for recognition by the splicing machinery and for splicing chemistry (reviewed in Wahl et al., 2009) (Fig1-2a). The 5' splice site (5'ss), typically a GU dinucleotide part of larger consensus, marks the 5' exon/intron junction in the pre-mRNA and is the donor site for chemistry. The 3' splice site (3'ss) is found at the 3'intron/exon junction and is an AG acceptor site. A branchpoint (bp) sequence near the 3' end of the intron contains an invariant adenosine residue essential for the catalytic step of the reaction. Although essential, the three sequence elements are not sufficient to define splice sites, as these are rather degenerate, and sequences flanking splice sites further act to contribute to splice site selection (discussed below).

The splicing reaction (Fig1-2b) progresses through two phosphoryl transfer reactions in which one phosphodiester bond is exchanged for another (Moore and Sharp, 1993). In the first step the 2' hydroxyl of the branchpoint adenosine attacks the 5'ss phosphate resulting in a free 5' exon and a 2'-5' phosphodiester bond making an intron-lariat-3' exon intermediate. In the second step, the 3' hydroxyl of the 5' exon attacks the phosphate of the 3'ss resulting in the ligation of the two exons and release of the lariat intron. This is similar to the self-splicing group II introns in that both have similar stereochemistries and occur through two transesterification reactions that are independent of NTP hydrolysis and suggests that pre-mRNA splicing may have evolved from these ancestors (Michel and Ferat, 1995). However, unlike splicing of group II introns, which occurs independently of protein co-factors, splicing of pre-mRNAs is promoted by the spliceosome consisting of both protein and RNA cofactors.

Five uridine-rich small nuclear RNAs (U snRNAs), U1, U2, U4, U5, U6 snRNAs, are associated with proteins to make up five small nuclear ribonucleoprotein participles (snRNPs) that assemble on the pre-mRNA substrate (reviewed in Wahl et al., 2009). As the spliceosome assembles, additional proteins transiently interact with the core snRNPs. In yeast the total number of proteins involved in some stage of the splicing cycle is ~90 (Fabrizio et al., 2009), while in higher metazoans its near 170 (Jurica and Moore, 2003; Wahl et al., 2009), making the spliceosome a very dynamic, protein-rich machine.

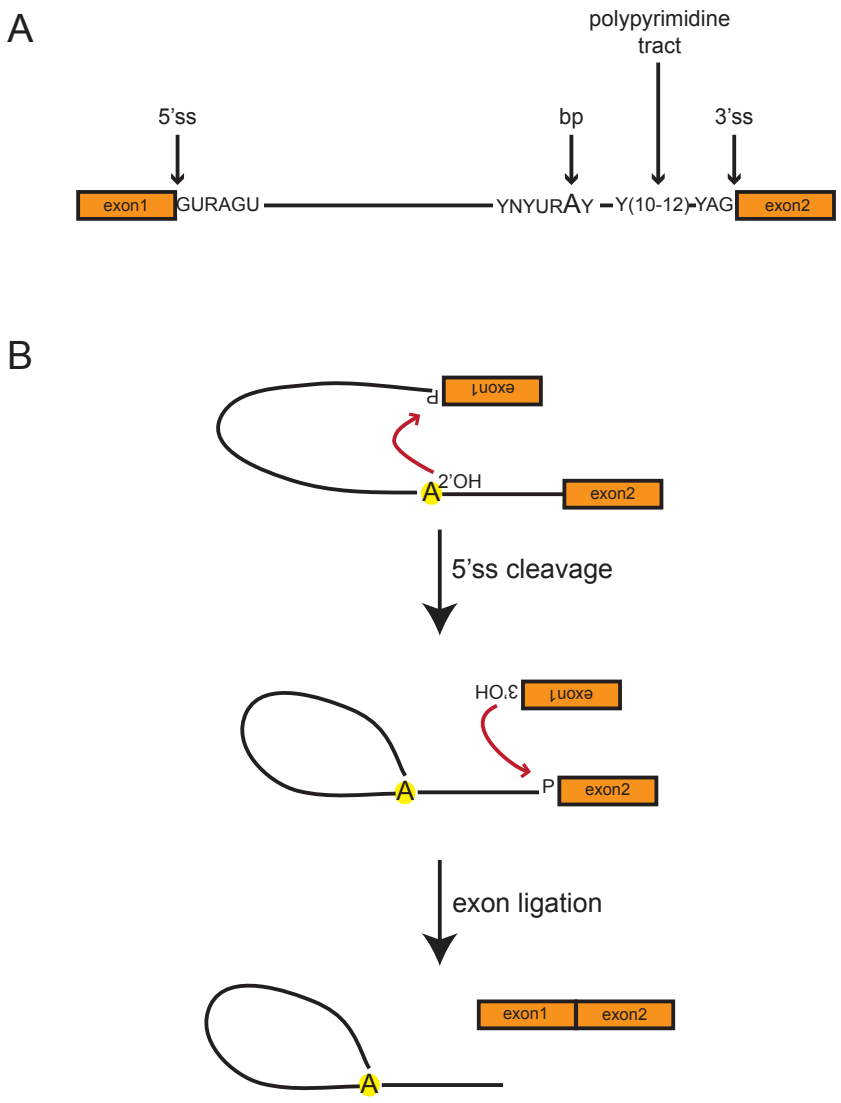

Fig1-2: Pre-mRNA splicing reaction.

(A) Three different sequence features are required for pre-mRNA splicing: 5' splice site (5'ss), branchpoint (bp), and 3' splice site (3'ss).The metazoan consensus (described in Wahl et al. (2009) Cell) of each is shown. N= any nucleotide; R= purine; Y=pyrimidine. (B) Pre-mRNA splicing progresses through two transesterification reactions. In the first step, the 2'OH of the branchpoint adenosine (A) attacks the phosphate of the 5'ss to produce a free 5' exon and a lariat intron-exon2 intermediate. In the second step the 3'OH of the free 5' exon attacks the phosphate of the 3' exon producing a ligated mRNA and a lariat intron.

The spliceosome assembly pathway has mainly been elucidated from in vitro experiments using several different model substrates. Non-denaturing gel electrophoresis of a splicing reaction in which a pre-mRNA is incubated with whole cell extract suggests that in vitro, spliceosome assembly occurs step-wise and distinct complexes accumulate sequentially. By this method, at least four distinct complexes can be resolved (Fig1-3).

Assembly of the spliceosome begins with the ATP-independent recognition of the 5'ss by U1 snRNP, a process promoted by the U1 snRNP-associated proteins and the base-pairing interaction between the 5'ss and U1 snRNA (reviewed in Rosbash and Seraphin, 1991). An auxiliary protein, BBP in yeast or SF1 in mammals, promotes recognition of the bp by U2 snRNP (Abovich and Rosbash, 1997; Berglund et al., 1998; Berglund et al., 1997). In mammals, another non-snRNP protein called U2AF binds the polypyrimidine tract, found between the bp and 3'ss, as a heterodimer consisting of 35kD and 65kD components to promote U2 snRNP binding (Singh et al., 1995; Zamore and Green, 1991). The stable association of U2 snRNP at the bp is the first ATP-dependent reaction that is stabilized through base pairing between the bp and U2 snRNA (reviewed in (Brow, 2002). Next, the tri-snRNP (a complex of U4/U6-U5 snRNPs) is recruited to the pre-mRNA and rearrangements within snRNAs and between the snRNAs and the pre-mRNA results in exchange of U1 snRNP for U6 snRNP and the dissociation of U4 snRNP and U1 snRNP from the splicing complex (reviewed in Ares and Weiser, 1995; Brow, 2002). This catalytically active spliceosome contains newly established base-pairings between U6 snRNA and the 5'ss as well as between U6 snRNA and U2 snRNA, bringing the 5'ss closer to bp to promote the first step of splicing (reviewed in Ares and Weiser, 1995; Staley and Guthrie, 1998). Additional rearrangements within the catalytically active spliceosome take place to position the U5 snRNA at the catalytic core bringing together the free 5' exon and the 3' exon to facilitate the second step of splicing (Konarska et al., 2006). After completion of the second step, the ligated mRNA is released as well as the snRNPs reconfigured and recycled for subsequent rounds of splicing.

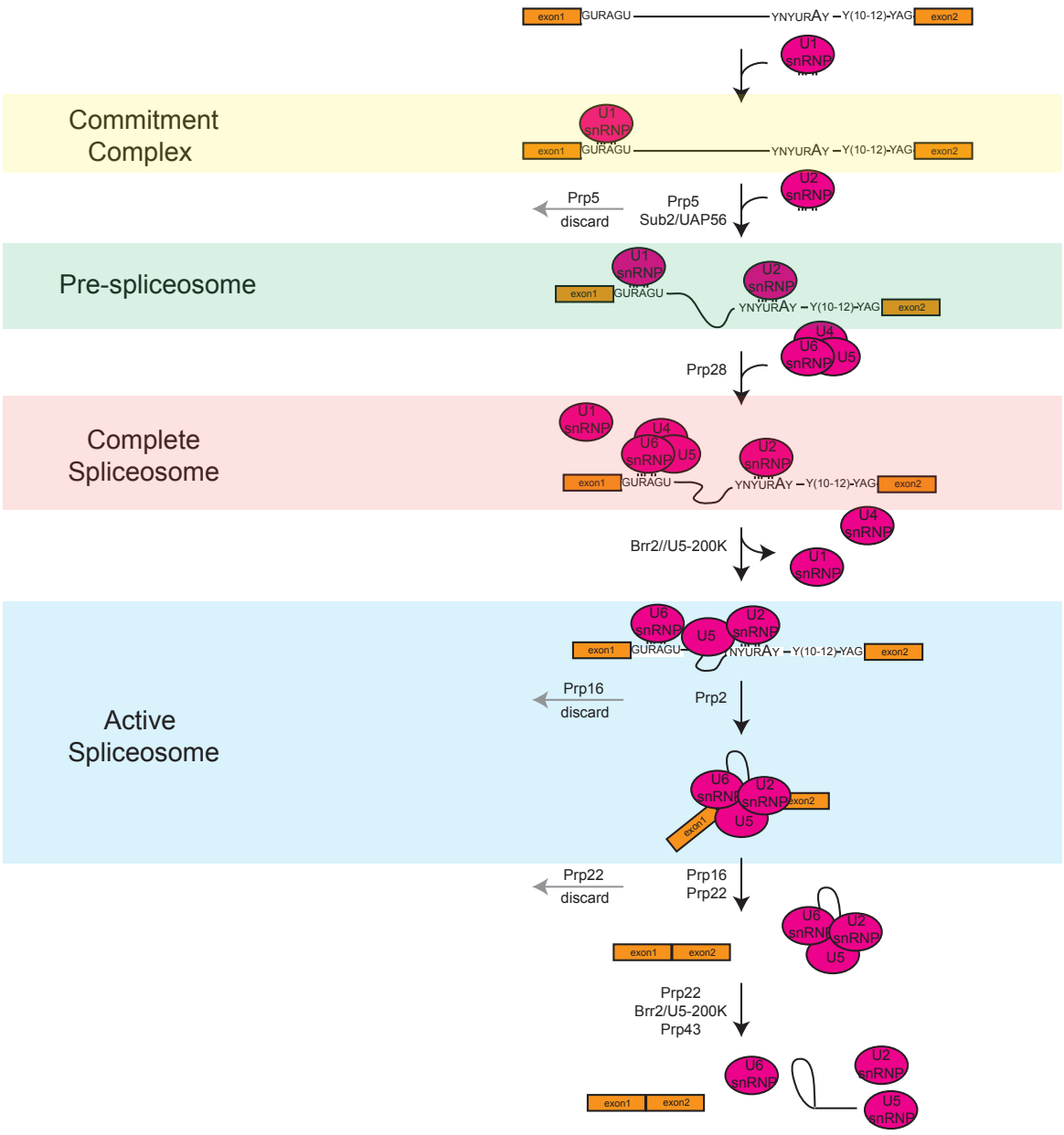

Fig1-3: Step-wise assembly of the spliceosome.

SnRNPs assemble on the pre-mRNA substrate sequentially. Non-denaturing gel electrophoresis of splicing reactions over time resolves four distinct splicing complexes, labeled on the left. Stable association of U1 snRNP at the 5'ss initiates commitment of the pre-mRNA to the splicing pathway. Binding of U2 snRNP to the bp forms the pre-spliceosome. Addition of the tri-snRNP to the pre-spliceosome forms the complete spliceosomal complex, but the subsequent removal of U1 snRNP and U4 snRNP is required to make catalytically active spliceomes. Eight DExD/H-box ATPases (Prp5, Sub2/UAP56, Prp28/U5-100K, Brr2/U5-200K, Prp2, Prp16, Prp22, and Prp43) contribute to sequential assembly of the spliceosome at the noted steps. Additionally, three of these (Prp5, Prp16, and Prp22) appear to ensure the fidelity of the splicing reaction.

The splicing reaction is driven by eight highly conserved RNA-dependent DExD/H-box helicases (Sub2/UAP56, Prp5, Brr2/U5-200K, Prp28/U5-100K, Prp2, Prp16, Prp22, Prp43) that act at specific steps of spliceosome assembly to promote the RNA-RNA, RNA-protein, and protein-protein rearrangements that drive the reaction forward (reviewed in Cordin et al., 2012; Staley and Guthrie, 1998). Sub2/UAP56 acts on the pre-mRNA (Kistler and Guthrie, 2001), while Prp5 acts on U2 snRNA to promote recognition of the bp and the bp-U2 snRNA interaction (Perriman and Ares, 2010; Perriman and Ares, 2007). Subsequently, Prp28/U5-100K promotes the exchange of the 5'ss from U1 snRNA to U6 snRNA during tri-snRNP addition (Chen et al., 2001; Staley and Guthrie, 1999). Unwinding of U4 snRNA/U6 snRNA depends on Brr2/U5-200K (Raghunathan and Guthrie, 1998). Prp2 activity is required before catalysis of the first step of splicing but after U4/U6 unwinding (Kim and Lin, 1996) and Prp16 acts after the first step but before the second step of splicing (Schwer and Guthrie, 1992). Prp22 is required during the second step of splicing (Schwer and Gross, 1998) and for release of the mRNA from the spliceosome after both splicing steps are complete (Company et al., 1991). Prp43 is involved in spliceosome disassembly and promoting release of the lariat intron (Arenas and Abelson, 1997; Martin et al., 2002), a step also promoted by Brr2/U5-200K (Small et al., 2006). These ATP-dependent helicases are key players in structural rearrangements and spliceosome dynamics.

In addition to their role in spliceosomal structural rearrangements, some of the DExD/H-box proteins appear to be involved in fidelity or correct splice site choice on the pre-mRNA. The degeneracy of the splice signals, especially in higher eukaryotes in which splice signals diverge greatly from the consensus, suggests that mechanisms must be in place to promote selection of the correct splice sites. "Kinetic proofreading" is one such proposed splicing fidelity mechanism (reviewed in Semlow and Staley, 2012) that functions during three main stages of spliceosome assembly: 1) during association of U2 snRNP with the bp through Prp5, 2) during first step catalysis through Prp16, and 3) during second step catalysis through Prp22. Base pairing of U2 snRNA with the bp depends on a structure within U2

snRNA (the branchpoint-interacting stem loop; BSL) that is antagonized by Prp5 (Perriman and Ares, 2010). The disruption of the BSL by Prp5 favors recognition of optimal bp sequences (Perriman and Ares, 2010; Xu and Query, 2007). Prp16 proofreading of suboptimal substrates occurs before the first step of splicing and appears to antagonize features that promote 5'ss cleavage (Burgess and Guthrie, 1993; Koodathingal et al., 2010). Similarly, Prp22 –dependent substrate discard seems to be in competition with second step of splicing and substrates with poor 3'ss characteristics are discarded through a Prp22-dependent mechanism (Mayas et al., 2006). Discard of substrates through Prp16 and Prp22 also employs the Prp43 helicase for spliceosome disassembly (Koodathingal et al., 2010; Mayas et al., 2010).

Alternative and regulated splicing

mRNA diversification through alternative splicing greatly expands the coding potential of the eukaryotic genome and is responsible for at least doubling the number of proteins that are encoded by genes. An extreme example is the *Drosophila melanogaster* gene *Down syndrome cell adhesion molecular (DSCAM)* which has the potential to generate 38,016 different mRNA isoforms due to multiple variants of 4 different exons (Schmucker et al., 2000). Recent transcriptome sequencing studies (Pan et al., 2008; Wang et al., 2008) indicate that greater than 95% of human multi-exonic genes are alternatively spliced and generate multiple mRNAs. The high extent of alternative splicing makes up for the "missing" protein-coding information in the human genome and contributes to the elaborate human proteome. In addition to adding proteomic diversity, alternative splicing mediates gene expression regulation through introduction of premature termination codons (PTCs, targeting the mRNA transcript for degradation) and through differential usage of UTRs which affects mRNA stability, localization, or translational efficiency.

In a multi-exon pre-mRNA there may be both constitutively (always included in the mRNA) and alternatively (differentially included in the mRNA) spliced exons. The most

common patterns of alternative splicing are cassette-exon inclusion or skipping, multiple cassette exons that are mutually exclusive, alternative 5' or 3' splice site choice, and intron retention (Fig1-4a; reviewed in Black, 2003; Braunschweig et al., 2013; Nilsen and Graveley, 2010). Differences in alternative splicing patterns may be due to features of the DNA (due to co-transcriptional splicing) and pre-mRNA (Fig1-4b). Adaptor proteins that interact with either chromatin remodelers or histone modifications seem to affect splice site choice by differentially recruiting spliceosomal components or altering Pol II transcription elongation rates across a gene (reviewed in Luco et al., 2011). Studies where Pol II elongation rates are altered with genetic mutations (de la Mata et al., 2003) or with drug treatment (de la Mata et al., 2010; Nogues et al., 2002) indicate that slow Pol II elongation rate promotes recognition of pre-mRNA splice sites in a weakly competitive intron (across a cassette exon) while rapid elongation rate promote skipping of the cassette exon as stronger splice sites downstream have become available (reviewed in Kornblihtt, 2007; Kornblihtt et al., 2013). A recent study (Shukla et al., 2011) shows DNA methylation at the alternative exon 5 in the *CD45* pre-mRNA represses inclusion of exon 5 by blocking binding of DNA-binding protein CTCF. When CTCF is bound to DNA, it serves as a roadblock for Pol II resulting in slowed elongation and inclusion of exon 5 (Shukla et al., 2011).

Key features of the pre-mRNA, such as the match to the splice site consensus and RNA secondary structure, also affect splice site selection (reviewed in Warf and Berglund, 2010). Splice site strength is a complex measure of accessibility of the splice site and complementarity between the pre-mRNA splice site and the snRNAs that recognize it. A splice site is considered strong if it is readily accessible to the snRNP and contains a high degree of base pairing with its complementary snRNA. RNA secondary structure can have both positive and negative effects on selection of a splice site. Secondary structures can inhibit splicing by masking of the essential recognition signals, such as in the example described by Blanchette and Chabot, 1997 when they encompass the splice site or structures can enhance splicing by reducing the effective distance between splice sites (such as Meyer

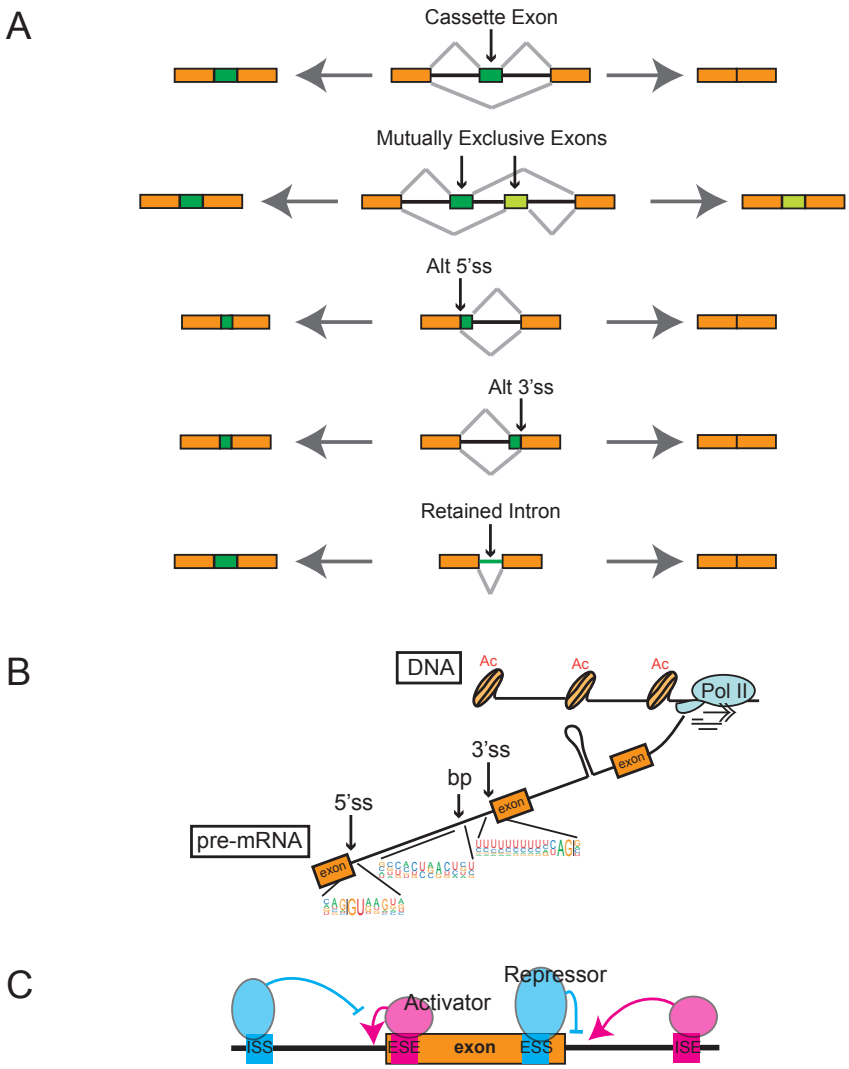

Fig1-4: Alternative splicing of pre-mRNA.

(A) Schematic representing types of alternative splicing. From top to bottom: Cassette exon inclusion/skipping, inclusion of mutually exclusive exons, alternative (Alt) 5'ss or 3'ss usage and retained intron which becomes an exon when not spliced. (B) Splicing regulation by features of the DNA (such as chromatin structure and modifications and Pol II elongation rate) and by features of the pre-mRNA such as splice site strength and secondary structure. (C) Splicing regulation by trans-acting splicing factors that modulate spliceosome recruitment. Blue factor is a repressor that binds intronic/exon splicing silencer (I/ESS) sequences and blocks local spliceosome assembly. Pink factor is an activator that binds intronic/exonic splicing enhancer (I/ESE) sequences and promotes spliceosome assembly.

et al., 2011). RNA secondary structure have been shown to affect pre-mRNA splicing indirectly by recruiting or blocking binding of regulatory splicing factors. For example, a structure encompassing the 5'ss of the yeast *RPL30* pre-mRNA recruits Rpl30 protein (Vilardell and Warner, 1994) to autoregulate splicing of the pre-mRNA. Binding of Rpl30 to the stem-loop structure in *RPL30* pre-mRNA, which strongly resembles the natural Rpl30 binding site in helix 34 in 25S rRNA (Vilardell et al., 2000), surprisingly allows U1 snRNP recognition of the 5'ss but blocks binding of the U2 snRNP at the bp (Macias et al., 2008). One possible explanation of this mechanism is that Rpl30 binding to the pre-mRNA disrupts an interaction between U1 snRNP and U2 snRNP.

In addition to features of the substrate, differences in alternative splicing may be due to trans-acting auxiliary splicing factors that bind the pre-mRNA to influence the accessibility of the splice sites and recruitment of the splicing machinery (reviewed in Black, 2003; Nilsen and Graveley, 2010) (Fig1-4c). Splicing factors that repress spliceosome recruitment recognize splicing silencer sequences found in either the intron or exon. Conversely, splicing factors that activate spliceosome recruitment recognize splicing enhancer sequences. The same splicing factor may be either an activator or a repressor and its role towards spliceosome assembly is often, but not always, determined by the location of its binding sequence with respect to the alternative exon (for example Erkelenz et al., 2013; Lim et al., 2011). The exact mechanisms by which different splicing factors confer splicing regulation remains unclear and likely differs between factors, but all have an RNA-binding domain for substrates recognition and another domain for protein-protein interaction that affects snRNP recruitment. Some splicing factors such as SR proteins and hnRNP proteins are expressed in all tissues but expression of other splicing factors can be tissue- or developmentally-regulated (reviewed in Kalsotra and Cooper, 2011).

Generally, changes in alternative splicing patterns between tissues or during development are attributed to changes in activity of these trans-acting factors. For example, expression of the splicing factor Mbnl1 increases four-fold in the adult heart compared to the

fetal heart, where Mbnl1 is barely expressed (Kalsotra et al., 2008; reviewed in Kalsotra and Cooper, 2011). Deletion of *MBNL1* in the adult heart reverts splicing of many developmentally regulated transcripts back to their embryonic splicing patterns. Much work has been dedicated to defining expression patterns of various splicing factors as well as the networks of transcripts directly regulated by a particular splicing factor (for example, Calarco et al., 2009; Du et al., 2010; Markovtsov et al., 2000; Ule et al., 2003; Warzecha et al., 2009; Zhang et al., 2008; reviewed in Braunschweig et al., 2013; Nilsen and Graveley, 2010).

Genome-wide studies of alternative splicing

Technological advances have made the challenging task of identifying tissue-specific splicing networks more feasible (reviewed in Licatalosi and Darnell, 2010). Most everything known about alternative splicing before the year 2000 came from single-gene or reporter-based studies. Sequencing of expressed sequence tags (ESTs) in the early 2000s estimated that 35-60% of all human genes were alternatively spliced (Modrek and Lee, 2002). The development of microarrays greatly improved the ability of researchers to evaluate alternative splicing across species, tissues, and upon genetic perturbations such as splicing factor deletion (Clark et al., 2002; Johnson et al., 2003; Pan et al., 2004). Microarrays are glass slides containing thousands of immobilized DNA fragments, called “probes”, that are complementary to portions of the genome (Schena et al., 1995; Shalon et al., 1996). To study changes in alternative splicing exon-junction microarrays, which contain probes complementary to mRNA junctions formed upon splicing of two exons together, allow for the simultaneous measurement of expression and splicing of many genes. Such studies greatly expand our knowledge of regulation of alternative splicing but are restricted by predefined probes from known sequences and may not detect novel alternative events (reviewed in Licatalosi and Darnell, 2010). Tiling microarrays (Shoemaker et al., 2001) try to circumvent this problem by using probes that completely cover or “tile” the genome. Most recently, high-throughput RNA sequencing (RNA-Seq) is used to generate sequencing reads of all cellular

RNA species, which after being mapped to the genome of origin can relate both gene expression levels and expression of all mRNA isoforms (reviewed in Blencowe et al., 2009; Wang et al., 2009). RNA-Seq data has added a breadth of information at nucleotide-level resolution about alternative splicing and polyadenylation variants of known genes as well as unannotated transcription all over a genome (for example Guttman et al., 2009; Pan et al., 2008; Wang et al., 2008).

To determine the effects a splicing factor has genome-wide, most studies compare splicing patterns of wild type cells to cells where the splicing factor is depleted or over-expressed. In these studies transcripts whose splicing changes in response to factor manipulation (depletion or overexpression) are ones regulated by the splicing factor. One of the caveats of such studies is discerning direct versus indirect effects of the factor (reviewed in Darnell, 2010; Licatalosi and Darnell, 2010). A direct effect of a splicing factor is one where the splicing factor binds to the pre-mRNA to influence its splicing whereas indirect effects are observed as a result of the primary splice decision. Comparing the affected transcripts for enrichment of a motif to which the splicing factor may bind followed by filtering for transcripts containing the motif, aids in recognition of direct targets (such as in Du et al., 2010; Zhang et al., 2008). Another method, Cross-linking and immunoprecipitation (CLIP), is used to study direct protein-RNA interactions (Licatalosi et al., 2008; Ule et al., 2003). In these studies, a splicing factor of interest is cross-linked to RNA sequences with which the factor interacts in vivo using UV-irradiation. Following purification of the splicing factor, the associated sequences are determined through microarray or RNA-Seq analysis and direct binding targets of the splicing factor can be observed (Licatalosi et al., 2008; Ule et al., 2006). Variations of CLIP, such as PAR-CLIP (Hafner et al., 2010), iCLIP (Konig et al., 2010), or CRAC (Bohnsack et al., 2012; Granneman et al., 2009) are also used to map splicing factor binding sites.

Combining CLIP-Seq type studies, to identify direct targets of the splicing factor, with microarray or RNA-Seq studies, to determine the effect on splicing the factor has, allows the

identification of networks of transcripts regulated by the splicing factor at a particular cell state (Fig1-5). Furthermore, these genome-wide studies have generated vast datasets that show alternative splicing patterns (Barbosa-Morais et al., 2012; Merkin et al., 2012; Pan et al., 2008; Wang et al., 2008) and splicing factor expression can be tissue- (for example Buckanovich et al., 1993; Calarco et al., 2009; Jin et al., 2003; Markovtsov et al., 2000; Underwood et al., 2005; Warzecha et al., 2009) or developmental stage-specific (for example Boutz et al., 2007; Gabut et al., 2011; Kalsotra et al., 2008) indicating that splicing is integrated within gene expression programs.

Yeast as a model system to study pre-mRNA splicing

Since the discovery of splicing (Ng and Abelson, 1980) and snRNAs (Ares, 1986; Wise et al., 1983) in the budding yeast *Saccharomyces cerevisiae*, many genetic and biochemical studies of pre-mRNA splicing have employed yeast as a model organism (Spingola et al., 1999). The mechanism of splicing and the core components of the splicing machinery are conserved from yeast to humans and pre-mRNA splicing is essential for growth of yeast, as it is in all eukaryotes (Wahl et al., 2009). Out of the ~6200 protein coding genes in yeast only ~300 contain introns. Although introns are much less prominent across the yeast genome compared to human, intron-containing genes are highly transcribed and make up about 30% of all transcripts in a yeast cell (Ares et al., 1999; Lopez and Seraphin, 1999; Warner, 1999). With the exception of a couple in-frame introns, splicing is required for production of a translatable non-PTC containing mRNA.

Variation in yeast splice signals is very limited and most introns (196/298) conform to the consensus (5'ss: GUAYGU; BP: UACUAAC; 3'ss:YAG) (Table 1-1). Ribosomal protein genes (RPGs) make up the largest intron-containing gene functional class (109 introns in 101 RPGs) in yeast and tend to have larger introns, with the distribution centered at about 400 nucleotides (Spingola et al., 1999). The remaining introns, with a length distribution centered at about 100 nucleotides (Spingola et al., 1999), are spread out across genes with various

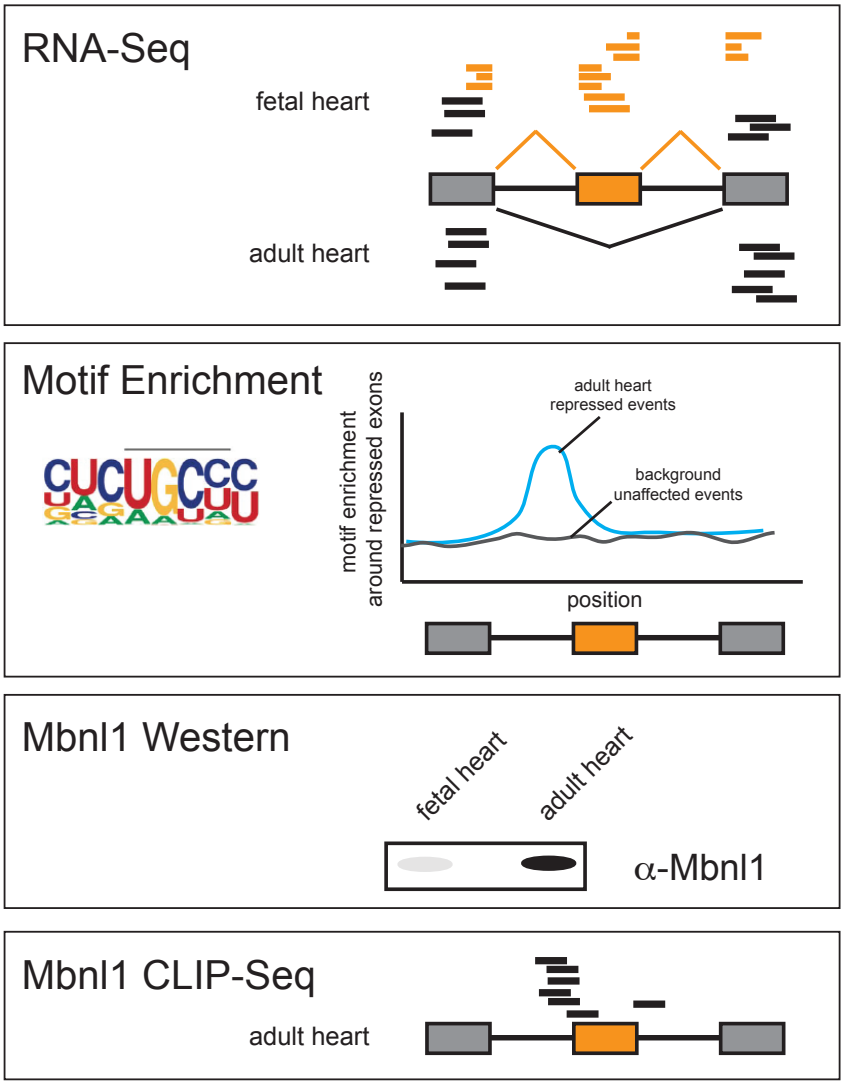

Figure 1-5: Splicing regulation is integrated with gene expression programs.

Genome-wide studies (such as RNA-Seq or microarrays) identify networks of transcripts that are co-regulated in a developmental or tissue-specific manner. One example transcript is shown. Reads in the central orange exon are observed only in the fetal heart indicating inclusion of the cassette exon. Bioinformatics analysis is used to identify a motif upstream of exons that become repressed during heart development. Western blot of Mbnl1 expression indicates the splicing factor Mbnl1 is only expressed in the adult heart. CLIP-Seq of Mbnl1 in the adult heart identifies direct binding sites for the protein.

functions (including 15 meiosis-specific genes with introns). The degree to which splicing of most yeast genes is regulated is unknown, but notable examples where regulation has been studied are described below.

Alternative splicing in yeast is mainly limited to regulation of intron removal that in turn determines whether the gene is expressed. Several regulatory mechanisms have been described in yeast. Splicing of four pre-mRNAs appears to be autoregulated by their gene product; two RPG pre-mRNAs: *RPL30* (discussed above; Macias et al., 2008; Vilardell and Warner, 1994, 1997; Vilardell et al., 2000) and *RPS14* (Fewell and Woolford, 1999; Li et al., 1995); and two other transcripts: *YRA1* (Preker et al., 2002; Rodriguez-Navarro et al., 2002b) and *DBP2* (Barta and Iggo, 1995). *YRA1* encodes a component of the RNA export machinery (Zenklusen et al., 2001) and participates in a negative autoregulatory feedback loop with its pre-mRNA. Autoregulation of *YRA1* splicing requires three unusual features found in its gene structure: 1) a long 5' exon (285 nucleotides) (Dong et al., 2007); 2) a large intron (776 nucleotides) (Dong et al., 2010; Dong et al., 2007; Preker and Guthrie, 2006) and 3) a non-canonical BP sequence (GACUAAC). Deletion of the *YRA1* intron renders the cells temperature-sensitive (Dong et al., 2007; Parenteau et al., 2008; Preker and Guthrie, 2006; Preker et al., 2002; Rodriguez-Navarro et al., 2002b; Zenklusen et al., 2001) demonstrating the importance of splicing regulation in cell viability. The mechanism of *YRA1* splicing autoregulation is unusual because excess Yra1 protein actually enhances the export of its own pre-mRNA before the first step of splicing can take place (Dong et al., 2007). *DBP2*, which encodes an RNA helicase involved in NMD (Bond et al., 2001), contains the largest intron (1002 nucleotides) thus far identified in *S. cerevisiae* at the 3'end of the gene. Barta and Iggo (Barta and Iggo, 1995) showed that a 552bp fragment within the *DBP2* intron is necessary for autoregulation of splicing and sufficient to confer Dbp2-regulation in a reporter construct. Interestingly, a portion of this fragment has the potential to form a stable secondary structure.

5'ss bp 3'ss combinations	# of occurrences	introns								
GUAUGU UACUAAC AAG	9	SEC17 (116=77+39)	YDL189W (99=82+17)	UBC5 (90=58+32)	REC114 (116=80+36)	LSM7 (96=62+34)	YOL047C (63=51+12)	YPL230W (75=52+23)	REC102 (97=52+45)	TFC3 (90=77+13)
GUAUGU AACUAAC CAG	7	YDR381C-A (194=49+145)	MTR2 (204=136+68)	MTR2 (213=136+77)	RPS21A (322=301+21)	TAD3-i1 (68=45+23)	YPR153W (134=90+44)	RPL19A (506=492+14)		
GUAAGU UACUAAC UAG	6	YBL059C-A (85=57+28)	YGR001C-i1 (62=43+19)	YIL156W-A (62=45+17)	PCC1 (75=51+24)	YIP3 (79=49+30)	SNC1 (113=51+62)			
GUAUGU GACUAAC CAG	5	SNR17B (130=118+12)	YBR230C (97=62+35)	IWR1 (70=46+24)	YPR098C (96=66+30)	SNR17A (157=145+12)				
GUAUGU CACUAAC CAG	5	UBC8 (123=77+46)	RPS25B (423=393+30)	PSP2 (362=197+165)	ERV41 (93=71+22)	MCM21 (83=64+19)				
GUAUGA UACUAAC UAG	5	BET1 (131=112+19)	RPL22A (389=334+55)	GCR1 (751=700+51)	RPS6A (394=357+37)	RPS11A (339=316+23)				
GUAUGC UACUAAC CAG	4	RPL40A (434=399+35)	RPL43B (275=242+33)	YNL050C (91=52+39)	HNT2 (89=53+36)					
GUAUGU AACUAAC UAG	4	PMI40 (93=74+19)	MTR2 (149=136+13)	TAD3-i2 (56=44+12)	YBR062C (82=49+33)					
GUAUGC UACUAAC UAG	3	PFD2 (88=61+27)	COF1 (179=47+132)	YBL059W (69=51+18)						
GUAAGU UACUAAC CAG	3	QCR10 (63=49+14)	SIM1 (487=458+29)	UBC9 (110=72+38)						
GUAUGU GACUAAC UAG	3	NYV1 (141=106+35)	YRA1 (766=746+20)	PTC7 (93=76+17)						
GUAUGU UGCUAAC UAG	2	RPL7B-i1 (409=387+22)	SRB2 (101=66+35)							
GUACGU GACUAAC UAG	2	YBR220C (421=389+32)	RPS9B (413=382+31)							
GUAAGU GACUAAC UAG	2	YOL048C (78=49+29)	YLR211C (59=49+10)							
GUAUGU UAUUAAC CAG	2	MOB1 (85=53+32)	PUS2 (80=67+13)							
GUAUGU CACUAAC UAG	2	MND1 (83=44+39)	PCH2 (113=77+36)							
GUAUGG UACUAAC CAG	2	PRP5 (167=148+19)	OSW2 (87=50+37)							
GUAUGA UACUAAC CAG	2	RPL43A (403=370+33)	RPS11B (511=492+19)							
GUACGU AACUAAC CAG	2	MTR2 (154=86+68)	MTR2 (163=86+77)							
GUAUGU UAUUAAC UAG	2	ERV1 (83=66+17)	FES1 (128=93+35)							
GUGAGU UACUAAC CAG	1	RPL20A (477=431+46)								
GUUUGU CACUAAC UAG	1	YMR194C-B (72=50+22)								
GUAUAU AACUAAC AAG	1	SPO22 (90=63+27)								
GUAAGU UAUUAAC CAG	1	YFR045W (72=48+24)								
GUAUGU UGCUAAC CAG	1	RPL7A-i1 (459=428+31)								
GUAUGA UACUGAC UAG	1	SUS1-i1(80=52+28)								
GUAUGU UACUAAU CAG	1	SCS22 (88=73+15)								
GUAUGU CGCUAAC UAG	1	YBR255C-A (94=59+35)								
GUAUGU AAUUAAC UAG	1	CIN2 (80=45+35)								
GUCAGU UACUAAC CAG	1	RPL30 (230=190+40)								
GUGAGU UACUAAC UAG	1	SRC1-2 (126=103+23)								
GUAAGU AACUAAC UAG	1	SPO1 (84=46+38)								
GUACGU CACUAAC CAG	1	RPS9A (501=473+28)								
GUACGU AACUAAC UAG	1	MTR2 (99=86+13)								
GUAGUA GACUAAC UAG	1	MER3 (152=69+83)								
GCAUGU UACUAAC CAG	1	COX5B (88=57+31)								
GUUCGU UACUAAC UAG	1	REC107 (80=66+14)								
GUAUGU UACUGAC CAG	1	BET4 (87=50+37)								
GUUAAG UACUAAC CAG	1	HOP2 (70=44+26)								
GUAUGU UACUAAU UAG	1	HRB1 (342=324+18)								
GUUCGU UACUAAC CAG	1	YPL109C (152=113+39)								
GUAUGU UAUUAAC AAG	1	SAE3 (86=71+15)								
GUAUGU GAUUAAC UAG	1	HPC2 (84=66+18)								
GCAAGU UACUAAC UAG	1	SRC1-1 (130=107+23)								
GUACGU UACUAAC AAG	1	OST5 (149=103+46)								
GUAUGC GACUAAC UAG	1	YCL002C (76=45+31)								
GUAUGU UACUGAC AAG	1	YKR005C (69=52+17)								
GUAAGU UACUAAU CAG	1	YLR445W (82=62+20)								
GUAUGU GACUAAC AAG	1	YLR199C (86=60+26)								

Table 1-1: Occurrence of non-canonical introns in yeast.

Combinations of non-consensus splice signals in known yeast intron-containing genes are shown. The underlined sequences depict variation from the consensus at either the 5'ss, bp, or 3'ss. The occurrence of each combination is shown and the gene containing the respective splice sequences. The number in the parenthesis next to the gene name indicates total intron length, distance from the 5'ss to the bp and distance from the bp to the 3'ss (total length= 5'ss:bp + bp:3'ss). Consensus sequence is 5'ss GUAYGU; bp UACUAAC; and 3'ss YAG; Y=pyrimidine. i1-1st intron; i2-2nd intron.

To date, Mer1 is the only developmental program-specific splicing factor known in yeast. Mer1 is expressed specifically during meiosis (Engebrecht et al., 1991) at which time it activates the splicing of four Mer1-responsive introns (Davis et al., 2000; Engebrecht et al., 1991; Munding et al., 2010; Nakagawa and Ogawa, 1999). Strikingly, all but four introns (out of 16) in the 15 intron-containing genes whose function is restricted to meiosis, contain splice signals that diverge from the consensus (Table 1-2). This makes meiotic intron-containing genes likely candidates for regulated splicing. One of the four exceptions containing consensus signals is *SPO70/AMA1*, which depends on Mer1 for splicing activation due to a splicing silencer sequence found downstream of its 5'ss (Spingola and Ares, 2000). The removal of this silencer sequence alleviates the dependence of this intron on Mer1 for efficient splicing (Spingola and Ares, 2000). In addition to the four Mer1-regulated introns, three other meiotic introns (*MEI4*, *PCH2*, and *SAE3*) seem to be dependent on other non-essential factors for their splicing. *MEI4* and *PCH2* depend on the non-essential U1 snRNP protein Nam8 (Munding et al., 2010; Qiu et al., 2011a) and *PCH2* and *SAE3* depend on Tgs1 (Qiu et al., 2011b), an enzyme that functions in capping of snRNAs (Mouaikel et al., 2002). Cases of alternative site usage and intron-retention have also been reported in yeast, however the underlying regulatory mechanisms are uncertain. For example, *SRC1* pre-mRNA is alternatively spliced at two non-canonical 5'ss (Davis et al., 2000; Rodriguez-Navarro et al., 2002a) (G<u>C</u>A<u>A</u>GU or GU<u>GA</u>GU) and both 5'ss are used at similar ratios in wild type cells (Grund et al., 2008). Splicing of the upstream 5'ss depends on an interaction between Hub1 (a ubiquitin-like modifier; Dittmar et al., 2002) with the tri-snRNP component Snu66 (Mishra et al., 2011). Src1 protein promotes transcription-coupled mRNA export, and two protein isoforms, differing in their C-terminus, with distinct functional capacities result after splicing of *SRC1* (Grund et al., 2008). *PTC7* pre-mRNA can encode a functional protein whether its intron is spliced or retained. Splicing or retention of the *PTC7* intron seems to be a nutrient-dependent decision that results in different localization of the Ptc7 protein (which is a type 2C protein phosphatase (Jiang et al., 2002; Ramos et al., 2000)).

Consensus:	*GUAYGU*	*UACUAAC*	*YAG*
gene	**5'ss**	**bp**	**3'ss**
DMC1	GUAUGU	UACUAAC	UAG
HOP2	GUUAAG/GUAUGU	UACUAAC/UACUAAC	CAG/CAG
MEI4	GUACGU	UACUAAC	CAG
MER2/REC107	GUUCGU	UACUAAC	UAG
MER3/HFM1	GUAGUA	GACUAAC	UAG
MND1	GUAUGU	CACUAAC	UAG
OSW2	GUAUGG	UACUAAC	CAG
PCH2	GUAUGU	CACUAAC	UAG
REC102	GUAUGU	UACUAAC	AAG
REC114	GUAUGU	UACUAAC	AAG
SAE3	GUAUGU	UAUUAAC	AAG
SPO1	GUAAGU	AACUAAC	UAG
SPO22/ZIP4	GUAUAU	AACUAAC	AAG
SPO70/AMA1	GUACGU	UACUAAC	CAG
YLR445W/GMC2	GUAAGU	UACUAAU	CAG

Table 1-2: Meiotic intron generally have non-consensus splice signals.

Genes with essential functions for meiosis and their splice signals are shown. Underlined sequences deviate from the consensus at either the 5'ss, bp or 3'ss. HOP2 has two introns. Consensus is 5'ss GUAYGU, bp UACUAAC, 3'ss YAG; Y=pyrimidine.

The most complex and poorly understood example of alternative splicing in yeast is found in the 5'UTR of the *MTR2* gene that codes for an essential mRNA export regulator (Kadowaki et al., 1994). This 5'UTR contains two canonical 5'ss, of which only the ORF-proximal is conserved in other *Saccharomyces* species, and three 3'ss, that give rise to six possible spliced mRNA isoforms as well as a functional unspliced mRNA. Usage of the ORF-distal 5'ss would code for a different Mtr2 protein that contains additional amino acids on its N-terminus. Of the six possible spliced isoforms, evidence for use of five of these has been provided by ESTs (Davis et al., 2000). The introns of *MTR2* 5'UTR may be representative of the constantly evolving genome where splice sites are lost or made in order to change the coding capacity or expression of a protein.

Genome-wide analysis of splicing in yeast

Early studies of splicing in yeast were aimed to identify intron-containing genes. After sequencing of the yeast genome (Levy, 1994), introns were predicted using only a few parameters, namely introns must be less than 1kb, found near the 5' end of the gene and contain splice site that conform to the consensus (Kalogeropoulos, 1995). Soon after, it became evident that many yeast introns contain non-canonical splice signals and introns may be found throughout the gene (Davis et al., 2000; Spingola et al., 1999). As a strategy to detect new introns, RNA species that accumulate in cells lacking the enzyme Dbr1, responsible for turnover of the excised lariat intron (Chapman and Boeke, 1991), were assayed relative to wild type (Spingola et al., 1999; Zhang et al., 2007). The *dbr1*Δ cells accumulate excised introns, which mark both transcription and splicing of a gene. The combined efforts of bioinformatic and experimental approaches have identified most spliced introns in the yeast genome; however new splice events are still being uncovered (Miura et al., 2006; Yassour et al., 2009) and more are likely to exist.

After the identification and validation of yeast intron-containing genes, splicing studies in yeast have employed splicing-sensitive microarrays (first described by Clark et al.,

2002) and RNA-Seq (first described by Nagalakshmi et al., 2008) approaches to capture global changes in splicing under various conditions. For example, splicing-sensitive microarray studies showed that perturbation in core components of the spliceosome have distinct effects on splicing of intron-containing genes (Burckin et al., 2005; Clark et al., 2002; Pleiss et al., 2007). Furthermore, splicing of some transcripts is more affected than others by the same mutation. Despite the variety of conditions and mutations tested, few studies have tried to make sense of the splicing networks encompassing the affected genes or the contribution towards the gene expression program.

Conclusion and aims

Pre-mRNA splicing serves a key point in regulation of gene expression by determining and fine-tuning both the content and level of expression of the mRNA transcript. Regulated splicing may be specific to a condition, tissue, or developmental stage and is typically attributed to changes in expression or function of a specific splicing factor. Many studies focus on defining splicing regulatory networks (also called splicing regulons) to identify the direct pre-mRNA targets that are co-regulated by the splicing factor. These studies provide a snapshot of splicing regulation at a particular cell state (reviewed in Kalsotra and Cooper, 2011).

Gene expression programs drive transitions from one cell state to another and must be regulated to ensure the coordinate expression of certain genes (reviewed in Ben-Tabou de-Leon and Davidson, 2007). Such regulation is achieved at multiple layers of the gene expression pathway from regulation of transcriptional networks to splicing networks to other post-transcriptional networks (such as those of microRNAs) (reviewed in Kalsotra and Cooper, 2011). A challenge in the field remains in understanding how networks are integrated with one another to collaboratively contribute to transitioning from one gene expression state to the next and defining cell identity.

The main objective of this thesis is to understand how splicing is integrated within gene expression programs. Particularly, how pre-mRNA splicing regulation is interlaced with the meiotic gene expression program in budding yeast. While the studies described in this thesis have been performed in yeast, the findings and principles presented should apply to any developmental program where transcription and splicing are changing.

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CHAPTER 2

INTEGRATION OF A SPLICING REGULATORY NETWORK WITHIN THE MEIOTIC GENE EXPRESSION PROGRAM OF *SACCHAROMYCES CEREVISIAE*

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Integration of a splicing regulatory network within the meiotic gene expression program of *Saccharomyces cerevisiae*

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Splicing regulatory networks are essential components of eukaryotic gene expression programs, yet little is known about how they are integrated with transcriptional regulatory networks into coherent gene expression programs. Here we define the *MER1* splicing regulatory network and examine its role in the gene expression program during meiosis in budding yeast. Mer1p splicing factor promotes splicing of just four pre-mRNAs. All four Mer1p-responsive genes also require Nam8p for splicing activation by Mer1p; however, other genes require Nam8p but not Mer1p, exposing an overlapping meiotic splicing network controlled by Nam8p. *MER1* mRNA and three of the four Mer1p substrate pre-mRNAs are induced by the transcriptional regulator Ume6p. This unusual arrangement delays expression of Mer1p-responsive genes relative to other genes under Ume6p control. Products of Mer1p-responsive genes are required for initiating and completing recombination and for activation of Ndt80p, the activator of the transcriptional network required for subsequent steps in the program. Thus, the *MER1* splicing regulatory network mediates the dependent relationship between the *UME6* and *NDT80* transcriptional regulatory networks in the meiotic gene expression program. This study reveals how splicing regulatory networks can be interlaced with transcriptional regulatory networks in eukaryotic gene expression programs.

[*Keywords*: Regulated splicing; regulons; splicing-sensitive microarray; epistasis]

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Cell identities and functional states arise from distinctive sets of expressed genes. Transitions from one state to another are achieved through activation of gene expression programs that lead to stable changes in the set of expressed genes. Programs are composed of regulatory networks, or regulons (Ben-Tabou de-Leon and Davidson 2007), that ensure coordinated expression of required groups of genes. Defining gene regulatory networks and obtaining insight into their relationships with each other is essential for understanding any developmental program.

Much work in this area has focused on transcription factors and the signaling pathways that activate them to promote coordinate transcription of groups of genes in a defined transcriptional regulon. Splicing regulatory networks may function in a parallel manner whereby splicing factors activate the coordinate splicing of specific transcripts, leading to changes in protein function important to progression of the gene expression program. A widely known cascade of splicing regulation occurs during sex determination in *Drosophila*, where Sex lethal (Sxl) promotes the productive splicing of *transformer* (*tra*) pre-mRNA. Tra protein (with Tra-2) then controls whether the male (no Tra) or the female (with Tra) form of the *doublesex* transcription factor is produced (Baker 1989; Lopez 1998; Black 2003). With the exception of this one example, little is known about how splicing and transcriptional regulators might control each other in complex programs of eukaryotic gene expression.

Meiosis in the budding yeast *Saccharomyces cerevisiae* is accompanied by a well-studied developmental gene expression program associated with transcriptional regulons (Chu et al. 1998; Primig et al. 2000). The program includes a transcriptional cascade that can be separated into at least three components: early meiotic genes regulated by Ume6p/Ime1p (Strich et al. 1994; Williams et al. 2002), middle meiotic genes activated by Ndt80p (Xu et al. 1995; Chu and Herskowitz 1998; Hepworth et al. 1998), and late meiotic genes (Mitchell 1994; Kassir et al.

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2003). As meiotic events such as chromosome synapsis and recombination take place, checkpoints mediated by phosphorylation of regulatory kinases ensure event completion and allow progression through meiosis (Hochwagen and Amon 2006). In the absence of progress, checkpoint activation causes a delay in the transcriptional program to coordinate meiotic cellular events with gene expression.

In addition to transcription, splicing is regulated during meiosis in yeast. Best understood is the activation of a small set of introns by the KH domain RNA-binding protein Mer1p (Nandabalan and Roeder 1995; Spingola and Ares 2000). *MER1* was first identified genetically by its contribution to spore viability, meiotic recombination, and synaptonemal complex (SC) formation (Engebrecht and Roeder 1989, 1990; Engebrecht et al. 1990), but turned out to be a splicing factor (Engebrecht et al. 1991). Its expression is induced during meiosis (Engebrecht and Roeder 1990) to activate the splicing of *MER2/REC107* (Engebrecht et al. 1991), *MER3/HFM1* (Nakagawa and Ogawa 1999), and *SPO70/AMA1* (Cooper et al. 2000; Davis et al. 2000) through an interaction with a conserved intronic enhancer sequence (5′-AYACCCYU-3′) (Spingola and Ares 2000). *NAM8/MRE2*, a component of the U1 snRNP, contributes to 5′ splice site recognition (Gottschalk et al. 1998; Puig et al. 1999) and is required for meiosis (Nakagawa and Ogawa 1997), in part through its role in splicing activation of Mer1p-responsive transcripts (Spingola and Ares 2000). Consistent with this, Mer1p also binds to the U1 snRNP (Spingola and Ares 2000) and its interactions with other spliceosome components have been enumerated (Spingola and Ares 2000; Spingola et al. 2004; Balzer and Henry 2008), but its mechanism of action remains unclear.

Despite increasing ability to define splicing regulatory networks (Ule et al. 2003; Zhang et al. 2008; Du et al. 2010), little is known about how transcriptional regulation is coordinated with splicing regulation and other cellular events in eukaryotic gene expression programs. In this study, we address two intimately connected problems. First, we want to understand how the Mer1p splicing regulatory network is connected to the transcriptional regulatory networks that operate in meiosis. Second, we want to understand the specific contributions of the genes in the Mer1p splicing network to the progress of meiosis and the meiotic gene expression program. Using splicing-sensitive microarrays, we compared splicing and mRNA levels in wild-type and *mer1Δ* cells after initiation of the meiotic program. In addition to observing inhibited splicing of the three known Mer1p-activated introns, we identified only one additional gene (*SPO22/ZIP4*) whose splicing is inhibited in *mer1Δ* cells. Surprisingly, both *MER1* and three of its four targets are under the control of Ume6p, the activator of the early meiotic genes (Strich et al. 1994; Steber and Esposito 1995; Williams et al. 2002). Proper function of Mer1p is necessary (through its contributions to the expression of Mer1p-responsive genes) for full activation of Ndt80p, the activator of the middle meiotic genes (Hepworth et al. 1998; Tung et al. 2000), suggesting a model in which the *MER1* splicing regulon bridges two major transcriptional regulons during meiosis.

Results

Deletion of MER1 *inhibits splicing of four introns in the yeast genome*

Mer1p is required for splicing of three pre-mRNAs (Engebrecht et al. 1991; Nakagawa and Ogawa 1999; Davis et al. 2000), but it is unclear how many more Mer1p-responsive introns might be lurking in the yeast genome. To observe the contribution of *MER1* to the meiotic gene expression program, we compared the global changes in mRNA levels and splicing during meiosis in synchronized wild-type yeast (SK1) to those of isogenic *mer1Δ* yeast using whole-genome splicing-sensitive microarrays (Fig. 1). As judged by their intron accumulation indexes (IAI) (see the Materials and Methods), only four meiotic genes (Fig. 1A, asterisks) show reduced splicing efficiency in *mer1Δ* as compared with wild-type cells. This is confirmed by RT–PCR (Fig. 1B) using RNA from the 5-h meiotic time point. The splicing efficiency of *MER2/REC107*, *MER3/HFM1*, *SPO22/ZIP4*, and *SPO70/AMA1* is substantially reduced in the absence of *MER1*. *MER2*, *MER3*, and *SPO70* pre-mRNAs are known to require Mer1p (Engebrecht et al. 1991; Nakagawa and Ogawa 1999; Davis et al. 2000), and here we show that *SPO22/ZIP4* pre-mRNA splicing efficiency also depends on Mer1p (Fig. 1C). The *SPO22* intron sequence contains a Mer1p intronic enhancer sequence (5′-AUACCCUU-3′) that closely matches the consensus 5′-AYACCCUY-3′ (Spingola and Ares 2000) 21 nucleotides downstream from the noncanonical 5′ splice site (GUAUAU instead of the canonical GUAUGU). We also tested several meiotically expressed intron-containing genes that appeared to have reasonable matches to the Mer1p enhancer near their 5′ splice sites using RT–PCR and found that none appeared to depend on Mer1p (Supplemental Table S1). Because we cannot strictly exclude the possibility that another Mer1p-responsive intron remains undetected in the genome, we tentatively conclude that the four known Mer1p-responsive genes identified thus far constitute the complete *MER1* splicing regulatory network.

Late meiotic gene expression is delayed by deletion of MER1

Mer1p is a splicing factor; thus, the direct effect of loss of *MER1* is the inhibition of efficient splicing of Mer1p enhancer containing pre-mRNAs. Although Mer1p could have yet-unknown functions, most downstream (indirect) effects of loss of *MER1* would presumably be due to compromised expression of the four Mer1p-responsive transcripts. To determine the indirect effects that loss of *MER1* has on the meiotic gene expression program, we compared total gene expression profiles of wild-type SK1 cells and isogenic *mer1*Δ cells during meiosis. The major differences in mRNA expression profiles affect the genes in two classes: the ribosomal protein (RP) transcripts (Fig. 1D) and the meiotic transcripts (Fig. 1E). Both the transcriptional repression of RP transcripts (Fig. 1D) and the transcriptional induction of early meiotic genes (Fig. 1E) remain unperturbed in cells lacking *MER1* compared with wild type. However, a block to progression through meiosis

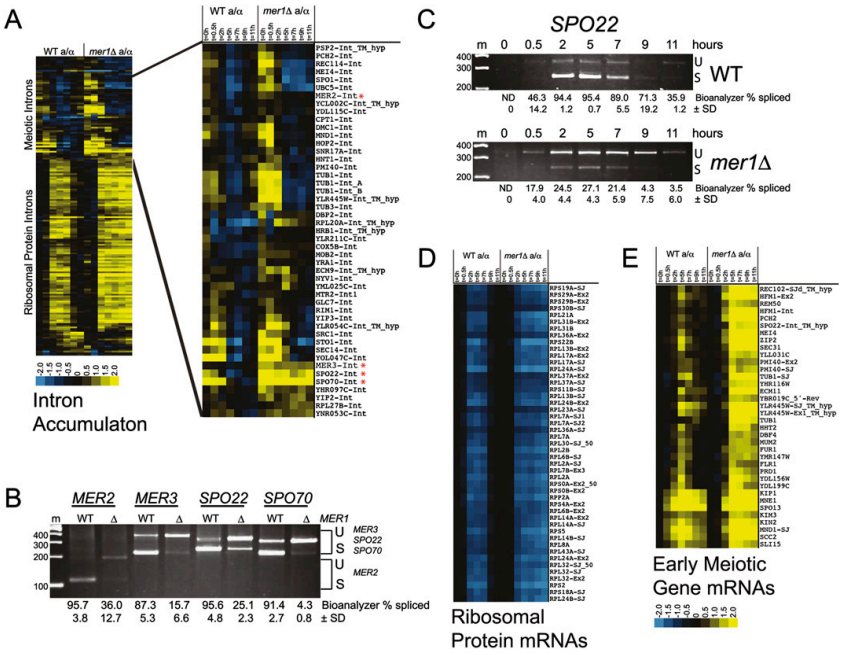

Figure 1. Meiotic gene expression in the absence of the Mer1p splicing factor. (*A*) Splicing changes as represented by intron accumulation indexes (Clark et al. 2002) during the time course of wild-type (*left* panel) and *mer1*Δ (*right* panel) meiosis. The asterisk (*) indicates introns whose splicing efficiency during meiosis is reduced in *mer1*Δ cells compared with wild type. Yellow represents an increase in the intron accumulation index, and thus a decrease in splicing efficiency. Blue represents an increase in splicing efficiency. (*B*) RT–PCR validation of *MER2*, *MER3*, *SPO22*, and *SPO70* splicing efficiencies in wild-type (WT) and *mer1*Δ yeast 5 h after induction of meiosis. (*C*) Expression and splicing of *SPO22* mRNA during meiosis in wild-type (WT) and *mer1*Δ strains. (*D*) RP gene expression during meiosis in wild-type (*left* panel) and *mer1*Δ (*right* panel) cells. Blue represents decrease in expression. (*E*) Expression of early meiotic genes in wild-type (*left* panel) and *mer1Δ* (*right*) cells. Yellow represents increase in expression. For *B* and *C*, "U" indicates unspliced pre-mRNA and "S" indicates spliced mRNA. Marker sizes are in base pairs. Splicing efficiency was calculated as described in the Materials and Methods.

in *mer1Δ* cells is evident by 9 h based on RP and meiotic gene expression profiles. RP gene expression fails to be activated by 9 h in *mer1Δ* cells (Fig. 1D, right panel). In addition, meiosis-specific transcripts remain high in late meiosis in the *mer1Δ* cells relative to wild type (Fig. 1E, right panel). We conclude that deletion of *MER1* affects the meiotic gene expression program by causing a delay in the reduction of meiotic transcript levels as well as a failure to activate RP expression in late meiosis. Because Mer1p is a splicing factor that promotes splicing of just four genes (Fig. 1A,B), we infer that the global delay in the gene expression program arises as an indirect consequence of failure to express adequately one or more of the Mer1p-responsive genes.

MER1 *and three of four Mer1p-responsive genes are activated by Ume6p*

Nutrient signals trigger the expression of early meiotic genes that convert the Ume6p transcription factor from its repressor form in vegetative cells to an activator of early meiotic genes (Mitchell 1994). The expression of *MER1* and its responsive genes (Fig. 1A) increases during the early wave of transcription. To determine whether Ume6p activates *MER1* and its responsive genes, we searched their promoters for the Ume6p-binding site (URS1) (Buckingham et al. 1990; Strich et al. 1994; Steber and Esposito 1995). We found the URS1 in the promoters of *MER1*, *MER3*, *SPO22*, and *SPO70*, but not *MER2* (Supplemental Fig. S1; Harbison et al. 2004). Consistent with this, *MER2* is not repressed during vegetative growth; its pre-mRNA is spliced only during meiosis when Mer1p is present (Engebrecht et al. 1991). Deletion of *UME6* in vegetative cells leads to derepression of meiotic genes during vegetative growth (Strich et al. 1994). We exploited this fact to test the role of Ume6p in expression of the *MER1* regulon as well as other meiotic intron-containing genes (Fig. 2; Table 1). A splicing-sensitive microarray experiment comparing vegetatively growing *ume6Δ* cells to wild type confirms derepression of *SPO22* (Williams et al. 2002) and also reveals new Ume6p-activated genes, *MER1* and *SPO70* (Table 1, shaded). To validate the array results, and to test *MER3* (for which array signals were not robust), we performed RT–PCR using RNA from *ume6Δ* and wild-type strains (Fig. 2A). *MER3*,

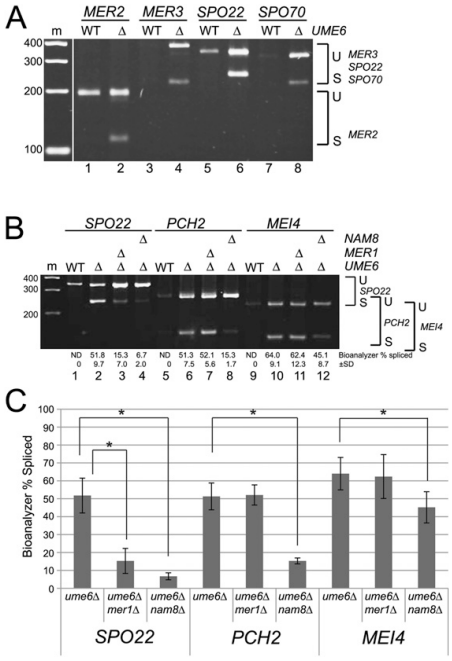

Figure 2. Derepression of meiotic genes in vegetative cells reveals splicing factor requirement for meiosis. (*A*) Expression and splicing of *MER2*, *MER3*, *SPO22*, and *SPO70* in wild-type (WT) and *ume6*Δ vegetative cells. (*B*) Expression and splicing of *SPO22*, *PCH2*, and *MEI4* in wild-type (WT), *ume6*Δ, *mer1*Δ*ume6*Δ, and *nam8*Δ*ume6*Δ vegetative cells. (*C*) Splicing factor dependence for efficient splicing of *SPO22*, *PCH2*, and *MEI4*. The asterisk (*) indicates a statistically significant difference in splicing efficiency ($\alpha = 0.05$) using a *t*-test (see the Materials and Methods). (U) Unspliced pre-mRNA; (S) spliced mRNA. Marker sizes are in base pairs. Splicing efficiency was calculated as described in the Materials and Methods.

SPO22, and *SPO70* transcription is derepressed in *ume6*Δ vegetative cells. Spliced transcripts from these genes as well as *MER2* are greatly increased in *ume6*Δ vegetative cells (Fig. 2A, lanes 2,4,6,8), indicating expression of *MER1*. Thus, we conclude that transcription of *MER1* and three of the four Mer1p-responsive genes is repressed by Ume6p in vegetative cells and is activated by Ume6p during meiosis. This means that the *MER1* splicing regulatory network is largely under the control of the Ume6p transcription factor.

An overlapping meiotic splicing regulon is controlled by Nam8p

The three previously identified Mer1p-responsive pre-mRNAs require both Mer1p and the U1snRNP protein Nam8p for splicing activation (Spingola and Ares 2000). To test whether splicing activation of *SPO22* also requires *NAM8*, we used vegetative *ume6*Δ cells containing or lacking either *MER1* or *NAM8*, and measured *SPO22* splicing efficiency (Fig. 2B, lanes 1–4). Splicing efficiency of *SPO22* is significantly reduced (Fig. 2C) in both *mer1*Δ*ume6*Δ and *nam8*Δ*ume6*Δ cells, indicating that, like the other Mer1p-responsive genes, *SPO22* splicing activation requires both *NAM8* and *MER1*.

We also tested other Ume6p-activated meiotic intron-containing genes using the *mer1*Δ*ume6*Δ and *nam8*Δ*ume6*Δ strains. We found that splicing efficiency of *PCH2* (Fig. 2B [lanes 5–8], C) is strongly dependent on *NAM8*, as is *MEI4*, albeit to a lesser but still statistically significant degree (Fig. 2B [lanes 9–12], C). Neither intron is affected by loss of *MER1*, because they lack the Mer1p enhancer. Transcriptional control of *NAM8* is distinct from that of *MER1*, since *NAM8* is expressed in both vegetative and meiotic cells and is not under Ume6p control (Ekwall et al. 1992). We conclude that a second meiotic splicing regulatory network is controlled by *NAM8*, and that this network overlaps with the Mer1p network but includes splicing events that do not require Mer1p.

A previous report described 13 meiosis-specific intron-containing genes based on tiling arrays (Juneau et al. 2007). We found additional genes whose expression is up-regulated during meiosis, and determined which of these are under Ume6p repression in vegetative cells (Table 1). The array experiment confirmed seven out of eight previously identified Ume6p-activated genes (Williams et al. 2002), including *SPO22*, and identified three new meiotic intron-containing genes regulated by Ume6p (*MND1*, *REC102*, and *SAE3*) (Table 1). Together with this new recognition that *MER3* and *SPO70* are under Ume6p control, we counted a total of 13 of 20 meiosis-induced intron-containing genes regulated by Ume6p.

Expression of the Mer1p-responsive genes is delayed relative to other Ume6p-activated genes

Induction of expression of Mer1p by Ume6p simultaneously with its responsive pre-mRNAs seems unusual, since the time needed for Mer1p translation would produce a delay in splicing and expression of the responsive genes. If true, for a period of time after Ume6p induction, Mer1p-responsive pre-mRNAs should accumulate while Mer1p protein is being produced. We measured Mer1p induction early in meiosis, and were first able to detect Mer1p 1 h after transfer to sporulation medium, increasing up to 2 h after induction of meiosis (Fig. 3A). Efficient Mer1p-dependent splicing was observed 2 h after transfer to sporulation medium, while unspliced transcripts were detected within 30 min (Fig. 3B). Furthermore, *MER2*, which is not under Ume6p control and is transcribed during both vegetative growth and meiosis (Engebrecht et al. 1991), exhibited a similar delay in splicing efficiency. A splicing delay was not evident for other intron-containing transcripts not under Mer1p control, such as *MEI4* (Fig. 3C). To examine this more closely, we performed RT–qPCR on RNA isolated at 30-min intervals after transfer to sporulation medium (Fig. 3D). Each of the Mer1p-responsive genes displayed a higher percentage of intron-containing transcript 30 min after onset of meiosis as compared with 1 h, when Mer1p first became evident. This experiment

Table 1. *The majority of meiotic intron-containing genes are transcriptionally activated by Ume6p*

Gene	Meiotic		ume6Δ vegetative		Reference
	Induction log ratio	Peak induction	Induction log ratio	Ume6 induced?	
AMA1/SPO70	5.57	9 h	1.34	+	Present study
MND1	4.30	5 h	1.45	+	Present study
SAE3	4.27	5 h	1.99	+	Present study
SPO22/ZIP4	4.12	5 h	2.88	+	Present study; Williams et al. 2002
DMC1	4.10	5 h	2.53	+	Present study; Williams et al. 2002
SRC1/HEH1	2.91	7 h	−0.21	–	
HOP2	2.75	5 h	2.95	+	Present study; Williams et al. 2002
URA2	2.73	30 min	0.20	–	
REC114	2.52	5 h	0.48	+	Williams et al. 2002
SPO1	2.20	5 h	1.12	+	Present study; Williams et al. 2002
MER1	2.12	5 h	0.60	+	Present study
PCH2	2.07	5 h	1.83	+	Present study; Williams et al. 2002
OSW2	2.07	7 h	0.34	–	
ECM9	1.89	7 h	−0.16	–	
REC102	1.78	5 h	1.50	+	Present study
MEI4	1.76	5 h	1.31	+	Present study; Williams et al. 2002
YLR445W	1.57	5 h	0.81	+	Present study; Williams et al. 2002
HFM1/MER3	1.53	5 h	0.21[a]	+	Present study
REC107/MER2	1.52	5 h	0.10	–	
PSP2/MRS15	1.14	7 h	−0.33	–	
PCC1	0.91	30 min	0.20	–	

[a]*MER3* does not meet the log ratio cutoff, but was validated as transcriptionally regulated by Ume6p (see Fig. 2A).
Twenty intron-containing genes become transcriptionally induced during meiosis; of these, 13 become induced in *ume6*Δ vegetative cells, as determined by either log ratio > 0.60 (or 1.5-fold increase in expression in *ume6*Δ compared with wild-type vegetative cells) or Williams et al. (2002). Shaded genes are part of the *MER1* regulon. *MER1* contains no intron.

revealed a splicing-dependent timing mechanism that separates expression of genes induced by a common transcription factor into two temporal components: those immediately expressed, and those delayed by the time necessary to translate sufficient splicing factor. This suggests that one contribution of the *MER1* splicing regulatory network to the gene expression program might be to promote appropriate timing of expression of a subset of meiotic genes.

Deletion of MER3 *and* SPO22 *delays* NDT80 *transcriptional induction*

After expression of Ume6p-induced genes, transcription of a second meiotic wave was triggered (Chu et al. 1998; Primig et al. 2000). This wave is regulated by the transcription factor Ndt80p and allows expression of middle meiotic genes, leading to exit from pachytene and entry into Meiosis I (Xu et al. 1995; Chu and Herskowitz 1998). Because the *MER1* splicing regulon is expressed as a consequence of the *UME6* transcriptional regulon, we wanted to ask how expression of the *MER1* regulon contributes (directly or indirectly) to the succeeding cellular events and the progress of the gene expression program. Functions of all four Mer1p-responsive genes have been studied, but their contributions to the meiotic gene expression program are unknown. Three of the four Mer1p-responsive gene products function during meiotic prophase. Mer2p/Rec107p is required for formation of double-stranded breaks (DSBs) to initiate recombination (Keeney 2001; Li et al. 2006); loss of *MER2* allows a rapid aberrant meiosis that bypasses the recombination pathway (Malone et al. 2004). Mer3p/Hfm1p is a recombination-specific DNA helicase (Nakagawa and Ogawa 1999; Nakagawa and Kolodner 2002; Mazina et al. 2004); in the absence of *MER3*, cells arrest in prophase due to the inability to resolve DSB intermediates (Nakagawa and Ogawa 1999). Spo22p/Zip4p promotes SC formation (Tsubouchi et al. 2006; Lynn et al. 2007); strains mutant for *SPO22* exhibit delayed progression through meiosis due to the defect in SC formation (Tsubouchi et al. 2006). The fourth gene product, Spo70p/Ama1p, is a meiosis-specific anaphase-promoting complex (APC) regulatory subunit that functions during chromosome segregation and spore formation (Oelschlaegel et al. 2005; Penkner et al. 2005; Diamond et al. 2009); *spo70*Δ cells arrest with segregated chromosomes but without spore formation (Rabitsch et al. 2001). Although Spo70p is absolutely required for spore formation (Rabitsch et al. 2001; Coluccio et al. 2004), its function in chromosome segregation is redundant with other APC regulatory subunits (Oelschlaegel et al. 2005; Penkner et al. 2005).

The phenotype of the *mer1*Δ strain is complex because loss of *MER1* results in the simultaneous reduction of expression of *MER2*, *MER3*, *SPO22*, and *SPO70*, each of which has distinct meiotic functions. To address this, we obtained deletions of each gene and separately assessed the contribution of each to the meiotic gene expression program by measuring mRNA levels of each mutant relative to wild type at 9 h after transfer to sporulation medium. Prophase-specific genes (Fig. 4A) have increased expression relative to wild type in each of the mutants, especially *mer3*Δ and *spo22*Δ, indicating blocked or delayed reduction of the Ume6p-activated transcripts in these strains.

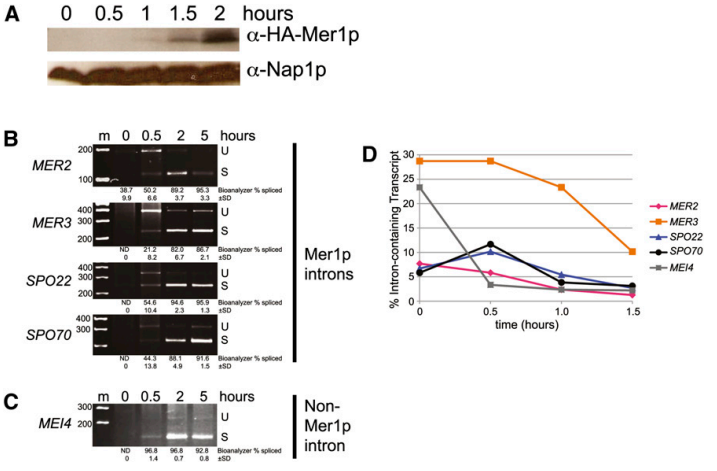

Figure 3. Accumulation of mRNA for Mer1p-responsive genes is delayed relative to other Ume6p-activated genes. (*A*) Western blot measuring Mer1p expression in wild-type cells early in meiosis. Nap1p was used as a loading control. (*B*) Measurement of expression and splicing of the Mer1p-responsive *MER2*, *MER3*, *SPO22*, and *SPO70* genes in wild-type cells at the indicated times after induction of meiosis. (*C*) Measurement of expression and splicing of the Mer1p-independent gene *MEI4* in wild-type cells at the indicated times after induction of meiosis. (*D*) Measurement of percent of intron-containing transcript (calculated as described in the Materials and Methods) as determined by RT–qPCR of *MER2*, *MER3*, *SPO22*, *SPO70*, and *MEI4* in wild-type cells at the indicated times after induction of meiosis. (U) Unspliced pre-mRNA; (S) spliced mRNA. Marker sizes are in base pairs. Splicing efficiency was calculated as described in the Materials and Methods.

Furthermore, expression of *NDT80*, the transcriptional activator of the middle genes (Fig. 4B), as well as important middle genes such as the B-type cyclins (*CLB1*, *CLB3*, *CLB4*, and *CLB5*) (Chu and Herskowitz 1998) and polo-like kinase *CDC5* (Clyne et al. 2003), is decreased in *mer3Δ and spo22Δ* cells (Fig. 4B). Other genes that function following the *NDT80* transcriptional wave (Chu et al. 1998), such as those required for active APC (Fig. 4C) or those involved in spore morphogenesis (Fig. 4D), display lower levels of gene expression compared with wild type in *mer3Δ* and *spo22Δ* strains. Consistent with Mer3p and Spo22p function in prophase, strains lacking these proteins do not enter the meiotic divisions and arrest before chromosome segregation at the pachytene checkpoint (Fig. 4E,F; for review, see Hochwagen and Amon 2006). Expression of genes required for completion of spore formation, such as *DIT1* and *DIT2* (Briza et al. 1994; Coluccio et al. 2004), is strongly reduced in the *spo70Δ* strain (Fig. 4D; see also Coluccio et al. 2004), indicating a delay or block in late gene expression. This block must occur after segregation but before spore formation, since *spo70Δ* cells arrest in meiosis with segregated chromosomes but no spores (Fig. 4E,F; see also Rabitsch et al. 2001; Coluccio et al. 2004).

Deletion of *MER2* does not block meiotic progression, but an aberrant meiosis takes place in which no DSBs form and aneuploid spores are produced at high frequency (Engebrecht et al. 1990; Cool and Malone 1992; Malone et al. 2004). We counted cell phenotypes in the *mer1Δ* strain at 9 h and found the majority (70.3%, 147 of 209) of *mer1Δ* cells resemble the *mer2Δ* phenotype and complete the meiotic gene expression program. A detectable fraction of *mer1Δ* cells arrests at positions similar to the arrest points of *mer3Δ* and *spo22Δ* (prophase) (11.0%, 23 of 209) or *spo70Δ* (segregated chromosomes but no spores) (18.7%, 39 of 209), suggesting that the phenotype of individual *mer1Δ* tetrads is influenced by stochastic events, such as whether a threshold level of Mer2p is produced through leaky splicing (Fig. 1B). Decreased expression of *NDT80* and Ndt80p-regulated genes in *mer3Δ* and *spo22Δ* cells shows that the gene expression program is halted in the absence of sufficient Mer3p or Spo22p. We conclude that the *MER1* splicing regulon is interposed between the *UME6* and *NDT80* transcriptional regulons.

Loss of Mer1p generates heterotypic effects on meiotic progression that are resolved by epistasis

Loss of Mer1p splicing factor leads to reduced expression of genes whose loss produces heterotypic block points in meiosis (Fig. 4). For example, reduced levels of either Mer2p or Spo70p would not be expected to trigger the pachytene checkpoint, whereas reduced levels of Mer3p or Spo22p would. To confirm this and evaluate checkpoint activation in the *mer1Δ* strain, we assayed the activation state of CDK (Cdc28p) by detecting inhibitory phosphorylation at Y19 (Leu and Roeder 1999) using a phospho-specific antibody. We observe strong, persistent Cdc28p phosphorylation at Y19 late in meiosis in *mer3Δ* and *spo22Δ* strains, and, to a lesser extent, in *mer1Δ* (Fig. 5A, lanes 4,8,10). In wild-type, *mer2Δ*, and *spo70Δ* strains, CDK is mostly unphosphorylated by 9 h into meiosis, indicating that these cells progress past pachytene (Fig. 5A, lanes 2,6,12). Presumably, the partial activation of the pachytene checkpoint in the *mer1Δ* strain is due to residual splicing of Mer1p-responsive transcripts in the absence of Mer1p (Fig. 1B; Engebrecht et al. 1991; Davis et al. 2000; Spingola and Ares 2000), eventually allowing slow progression past the checkpoint and explaining the delayed gene expression program. This partial activation of the checkpoint likely occurs in subpopulations of *mer1Δ* cells that lack adequate Mer3p or Spo22p but produce sufficient Mer2p to initiate DSBs. Other subpopulations that produce inadequate Mer2p would immediately bypass the checkpoint because DSBs would not form in those cells.

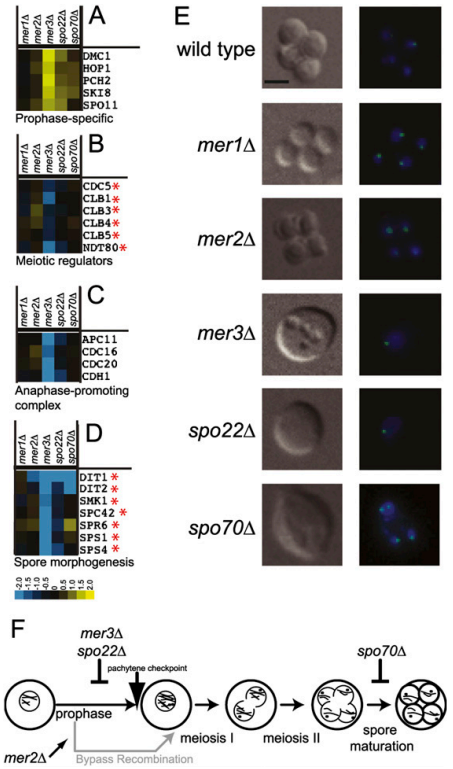

Figure 4. *MER1* regulon expression is required for induction of *NDT80* and Ndt80p-regulated genes. (*A*) Expression of genes whose products function during prophase in *mer1Δ*, *mer2Δ*, *mer3Δ*, *spo22Δ*, and *spo70Δ* compared with wild type 9 h after induction of meiosis. (*B*) Same as *A* for genes whose products are regulators of the meiotic divisions. (*C*) Same as *A* for genes whose products function in the APC. (*D*) Same as *A* for genes whose products function in spore morphogenesis. Yellow represents increase in expression, while blue represents decrease in expression relative to wild type. The asterisk (*) indicates genes shown by Chu and Herskowitz (1998) or Clyne et al. (2003) to be regulated by Ndt80p. (*E*) Sample pictures of major phenotypes of wild-type, *mer1Δ*, *mer2Δ*, *mer3Δ*, *spo22Δ*, and *spo70Δ* cells 9 h after induction of meiosis. On the *left* are differential interference contrast micrographs, and on the *right* are superimposed fluorescence micrographs of DNA stained with DAPI (blue) and CenV-GFP (green). Bar, 2 μm. (*F*) Diagram of meiotic events and execution points of Mer1p-responsive gene deletions.

To test this idea, we constructed double deletions within the *MER1* regulon to assess epistasis (Fig. 5B). Double mutants of *mer3Δ* or *spo22Δ* with *mer1Δ* showed reduced activation of the checkpoint (Fig. 5B, lanes 2,6), the same as the *mer1Δ* single mutant (Fig. 5A, lane 4), rather than the strong checkpoint activation observed in the *mer3Δ* or *spo22Δ* single mutants. Double mutants of *mer3Δ* or *spo22Δ* with *mer2Δ* showed little or no detectable pachytene checkpoint activation (Fig. 5B, lanes 4,8), the same as the *mer2Δ* single mutant (Fig. 5A, lane 6). The meiotic arrest points of the *mer1Δ* strain resemble the *mer2Δ* mutant (Fig. 4E). Likewise, the arrest points of the *mer3Δmer1Δ* and *mer3Δmer2Δ* resemble the *mer2Δ* single mutant, rather than *mer3Δ* (Fig. 5C). These results indicate that both *mer1Δ* and *mer2Δ* are epistatic to *mer3Δ* and *spo22Δ* with respect to pachytene checkpoint activation. Thus, loss of Mer1p leads primarily to meiotic events that arise as a consequence of limited expression of Mer2p. Furthermore, this experiment shows that the successful expression of Mer3p and Spo22p is monitored by the pachytene checkpoint, ensuring that the activity of the *MER1* regulon leads to *NDT80* expression.

Discussion

In this study, we define the *MER1* splicing regulatory network as consisting of Mer1p splicing factor and Mer1p-responsive pre-mRNA transcripts from four genes: *MER2/REC107*, *MER3/HFM1*, *SPO22/ZIP4*, and *SPO70/AMA1* (Fig. 1). Deletion of *MER1* reduces splicing efficiency of these four pre-mRNAs and causes a cascade of defects in the transcriptional program, including prolonged high levels of Ume6p-activated gene transcripts and a delay in induction of middle and late gene transcripts. Surprisingly, *MER1* and all but one of its responsive genes are under the control of the Ume6p transcription factor (Fig. 2). This arrangement divides Ume6p-controlled genes into two waves, one of which (including the Mer1p-responsive genes) is delayed in mRNA expression by the amount of time necessary to accumulate Mer1p after Ume6p-mediated activation (Fig. 3). Function of the *MER1* splicing regulon is necessary, in turn, for the expression of the

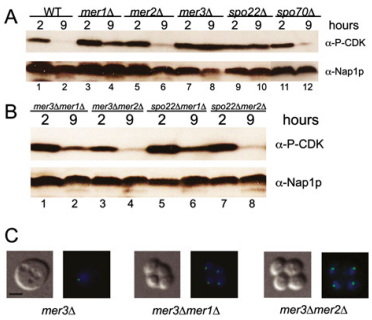

Figure 5. Pachytene checkpoint activation persists in *mer3Δ* and *spo22Δ*, and, to a lesser degree, in the *mer1Δ* strain. (*A*) Western blot measuring phosphorylation state of CDK on Y19 in wild-type (WT), *mer1Δ*, *mer2Δ*, *mer3Δ*, *spo22Δ*, and *spo70Δ* strains 2 h and 9 h after induction of meiosis. (*B*) Same as *A* using *mer3Δmer1Δ*, *mer3Δmer2Δ*, *spo22Δmer1Δ*, and *spo22Δmer2Δ* strains. Nap1p was used as a loading control in *A* and *B*. (*C*) Sample pictures of major phenotypes of *mer3Δ*, *mer3Δmer1Δ*, and *mer3Δmer2Δ* strains at 9 h after induction of meiosis. On the *left* are differential interference contrast micrographs, and on the *right* are superimposed fluorescence micrographs of DNA stained with DAPI (blue) and CenV-GFP (green). Bar, 2 μm.

NDT80 transcriptional regulon (Fig. 4). Specifically, compromising expression of either of two Mer1p-responsive genes, *MER3* and *SPO22*, blocks *NDT80* expression (Fig. 4) and triggers the activation of the pachytene checkpoint (Fig. 5), resulting in prophase arrest. Although loss of *MER1* reduces expression of all four genes, it appears that the consequent loss of Mer2p in the *mer1Δ* mutant accounts for much of the phenotype (Fig. 4). We show that the *MER1* splicing regulon is primarily under the control of one transcription factor (Ume6p) and is required for the activation of another (Ndt80p), and thus bridges two key transcriptional regulons during the meiotic gene expression program (Fig. 6).

How does splicing regulation contribute to meiotic gene expression?

Although only ~300 yeast genes have introns, the presence of introns is strongly associated with gene functional class. After cytoplasmic RP genes (103 introns in 100 genes), the largest functional class of yeast intron-containing genes are meiotically induced genes, most of which are under Ume6p transcriptional control (13 of 20 meiotic intron-containing genes) (Table 1). One explanation for this might be that introns help keep meiotic genes from being expressed in vegetative cells, adding an additional layer of protection in the event of incomplete transcriptional repression (Juneau et al. 2007). Two findings suggest that such effects may be more subtle or only enforced on evolutionary time scales. First, *ume6Δ* cells grow reasonably well, given the loss of nonmeiotic functions of Ume6p, while actively transcribing and splicing early meiotic introns (Fig. 2). Second, only one meiosis-specific splicing factor has been found (Mer1p) (Engebrecht et al. 1991), and it activates the splicing of only four pre-mRNAs (Fig. 1; Engebrecht et al. 1991; Nakagawa and Ogawa 1999; Davis et al. 2000). It is possible that Mer2p and Spo22p represent the key regulatory subunits of their respective protein complexes, and that the function of these complexes is critically dependent on Mer1p-activated expression of *MER2* and *SPO22*, but additional experiments would be required to demonstrate this.

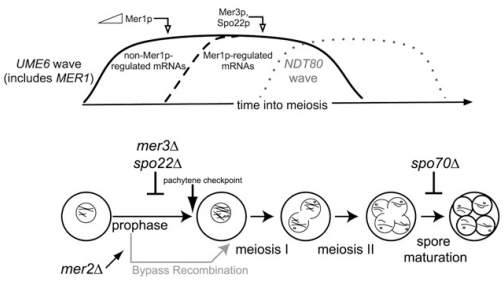

Figure 6. The *MER1* regulatory network and the meiotic gene expression program. The *UME6* expression wave is divided into two temporal components: an early component including non-Mer1p-regulated mRNAs (including Mer1p mRNA itself), and a later component including the mRNAs whose splicing is dependent on Mer1p. Loss of expression of either of two Mer1p-responsive genes (*MER3* and *SPO22*) arrests the cells at the pachytene checkpoint, which must be passed in order for induction of the subsequent *NDT80* expression wave to proceed. However, loss of Mer1p-responsive gene *MER2* bypasses recombination and the pachytene checkpoint due to the absence of DSBs. This allows completion of meiosis, even in the absence of *MER3* or *SPO22*. Loss of Mer1p-responsive gene *SPO70* arrests the cells after chromosome segregation but before spore formation, later in the meiotic gene expression program. Note that the timing of peak RNA expression precedes the execution points for several of the proteins, presumably due to regulatory events at other levels.

We favor the hypothesis that introns in meiotic genes allow for temporal expression regulation during meiosis. As cells shift into the meiotic gene expression program, there appears to be a general increase in splicing efficiency for meiotic genes (Fig. 1; Juneau et al. 2007) as well as for intron-containing genes that are expressed in both vegetative growth and meiosis (Fig 1). The mechanism of this increase in splicing efficiency during meiosis is unknown, but must be independent of *MER1* function, since the residual splicing of Mer1p-responsive genes observed in the absence of *MER1* also increases at this time (Fig. 1).

What is the specific value of the *MER1* regulon to the timing of the meiotic gene expression program? By inducing transcription of *MER1* and its responsive genes with the same transcriptional regulator, the cell creates a timed delay in expression of all Mer1p-responsive genes relative to other Ume6p-induced genes. This is distinct from the *NAM8* splicing regulon, which includes Ume6p-induced *PCH2* and *MEI4* (Table 1). *NAM8* is transcribed during both vegetative growth and meiosis and is not regulated by Ume6p (Ekwall et al. 1992). Although Nam8p function is essential for meiosis (Nakagawa and Ogawa 1997), the *NAM8* splicing network produces no delay in expression of *PCH2* or *MEI4* (Fig. 3). The special nature of the *MER1* splicing regulon divides the expression of coinduced genes into two components: an early wave that is independent of the splicing factor, and a delayed wave that is dependent on the splicing factor. Thus, the existence of a splicing regulatory network can contribute to the coordination of gene expression in time by creating secondary waves of splicing-dependent expression within large waves of transcriptional regulation (Fig. 6).

Transcriptional regulons are interlaced with splicing regulons

The meiotic gene expression program requires both transcriptional (*UME6* and *NDT80*) and splicing (*MER1*) networks for progression, as failed expression of these program regulators blocks meiosis (Engebrecht and Roeder 1990; Steber and Esposito 1995; Xu et al. 1995). We asked how the splicing regulatory network is integrated with each transcriptional regulatory network. Using genomics and genetics, we found that the *UME6* transcriptional network activates the expression of the *MER1* splicing network, which in turn is required for activation of the subsequent *NDT80* transcriptional network.

An intriguing characteristic of the *MER1* regulon is that it has evolved a complex relationship with the *NDT80*

transcriptional regulon. Loss of Mer1p expression does not completely block *NDT80* induction or inhibit meiotic progress in the same way in every cell. Splicing of Mer1p-responsive transcripts occurs, but is much less efficient in *mer1*Δ cells, allowing some mRNA from each of the four Mer1p-responsive genes to be made, presumably resulting in partially inadequate levels of Mer2p, Mer3p, Spo22p, and Spo70p. Depending on stochastic events, these proteins may be limiting in different cells attempting the meiotic program. If Mer2p is limiting, no DSBs will be made, and thus neither Mer3p nor Spo22p will be required, leading to recombination bypass, *NDT80* induction, and mostly successful chromosome segregation (except that spore viability suffers due to increased nondisjunction in the absence of recombination) (Roeder 1997).

In those cells where Mer2p is not limiting, DSBs are formed, but limiting amounts of Mer3p or Spo22p (or both) result in delays at the pachytene checkpoint and delayed *NDT80* induction (Tung et al. 2000) until adequate levels of the missing protein can accumulate to pass the checkpoint. This explains the leaky, mixed phenotype of *mer1*Δ tetrads, and insinuates splicing regulation into both the initiation and resolution steps of recombination, the key checkpoint-regulated step in meiosis. The evolutionary importance of this is underscored by the limited number of genes in yeast that still require splicing—not to mention regulated splicing—for their expression, but why it is important seems obscure. Nonetheless, the nature and function of the Mer1p-responsive genes ensure that correct regulated splicing must occur for *NDT80* induction and efficient, accurate meiosis to take place.

Implications

Developmental programs progress through tightly coordinated gene regulatory networks. Completely defining a gene regulatory network in complex systems is challenging, since the main experimental approach involves determining the effect of loss of function of the master regulator. Such experiments produce complex phenotypes comprised of direct effects and a cascade of indirect effects that must be distinguished. Even for the well-studied *Drosophila* sex determination pathway, in which expression of the master regulator Sxl ultimately leads to a male or female form of the transcription factor Dsx (Baker 1989; Lopez 1998; Black 2003), we cannot begin to explain the integration of observed sex-specific transcription and splicing (Robida et al. 2007; Telonis-Scott et al. 2009). Part of this is due to Sxl regulation of translation as well as splicing (Penalva and Sanchez 2003), and another part is due to incomplete understanding of the sets of genes that respond to Tra and Dsx, and what the effects of those might be on sex-specific transcription and splicing.

Our study shows that, even for the relatively simple *MER1* splicing regulatory network, such downstream effects can be at cross-purposes and difficult to dissect. Sorting true responsive genes from indirectly activated genes will require comparison of large sets of perturbations, as well as the identification of sequence features that mediate action of the master regulator(s). Finally, more effort is needed to relate transcription and splicing regulatory networks to each other. Discerning higher-level dependence relationships will help identify and attribute many secondary events to specific primary events. We will need to know which transcription factors regulate the expression of which splicing factor genes, which splicing factors regulate expression of which other splicing factors, and how alternative splicing of transcription factor mRNAs affect transcription factor function.

Materials and methods

Strains

A complete list of strains is in Supplemental Table S2. Briefly, all vegetatively grown haploid strains were derived from the yeast deletion set background (Winzeler et al. 1999). All experiments involving meiosis used the high-meiotic synchrony strains with the SK1 background (Primig et al. 2000). Diploid single-mutant SK1 strains were constructed by cassette-based gene replacement, followed by sporulation and verification of the deletion by PCR. Diploid double-mutant SK1 strains were constructed by cassette-based gene replacement in the heterozygous knockout of each single deletion, followed by sporulation and verification of the double deletion by PCR. HA3-*MER1* was constructed by N-terminally tagging *MER1* under its native promoter with three copies of the HA epitope (HA3) marked by *TRP1* (Wach et al. 1997; Longtine et al. 1998). All derivatives in this study harbor a tet operator array near the centromere of chromosome V and express the tet repressor-GFP fusion protein to allow for fluorescence detection of chromosome V segregation (Michaelis et al. 1997).

Media, culture conditions, and induction of meiosis

Standard methods for yeast culture were used (Sherman 1991) at 30°C. SK1 cells were induced for synchronous meiosis as described in Padmore et al. (1991). Briefly, cells were streaked from frozen stocks to YP-glycerol (3%) plates, and then single colonies from glycerol were streaked to YPD plates. After 2 d on YPD, a single colony was inoculated into 5 mL of YPD, and, 30 h later, 50 mL of YPA (1% potassium acetate, 1% yeast extract, 2% peptone) was inoculated to OD_{600} = 0.25 and shaken for 14 h. After growth in YPA, cells were washed with water and suspended in SPM (1% potassium acetate, 0.02% raffinose), defined as time 0 of meiosis. Aliquots were taken at 30 min, 2 h, 5 h, 7 h, 9 h, and 11 h for RNA or protein preparation; spun down at room temperature; flash-frozen in liquid nitrogen; and stored at −80°C.

RNA isolation

RNA was isolated as described in Rio et al. (2010). Total meiotic RNA was extracted according to method 2 to ensure uniform RNA extraction from late spore stages. Total vegetative RNA was prepared according to method 1.

Microarray analysis

Microarray analysis was done as described previously (Clark et al. 2002; Burckin et al. 2005). Our arrays are printed in-house and contain ~20,000 spots containing oligonucleotides for all yeast genes (in duplicate) and intron, splice junction, and second exon probes for all intron-containing genes (in quadruplicate) (Burckin et al. 2005). Data from four independent meiotic time courses were combined as follows. Each time point from each replicate meiotic

time course was hybridized to a pair of dye-swapped arrays using an arbitrary reference pool of RNA comprised of 50% time 0 RNA plus 10% each of time 2 h, 5 h, 7 h, 9 h, and 11 h. After normalization and removal of outlier arrays, the data was zero-subtracted (to eliminate variation derived from the reference pools) and averaged. To evaluate splicing changes, we used the IAI, which is derived by subtracting the log ratios of the second exon signals from the intron signals in order to normalize for changes in transcript level on a gene-by-gene basis (Clark et al. 2002). The data presented in Table 1 came from an experiment in which RNA from the *ume6*Δ strain was compared with wild type grown in YPD, and represent the average of a dye-swapped pair of arrays. For the experiment comparing the 9-h time expression pattern of deletion of each member gene in the *MER1* regulon (Fig. 4), we compared 9-h RNA from each mutant to 9-h RNA from wild-type cells, again as the average of dye-swapped pairs. To produce the images in Figures 1 and 4, we used Gene Cluster 3.0 (de Hoon et al. 2004) and Java Treeview (Saldanha 2004). Array data was released through the Gene Expression Omnibus under accession number GSE24686.

RT–PCR and qPCR

RNA was extracted from at least three biological replicates. Reverse-transcribed RNA (cDNA) was amplified using the primers in Supplemental Table S3. Semiquantitative RT–PCR was carried out by limiting cycle numbers to 20 and using cDNA derived from 300 ng of total RNA. PCR products were first analyzed by agarose gel electrophoresis. To obtain estimates of splicing efficiency, we used the Agilent 2100 Bioanalyzer to determine molar amounts of each PCR product and estimated splicing efficiency as follows: percent spliced = [(molarity of spliced peak)/(molarity of unspliced peak + molarity of spliced peak)] * 100. Bioanalyzer percent spliced values from triplicate biological replicates were averaged and the standard deviations are shown. To test for significant differences in splicing between samples (as in Fig. 2C), a paired, two-tailed *t*-test was performed and $P < 0.05$ was considered significant. qPCR was performed using a commercially available master mix (Fermentas) and qPCR primers described in Supplemental Table S3. The graph shown in Figure 3D is a measure of percent of intron-containing RNA from 0 h to 1.5 h every 30 min after the onset of meiosis. This analysis used two primer sets for each gene: one pair for intron-containing pre-mRNA (spanning the 3′ splice site) and one set for total RNA (within the second exon). Primer pair amplification efficiencies were confirmed to be >1.95. Threshold cycles were determined using reactions containing the same amount of cDNA and the percent of intron-containing RNA = $2^{(-\Delta\Delta Ct)} * 100$, where $\Delta\Delta Ct = (Ct_{inF-exR} - Ct_{exF-exR})_{geneX}$.

Western blotting

Frozen cell pellet aliquots from the 0-h, 0.5-h, 1-h, and 1.5-h time points (Fig. 3A) or from the 2-h and 9-h time points (Fig. 5) were prepared as in Rudner et al. (2000). After electrophoresis on SDS-containing 15% acrylamide gels, samples were transferred to nitrocellulose membrane. The blot in Figure 3A was blocked in 3% milk in PBST buffer containing 387 mM NaCl total and was incubated overnight at 4°C in blocking buffer containing 1:1000 α-HA.11 monoclonal antibody (Covance). The blots in Figure 5 were blocked in TBST containing 5% BSA and incubated overnight at 4°C in blocking buffer containing 1:1000 α-phospho-cdc2 (Tyr 15) (Cell Signaling Technology) for Cdc28p-Y19 detection, visualized (see below), then stripped and reprobed overnight with 1:2000 α-Nap1 (affinity-purified rabbit polyclonal raised against $Nap1p_7$; a gift from Doug Kellogg, University of California at Santa Cruz) as a loading control. Primary antibody was detected with HRP-conjugated sheep anti-mouse secondary antibody (for HA detection) (GE Healthcare) or donkey anti-rabbit secondary antibody (for phospho CDK and Nap1p detection) (Santa Cruz Biotechnology) and was visualized with ECL Plus (GE Healthcare).

Microscopy

Aliquots (100 μL) from the 9-h time point were fixed with formaldehyde for 1 h at room temperature. Samples were DAPI-stained and visualized with a Leica DM5500 microscope (Leica Microsystems) using DIC, as well as GFP and DAPI channels.

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Supplemental Table and Figure Legends

Table S1. Candidate Mer1p-responsive genes. List of genes with introns containing a match to the Mer1p enhancer sequence near their 5' splice site (known responsive genes shaded). Indicating distance between 5' splice site and enhancer sequence (dist), match to the enhancer (score), conservation in seven yeast species (consv) (Very>Partly>Not conserved), meiotic expression (i, induced; x, expressed), and whether or not transcript splicing is dependent on Mer1p. We have eliminated three of these as candidates (*SNR17A*, *CPT1*, and *HOP2*) using quantitative PCR. The others show little or no difference in splicing in arrays comparing wild type with *mer1*Δ.

Table S2. Yeast Strains. PCR-based gene replacement was used to generate the non-purchased SK1 deletion mutants, whereby the targeted gene was replaced with either the *TRP1* gene from *S. cerevisiae* or *kHIS3* from *S. kluyveri* (Wach et al. 1997; Longtine et al. 1998). Gene deletions were confirmed by PCR. SK1 (K8409) and *spo22*Δ were purchased from ATCC.

Table S3. RT-PCR and RT-qPCR primers.

Figure S1. The URS1 regulatory sequence is found in the promoters of *MER1*, *MER3*, *SPO22* and *SPO70*. (A) URS1 consensus motif and URS1 sequences found in promoters of *MER1*, *MER3*, *SPO22*, and twice in *SPO70*. (B) UCSC Genome Browser screen shots of promoter regions of *MER1*, *MER3*, *SPO22*, and *SPO70* containing URS1 motif.

Table S1: Canditate Mer1p-responsive genes

ORF/gene	Seq containg match to YRYACCYY	dist	Score	consv	meio	true
YBR119W/MUD1	gtatgta**TATACCTT**gtaattta	8	ND	P	i	
SNR17A/snoRNA U3	gtatgtaa**TATACCCC**aaacattt	9	5.67	V	x	n
YNL130C/CPT1	gtatgttgcttatcttat**TGCACCCT**aaatcttc	19	7.79	P	x	n
YJR021C/REC107	gttcgtaccaacacagtg**CATACCCT**caagtttt	19	7.59	P	i	y
YGL251C/MER3	gtagtaacgaagcttagc**AACACCCT**tatcagttt	19	7.09	V	i	y
YIL073C/SPO22	gtatataacaaaatgcaaaa**CATACCCT**tattaact	21	7.68	V	i	y
YDR305C/HNT2	gtatgcactctcatatgtttttt**TGTACCCC**attcgcac	24	7.79	V	i	
YGR225W/AMA1	gtacgttattaagagcttatgctttca**CATACCCT**tttctggt	28	7.92	V	i	y
YGL033W/HOP2	…gctcatcaaataccgccattactaacaat**TGTACCCC**gggtattt	49	6.97	P	i	n
YBR089C-A/NHP6B	…gtagtatcctctaaaggactgctgttctg**TGCACCCC**cttcc	56	6.97	N	i	

Table S2: Yeast Strains

STRAIN	GENOTYPE
SK1	K8409: MATa/MATalpha HO/HO URA3-tetR-GFP/URA3-tetR-GFP URA3:tetO224/URA3:tetO224 REC8-HA3/REC8-HA3 his3::hisG/his3::hisG trp1Δ/trp1Δ (ATCC:MYA-2089)
SK1-HA-MER1	K8409 TRP1:HA3-MER1/TRP1:HA3-MER1
*mer1*Δ	K8409 mer1::kHIS3/mer1::kHIS3
*mer2*Δ	K8409 mer2::kHIS3/mer2::kHIS3
*mer3*Δ	K8409 mer3::kHIS3/mer3::kHIS3
*spo22*Δ	MATa/MATalpha HO/HO Promoter of URA3-tetR-GFP/Promoter of URA3-tetR-GFP URA3:tetO224/URA3:tetO224 REC8-HA3/REC8-HA3 his3::hisG/his3::hisG yil073c ::HISMX6/ yil073c ::HISMX6 (ATCC: MYA-1937)
*spo70*Δ	K8409 spo70::kHIS3/spo70::kHIS3
*mer1*Δ*mer3*Δ	K8409 mer1::TRP1/mer1::TRP1 mer3::kHIS3/mer3::kHIS3
*mer1*Δ*spo22*Δ	K8409 mer1::TRP1/mer1::TRP1 spo22::kHIS3/spo22::kHIS3
*mer1*Δ*spo70*Δ	K8409 mer1::TRP1/mer1::TRP1spo70::kHIS3/spo70::kHIS3
*mer2*Δ*mer3*Δ	K8409 mer2::TRP1/mer2::TRP1 mer3::kHIS3/mer3::kHIS3
*mer2*Δ*spo22*Δ	K8409 mer2::TRP1/mer2::TRP1 spo22::kHIS3/spo22::kHIS3
*mer2*Δ*spo70*Δ	K8409 mer2::TRP1/mer2::TRP1 spo70::kHIS3/spo70::kHIS3
WT-BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0
*ume6*Δ	BY4741 ume6::KanMX6
*ume6*Δ*mer1*Δ	BY4741 ume6::KanMX6 mer1::KanMX6
*ume6*Δ*nam8*Δ	BY4741 ume6::KanMX6 nam8::KanMX6

Table S3: RT-PCR and RT-qPCR primers

Gene	Sequence
MER2 F1:	5' ACCAGCTACTGGAACAAGAT 3'
MER2 R1:	5' TCGATAACATTGCTGTTGAC 3'
MER3 F1:	5' GTTTGATCGCCTCGGTACAG 3'
MER3 R1:	5' AATTATCGTCTTTGTCGAAGAATTGC 3'
SPO22 F1:	5' TCAGACCACAACGTTAACTC 3'
SPO22 R1:	5' TCCATAGACTTGATGCTGCA 3'
SPO70 F1:	5' GTGAGCCTCTTTGAAATAAAGAGTTT 3'
SPO70 R1:	5' GTTTATCCAAGTCGGAAATATCCC 3'
MEI4 F1:	5' GAGGCAAACTGGAAGATATG 3'
MEI4 R1:	5' AGAGCACCTACATCTTCGAC 3'
PCH2 F1:	5' CAAGATCAACTGGAGTCAAG 3'
PCH2 R1:	5' TCGTCTACAGGAAATGTCCG 3'
qPCR *MER2*-inF	5' TTCATTTTCTTCCAAAACACATTTT 3'
qPCR *MER2*-exF	5' GATTTGGCTTCCCAGATTGA 3'
qPCR *MER2*-exR	5' CCGTCTCATGCTGCTTGTTA 3'
qPCR *MER3*-inF	5' GGAAATGCAACCAAAAGTGG 3'
qPCR *MER3*-exF	5' TGACTTTAACGACCAGTCTGCTAC 3'
qPCR *MER3*-exR	5' TGTCGAAGAATTGCAGACCA 3'
qPCR *SPO22*-inF	5' TCTGGACGAGCAATAGCAAC 3'
qPCR *SPO22*-exF	5' ATCGCAAGTTTATGCGGCTA 3'
qPCR *SPO22*-exR	5' CTTGATGCTGCATTTTCCAA 3'
qPCR *SPO70*-inF	5' AAGAGCTTATGCTTTCACATACCC 3'
qPCR *SPO70*-exF	5' GAATGAACATGCAAACCTGCT 3'
qPCR *SPO70*-exR	5' CAAAGACTTCGACCAAGGACA 3'
qPCR *MEI4*-inF	5' ACGTGAAATTGTCACATCCTT 3'
qPCR *MEI4*-exF	5' CCAGGAATCCTACGTTGTGG 3'
qPCR *MEI4*-exR	5' AGGCGCAACCCATTTGTAT 3'

A

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URS1 site   5'-AGCCGCCGA-3'
MER1        5′-ATTTTAGCCGCCGACAGTGTTA-3′
MER3        5′-TAAATACCCGCCGAGCCTGCAT-3′
SPO22       5′-CAATTAGCCGCCGAAGTTTGTA-3′
SPO70       5′-AAGGCAGACGCCGAAAAATACA-3′
SPO70       5′-ATTTTTGGCGCCTTAAAAATGG-3′
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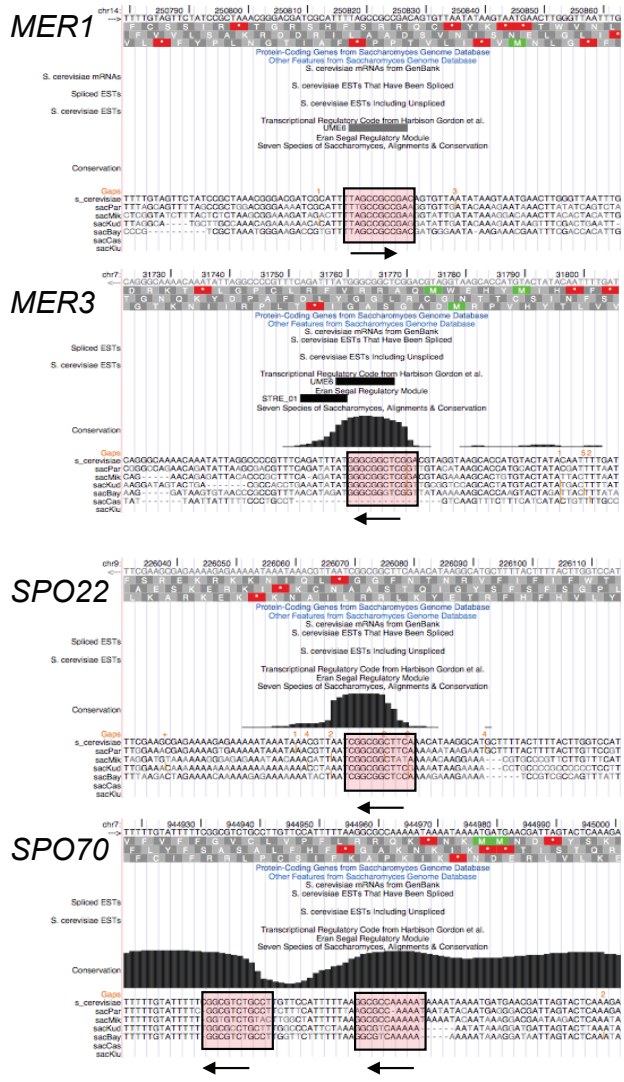
B



CHAPTER 3

CHANGE IN COMPETITION BETWEEN PRE-MRNAS FOR THE SPLICING MACHINERY DRIVES GLOBAL REGULATION OF SPLICING

Change in competition between pre-mRNAs for the splicing machinery drives global regulation of splicing

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Summary

During meiosis in yeast, global splicing efficiency increases and then decreases. Here we provide evidence that splicing improves due to reduced competition for the splicing machinery. The timing of this regulation corresponds to repression and reactivation of ribosomal protein genes (RPGs) during meiosis. In vegetative cells RPG repression by rapamycin treatment also increases splicing efficiency. Down-regulation of the RPG-dedicated transcription factor gene *IFH1* genetically suppresses two spliceosome mutations *prp11-1* and *prp4-1*, and globally restores splicing efficiency in *prp4-1* cells. We conclude that the splicing apparatus is limiting and pre-mRNAs compete. Splicing efficiency of a pre-mRNA therefore depends not just on its own concentration and affinity for limiting splicing factor(s) but also on those of competing pre-mRNAs. Competition between RNAs for limiting RNA processing factors appears to be a general condition in eukaryotic cells important for function of a variety of post-transcriptional control mechanisms including miRNA repression, polyadenylation and splicing.

Introduction

Pre-mRNA splicing is a fundamental step of eukaryotic gene expression. It can vary in complexity from removal of a single intron to elaborate patterns of alternative splicing that create multiple distinct mRNAs. This complex set of mRNAs diversifies the functionalities of proteins that can be produced from a gene. Alternative splicing patterns arise from differences in key pre-mRNA features such as splice site strength (Roca et al., 2005; Yeo and Burge, 2004), secondary structure (Hiller et al., 2007; Howe and Ares, 1997; Kreahling and Graveley, 2005; Plass et al., 2012; Shepard and Hertel, 2008), or transcription elongation rates (de la Mata et al., 2003; Howe et al., 2003; Kornblihtt, 2005; Roberts et al., 1998), as well as to trans-acting splicing factors that bind pre-mRNA to differentially enhance or repress spliceosome recruitment (Black, 2003; Nilsen and Graveley, 2010). The regulation of alternative splicing is generally attributed to the changing activities of trans-acting splicing factors that control the likelihood of local spliceosome assembly.

Recent studies have attempted to capture the regulatory networks for individual splicing factors, usually by depleting or overexpressing a specific splicing factor and measuring changes in alternative splicing across the genome. Combining analyses of the global differences in tissue-specific alternative splicing (e. g., Barbosa-Morais et al., 2012; Merkin et al., 2012; Pan et al., 2008; Pan et al., 2004; Sugnet et al., 2006; Wang et al., 2008), tissue-specific splicing factor expression (e. g., Buckanovich et al., 1993; Calarco et al., 2009; Jin et al., 2003; Markovtsov et al., 2000; Underwood et al., 2005; Warzecha et al., 2009), and changes in splicing factor expression and splicing during differentiation (e. g., Boutz et al., 2007; Gabut et al., 2011; Kalsotra et al., 2008) reveals that alternative splicing is deeply integrated into the gene expression programs that define cell identity and state. To understand gene expression, splicing regulatory networks must be connected with transcriptional and post-transcriptional regulatory networks (reviewed in Kalsotra and Cooper, 2011) such as those of miRNAs, so the contribution of splicing regulation to a change in cell

identity or state can be understood. A largely ignored aspect of splicing regulation concerns systems-level accounting of substrate concentrations and availability of required factors. Recent reports suggest competition phenomena in splicing (Berg et al., 2012; Du et al., 2010; Kaida et al., 2010; Kanadia et al., 2003; Yin et al., 2012) indicating that splicing may also be regulated by changes in competition for a fixed level of factor activity.

In a previous study of meiosis in *Saccharomyces cerevisiae*, we identified relationships between two transcriptional regulatory networks and the Mer1 splicing regulatory network, and examined the roles of the four target transcripts controlled by the Mer1 splicing factor (Munding et al., 2010). We also observed a general increase in splicing efficiency during meiosis (see also Juneau et al., 2007) that we could not assign to any particular trans-acting factor. Here we identify the molecular basis for this improvement and provide evidence that the global increase in splicing efficiency is due to relief of competition for the splicing apparatus that occurs during the programmed repression of ribosomal protein genes (RPGs) early in meiosis. This phenomenon is not restricted to meiosis since blocking RPG transcription with rapamycin in vegetative cells also improves splicing of other transcripts. Down-regulating transcription of RPGs suppresses temperature sensitive (ts) growth of the *prp4-1* and *prp11-1* spliceosome mutations, and rescues splicing defects for nearly all intron-containing genes. These results imply that competition for a limiting splicing machinery can be exploited to control splicing of less competitive substrates through transcriptional control of the overall substrate pool.

Results

A global increase in splicing efficiency during meiosis

In *S. cerevisiae*, splicing of numerous meiosis-specific transcripts improves early in meiosis (Juneau et al., 2007; Munding et al., 2010), including four that depend on the meiosis-specific splicing factor Mer1 (Cooper et al., 2000; Davis et al., 2000; Engebrecht et

al., 1991; Munding et al., 2010; Nakagawa and Ogawa, 1999). In our previous study, strain SK1 was induced to enter a rapid synchronous meiosis and RNA was isolated at times after, and analyzed on splicing-sensitive microarrays (Munding et al., 2010). In addition to meiotic transcripts, we noticed that many non-meiotic transcripts also showed improved splicing. To investigate this we examined the 156 intron-containing genes (ICGs) whose expression does not decrease more than 2-fold during mid-meiosis (55% of total ICGs; Fig 1). We detect improved splicing by a decrease in Intron Accumulation Index (IAI, a measure of the change in ratios of intron signal to exon 2 signal between two samples, Clark et al., 2002). Splicing improves during mid-meiosis and then declines (Fig1A, blue color indicates reduced IAI, interpreted as improved splicing, numerical data in Table S1).

To determine a threshold for calling a change in splicing efficiency, we assessed noise in the data by estimating variation in the IAI distribution between replicate samples that should not show splicing changes (see Experimental Procedures, Fig 1B, control distribution, Table S1). We compared the distribution of IAI changes between time zero and the indicated time point for the set of 156 IGCs to this control (background) distribution (Fig 1B). It is clear that the splicing efficiency globally increases in mid-meiosis, peaking at 5 hrs. Of the 156 genes 61 (39%) improve in splicing efficiency by at least 1.4-fold at two of three mid-meiotic time points (2h, 5h, or 7h, Fig 1C). Among the genes whose splicing improves during mid-meiosis, most (48/61) are constitutively expressed without known meiosis-specific functions and 33/61 are not transcriptionally upregulated during meiosis (Fig 1C). Only a few genes (10/156, 6%) appear to decrease in splicing efficiency more than 1.4 fold, which is fewer than expected by chance given the control distribution (Fig 1B, C). We confirmed these results by RT-qPCR for two meiotically-induced and two constitutively expressed genes (Fig 1D). We conclude that splicing efficiency for both meiotic and constitutively expressed ICGs globally increases during mid-meiosis. We hypothesize that a splicing regulatory mechanism not specifically restricted to meiotic transcripts is active during mid-meiosis to activate splicing globally.

Splicing is less efficient when ribosomal protein genes are expressed

Meiosis in yeast is triggered in part by nutrient signaling (Mitchell, 1994; Neiman, 2011), which also leads to transcriptional repression of RPGs (Chu et al., 1998; Gasch et al., 2000; Munding et al., 2010; Primig et al., 2000; Warner, 1999). RPGs represent the largest functional class of ICGs in *S. cerevisiae* (101 of 293 ICGs are RPGs). Given their high expression levels, RPG pre-mRNAs comprise fully 90% of the splicing substrates in a vegetative cell (Ares et al., 1999; Lopez and Seraphin, 1999; Warner, 1999). After their collective repression early in meiosis, RPGs are reactivated in late meiosis (Chu et al., 1998; Munding et al., 2010; Primig et al., 2000), even though the starvation conditions continue. We wondered whether the increase in splicing efficiency during meiosis might be due to the reduction of RPG pre-mRNAs that normally occupy the spliceosome during vegetative growth. This idea is consistent with the timing of both improved splicing efficiency during RPG repression early in meiosis, and loss of efficient splicing during RPG reactivation at about 9 hours (Fig 1A, B). Based on this, we tested the hypothesis that RPG expression reduces the splicing efficiency of other pre-mRNAs.

As a first test, we asked whether splicing of meiotic transcripts normally only expressed in the absence of RPG expression, is less efficient during vegetative growth when RPGs are highly expressed. Meiotic genes are repressed during vegetative growth by the transcriptional regulator *UME6* (Mitchell, 1994; Munding et al., 2010; Strich et al., 1994; Williams et al., 2002). Thus we evaluated splicing in vegetative *ume6*Δ cells, where derepressed meiotic genes and RPGs are simultaneously expressed (Fig 2A). Transcripts from *SPO22*, *MEI4*, and *PCH2* are highly expressed and efficiently spliced during meiosis (Fig 2A, lanes 1, 4, 7), and are not expressed in wild type vegetative cells (Fig 2A, lanes 2, 5, 8). Deletion of *UME6* in vegetative cells allows expression and some splicing of *SPO22*, *MEI4*, and *PCH2* (Fig 2A, lanes 3, 6, 9), however splicing is much less efficient in vegetative

cells where RPGs are expressed. Quantification confirms that splicing is reduced by 25-45% during vegetative growth as compared to mid-meiosis (Fig 2B).

Splicing improves globally when RPGs are repressed

If poor splicing efficiency of meiotic transcripts in vegetative *ume6*Δ cells (Fig 2) is due to RPG expression, then splicing should improve upon repression of RPGs. RPG transcription is promoted by nutrients through the conserved protein kinase TOR (Cardenas et al., 1999; Hardwick et al., 1999; Powers and Walter, 1999). TOR is inactivated by rapamycin (Heitman et al., 1991), leading to rapid RPG repression (Hardwick et al., 1999; Powers and Walter, 1999). We treated vegetative *ume6*Δ cells with rapamycin (200ng/mL) and monitored RPG pre-mRNA and mRNA levels as well as pre-mRNA and mRNA from non-RPGs. Steady state levels of RPG pre-mRNAs drop immediately upon rapamycin addition with an initial half-life of less than 7 minutes (Fig 3A), likely due to the combination of transcription inhibition and continued splicing of transcripts initiated before rapamycin addition. RPG mRNAs decay more slowly with half-lives similar to those reported by others (Holstege et al., 1998; Li et al., 1999; Wang et al., 2002). As RPG transcription stops and RPG pre-mRNAs disappear, splicing efficiency of non-RPG pre-mRNAs increases (Fig 3B). Within 7 minutes, splicing efficiency is detectably improved. The rapamycin-induced improvement in splicing is mediated through TOR because cells lacking the *FPR1* gene, which encodes a cofactor required for rapamycin binding to TOR (Heitman et al., 1991; Lorenz and Heitman, 1995), do not show improved splicing efficiency after rapamycin treatment (Fig S1A). Most unspliced pre-mRNAs are decayed by NMD (Burckin et al., 2005; Sayani et al., 2008) after export to the cytoplasm (Kuperwasser et al., 2004). To exclude the possibility that rapamycin mimics improved splicing by somehow increasing the efficiency of NMD, we performed the same experiment in cells deleted of the essential NMD factor Upf1 (Leeds et al., 1991). In these cells, the steady state levels of unspliced transcripts are much

higher than in wild type (Fig S1B); nonetheless, treatment with rapamycin still results in dramatically increased splicing efficiency (Fig S1C).

To explore the transcriptome-wide effect on splicing after RPG repression, we performed RNA sequencing (RNA-seq). We evaluated expression of intron-containing RNA (measured by intronic reads) and total RNA (measured by exon 2 reads) of both RPGs and non-RPGs in cells treated with rapamycin for 10 and 60 minutes (Fig 3C). RPG pre-mRNAs decrease to ~20% of initial levels within 10 minutes of rapamycin treatment, whereas total RPG RNA (mostly mRNA) remains high and falls substantially only after 60 minutes of treatment (Fig 3C, left panel). In comparison, non-RPG expression remains relatively unchanged during the time course (Fig 3C, right panel). We evaluated splicing in cells treated with rapamycin for 10 minutes relative to untreated cells, using a cut-off of 1.25-fold change in splicing ($|IAI| \geq 0.3$), threshold established using control distribution, see Experimental Procedures, Fig S1D). Of the 116 ICGs whose expression changes less than 2-fold upon rapamycin treatment, 68 improve in splicing efficiency by at least 25% (Fig 3D, Fig S1D). Thus in both vegetative and meiotic cells, RPG expression is associated with inefficient splicing of other transcripts.

Down-regulation of an RPG-dedicated transcription factor suppresses spliceosomal defects

While searching for a way to manipulate RPG expression without rapamycin, we found a report from John Woolford's lab of extragenic "supersuppressors" that rescued multiple different spliceosomal mutations (Maddock et al., 1994). One class of such suppressors fell in the *SPP42* gene, now also known as *FHL1*, since shown to encode one of several transcription factors dedicated primarily to RPG transcription (Martin et al., 2004; Rudra et al., 2005; Schawalder et al., 2004; Wade et al., 2004; Zhao et al., 2006). Our hypothesis that pre-mRNAs compete for a limiting splicing apparatus prompted a new interpretation of their suppressor results. If RPG pre-mRNAs compete with essential pre-

mRNAs, then competition might be exacerbated in a strain with a compromised spliceosome, for example the ts *prp4-1 and prp11-1* strains (Galisson and Legrain, 1993; Hartwell, 1967). Furthermore if ts growth is a consequence of failure to splice growth rate limiting pre-mRNAs, this defect might be suppressed by relieving the competition for the compromised splicing machinery. The ability of *spp42-1* to suppress multiple different splicing mutations (Maddock et al., 1994) and its subsequent identification as a dedicated RPG transcription factor suggested it reduced RPG expression and relieved competition.

To test the idea that down-regulation of an RPG-dedicated transcription factor might suppress different ts spliceosome mutations, we constructed strains carrying either the ts *prp4-1* or *prp11-1* alleles and a glucose-repressible promoter controlling expression of the dedicated RPG transcription factor encoded by *IFH1*, a protein required by *FHL1/SPP42* to promote RPG transcription (Rudra et al., 2005; Schawalder et al., 2004). *PRP4* encodes a protein in the U4/U6 snRNP, which enters the spliceosome as part of the U4/U6-U5 trisnRNP, whereas *PRP11* encodes a subunit of the U2-associated SF3a complex that establishes U2 snRNP association with the intron branchpoint at an early step (see Will and Luhrmann, 2011 for review). These two proteins contribute to very different steps in the splicing pathway. The *prp4-1; GAL-IFH1* and the *prp11-1; GAL-IFH1* strains grow similarly to their corresponding *IFH1* strains at permissive temperature (26ºC) on glucose medium. But at the non-permissive temperature (30ºC for *prp4-1; IFH1* and 33ºC for *prp11-1; IFH1*), both ts mutations are suppressed by down-regulation of *IFH1*, as signified by improved growth on glucose-containing media (Fig 4A). Using qPCR, we find that at 26ºC on glucose, *prp4-1; GAL-IFH1* cells express reduced levels of *IFH1* and RPG mRNAs (Fig 4B). These genetic observations suggest that a modest decrease in the RPG pre-mRNA pool rescues growth defects of the *prp4-1* strain by improving splicing of other essential transcripts.

To confirm this interpretation we performed RNA-seq and examined the global effect of *IFH1* down-regulation on splicing of other transcripts. We compared the splicing efficiencies of genes whose expression does not change more than 2-fold in *prp4-1; GAL-IFH1* cells to those of *prp4-1; IFH1* cells. Of the 225 ICGs whose expression does not change, fully 93% improve in splicing efficiency by at least 1.25-fold in *prp4-1; GAL-IFH1* cells (Fig 4C). This includes most RPG splicing events (88/93) as well as non-RPG splicing events (121/132). Validation for several genes by RT-qPCR shows that splicing is restored by down-regulation of *IFH1* (Fig 4D). Thus we conclude that subtle down-regulation of a dedicated RPG transcription factor can rescue spliceosomal defects through an unusual suppression mechanism. We infer that by reducing the overall load of RPG pre-mRNAs, the demand on the compromised spliceosome is sufficiently relieved to allow a level of splicing of other less efficiently spliced essential transcripts. The RNA-seq data incidentally revealed that the mutant Prp4-1 protein has the substitution F320S in a WD repeat domain (data not shown).

To exclude the possibility that the increase in splicing efficiency observed in these three conditions (meiosis, rapamycin treatment, and *IFH1* down-regulation) is associated with improved expression of the splicing machinery, we evaluated expression of the five snRNAs and 110 genes encoding splicing proteins in all three treatments (Table S2). Although expression differs across conditions, no global up-regulation of the splicing apparatus is observed under any condition. Furthermore there is no single gene whose expression is correlated with splicing improvement in all conditions (Table S2). We conclude that pre-mRNAs compete with each other for a limiting splicing apparatus and that increased splicing efficiency is associated with relief of competition by reduced RPG expression.

Pre-mRNA substrates compete at an early step of spliceosome recruitment

Inspection of the splice sites in pre-mRNAs that compete poorly revealed many with canonical sequences and no convincing enrichment for any single unusual feature. To explore whether substrates that diverge from splicing signal consensus vary in their competitive ability, we used ACT1-CUP1 reporters (Lesser and Guthrie, 1993) containing mutations in the 5' splice site (5'ss), branchpoint (bp), and 3' splice site (3'ss, Fig 5A). We tested the effect of rapamycin treatment on reporter splicing efficiency in vegetative cells, expecting that a substrate altered in a feature required for competition would show the most improvement in response to RPG repression. Of the seven different mutants tested, only two branchpoint mutants (C256A and A259C) improved in splicing efficiency after treatment with rapamycin (Fig 5B). We separately evaluated first and second step splicing efficiency and find that rapamycin significantly improves the first step for both C256A and A259C mutant pre-mRNAs (Fig 5C). Other substrates with first step defects, such as the 5'ss mutant U2A, did not significantly improve (Fig 5B). While A259C also shows second step improvement, this effect is likely a consequence of the 2-fold improvement in the first step. The 3'ss mutant U301G (defective in second step catalysis) showed no significant improvement (Fig 5B). This experiment indicates that competition is likely to involve factors acting with the intron branchpoint to commit the pre-mRNA to completion of splicing.

Discussion

These results provide strong evidence that pre-mRNAs compete for the splicing apparatus. For this reason, changes in the composition of the pre-mRNA pool in the nucleus have significant impact on splicing regulation. By manipulating the composition of the pool of competing pre-mRNAs through transcription (Figs 3 and 4) we show that the balance of splicing competition is important for cell function. The ability of competing RNAs to influence splicing by a "trans-competition control" mechanism appears related to a larger group of phenomena described in vertebrate cells in which competition between RNAs for a limiting

regulatory factor leads to global changes in gene expression. This mechanism is established for miRNA regulation, whereby repression of an mRNA by a miRNA is affected by the level of other competing RNAs (called “competitive endogenous RNAs,” ceRNAs; Salmena et al., 2011). This process, first described in plants and called “target mimicry” (Franco-Zorrilla et al., 2007), also regulates muscle development (Cesana et al., 2011), and affects cancer progression (Poliseno et al., 2010) in animals. Our results indicate that a parallel mechanism is at work in splicing regulation, whereby pre-mRNAs compete for a limiting splicing machinery, and splicing of many introns is influenced by changes in the composition of the transcript pool. In the case of splicing, the competing RNAs are also substrates, rather than inert decoys.

Evidence that splicing regulation is subject to the composition of a pool of endogenous competing RNAs is not limited to yeast. In models of the human disease myotonic dystrophy, abnormal expression of a CUG repeat expansion RNA acts as a ceRNA for the MBNL1 splicing factor, mimicking a loss of MBNL1 function in splicing (Du et al., 2010; Kanadia et al., 2003; Miller et al., 2000), indicating that pre-mRNAs compete for MBNL1. Similarly sno-lncRNAs have been identified as a kind of ceRNA for pre-mRNAs dependent on the splicing factor RBFOX2 (Yeo et al., 2009; Yin et al., 2012). Under conditions where sno-lncRNAs are depleted (such as in Prader-Willi syndrome, Yin et al., 2012) competition for RBFOX2 is relieved. A third example involves the U1 snRNP, which appears limiting for an activity that influences polyadenylation site selection (Berg et al., 2012; Kaida et al., 2010). When the levels of pre-mRNA increase, the spectrum of polyA sites utilized in the cell changes, creating mRNAs with alternative 3’UTRs, with each pre-mRNA presumably acting as a ceRNA for all the others. Thus understanding post-transcriptional gene regulation requires accounting of changes in the levels of the limiting regulatory factor as well as changes in composition of the larger transcript pool that affect competition for that limiting factor.

What conditions are required for trans-competition control?

Splicing can be regulated by changes in physical levels, specific activity or localization of splicing factors that control the rate-limiting step of splicing in a transcript specific fashion (Black, 2003; Nilsen and Graveley, 2010). Trans-competition control accounts for changes in splicing factor activity observed by altering the effective load of pre-mRNAs that also employ the limiting factor or other RNAs that occupy the factor. Thus splicing regulation may be achieved by either changing the abundance of a limiting factor (or exchanging one limiting factor for another) or by altering the dynamics of competition by changing the composition of the RNA pool (Fig 6A). These systems-level considerations argue that understanding the demand for the splicing machinery and how pre-mRNA competition changes during development will be required to integrate regulatory networks into their gene expression programs. In mammalian systems, induction of gene expression programs can result in large changes in the composition of the transcript pool (Berg et al., 2012), altering competition for the splicing machinery. Under such conditions, the competitive advantage of alternative exons for the splicing machinery may be decreased, resulting in a shift of mRNA isoforms.

The principles of trans-competition control can be explained using a modification of the general Michaelis-Menten model for competitive inhibition where two different substrates (S_1 and S_2) compete (Fig 6B). In this case, when the spliceosome is limiting, the amount of mRNA product P_1 depends on both the concentration of pre-mRNA S_1 ([S1]) and its splicing rate (k1) as well as the concentration ([S2]) and splicing rate (k2) of the competing pre-mRNA substrate (Fig 6B and S2). This simple model shows that splicing regulation can be achieved by altering the competitive status of a target pre-mRNA through modulation of the levels of other RNAs that compete for a limiting factor. In a cell there are thousands of competing introns, each with its own affinity for the spliceosome; as the concentration of any one of

them changes, the splicing efficiency of all the others then must change as well. Similar to the queuing theory (Cookson et al., 2011), where degradation of unrelated proteins dependent on a common enzyme become coupled due to competition for the enzyme, change in the demand for the spliceosome couples pre-mRNAs whose splicing is affected after a change to the pool of substrates.

Functional importance of trans-competition control.

The inverse relationship of RPG expression and splicing of meiotic transcripts may contribute to the meiotic and vegetative gene expression states. Repression of RPGs might promote the meiotic state by allowing sufficient splicing of meiotic transcripts. Conversely, expression of RPGs inhibits splicing of meiotic transcripts, thereby promoting the vegetative state. Strong evidence for the functional importance of balanced competition comes from suppression of splicing defects upon down-regulation of RPGs (Fig 4). Rescue of the ts phenotype of *prp4-1* and *prp11-1* arises from poor splicing of essential pre-mRNAs because they are outcompeted by RPG pre-mRNAs. Restoring the competitive balance decreases the demand on the splicing machinery by reducing the load represented by intron-containing RPGs allows improved splicing of essential non-RPG pre-mRNAs that then increases viability of the *prp4-1* and *prp11-1* strains.

A number of human diseases are associated with missense mutations in core spliceosome components (reviewed in Padgett, 2012), such as Prp8 and Prp31 (retinitis pigmentosa) and SF3B1 (myelodysplastic syndrome and chronic lymphocytic leukemia). These cases may mirror the subtle loss of splicing capacity observed for the *prp4-1* and *prp11-1* mutations and alter the competitive landscape for splicing, contributing to disease. Different pre-mRNAs clearly have distinct dependencies on conserved components of the splicing machinery (Burckin et al., 2005; Clark et al., 2002; Park et al., 2004; Pleiss et al., 2007), suggesting transcripts may compete for different limiting factors depending on the context. Thus the key to understanding why certain mutations in conserved splicing factor

genes lead to specific diseases may lie in the nature of the composition of the transcript pool in the specific cell type affected, and which pre-mRNA molecules suffer under the altered competitive situation.

Experimental Procedures

Strains and plasmids

Strains are listed in Table S3. *GAL-IFH1* strains were constructed (Longtine et al., 1998; Wach et al., 1997) and verified by PCR, so that the *GAL1* promoter (marked by the *Saccharomyces kluveri HIS3* gene) was placed upstream of *IFH1*. Strains carrying the *prp4-1* or the *prp11-1* mutations were provided by S. Ruby (Ruby et al., 1993). The *prp4-1; GAL-IFH1* and the *prp11-1; GAL-IFH1* strain were constructed by crossing to the *GAL-IFH1* strain. ACT1-CUP1 reporter plasmids (Fig 5) are from (Lesser and Guthrie, 1993).

Media and culture conditions

Standard methods for yeast culture conditions were used (Sherman, 1991). Rapamycin was added cells grown to OD_{600}≈0.5 at 200ng/mL for the indicated time. All yeast strains were grown at 30ºC unless otherwise indicated.

RNA isolation

RNA was isolated as described in (Rio et al., 2010). Total meiotic RNA was extracted according to Method 2 to ensure uniform RNA extraction from late spore stages. Total vegetative RNA was prepared from cells grown to OD_{600}=0.5 according to Method 1.

Transcriptome profiling

Microarray data (Munding et al., 2010) is from Gene Expression Omnibus, accession number GSE24686. RNA-Seq data in Fig 3 is from two independent rapamycin time courses. RNA-Seq data in Fig 4 represents one culture from each strain (grown to OD_{600}≈0.5 in YPD at 26ºC). RNA-Seq data has been released through the Gene Expression Omnibus under

accession number GSE44219. Additional experimental details are included in Supplemental Information.

RT-PCR and qPCR

Reverse transcribed RNA (cDNA) was amplified using the primers in Table S4. Semi-quantitative RT-PCR was carried out by limiting cycle numbers to 21 and using cDNA derived from 300ng of total RNA. Estimates of splicing efficiency used the Agilent 2100 Bioanalyzer. qPCR was preformed using a master mix (Fermentas). Additional experimental details are included in Supplemental Information.

Primer Extension

At least 3 colonies of BY4741 transformed with each ACT1-CUP1 reporter plasmid were grown to OD=0.5 in SCD medium lacking leucine. 5µg of total RNA was annealed to 0.1ng of PE1 primer (5'-CCTTCATTTTGGAAGTTA-3') and primer extended as previously described (Perriman and Ares 2007). Extension products were analyzed on a Typhoon imaging system (GE Healthcare). 1^{st} step splicing efficiency was calculated as (M+L)/(M+L+P); 2^{nd} step splicing efficiency was calculated as M/(M+L); total splicing efficiency was calculated as M/(M+L+P) where M is mRNA, L is lariat intermediate, and P is pre-mRNA.

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Figure Legends

Figure 1. Splicing efficiency improves globally during mid-meiosis. (A) Top Panel: Changes in splicing efficiency during the meiotic time course as represented by Intron Accumulation Indexes. Increased intron accumulation (yellow) represents a decrease in splicing efficiency, while decreased intron accumulation (blue) indicates an increase in splicing efficiency. See Table S1 for data file. Bottom Panel: Changes in RPG gene expression during the meiotic time course. Purple represents a decrease in gene expression. (B) Distribution of intron accumulation indexes from the microarray data at 2, 5, 7, and 9h meiotic time points relative to the zero time point, and a control distribution from self comparison of replicates (see Experimental Procedures). Red line marks 40% increase in splicing efficiency (IAI < -0.5) used as a threshold for significant splicing change. Numbers in red indicate the fraction of events in each distribution that exceeded the threshold. P-values are derived from a one-tailed t-test comparison of the individual 2, 5, 7, or 9h distributions to the control. (C) Classification of splicing changes at mid-meiotic time points (2, 5, and 7 h) for the 156 events whose expression does not decrease more than 2-fold during mid-meiosis. Bold letters indicate splicing change. “NC” indicates no change. “Txn UP“ indicates genes that are transcriptionally induced ≥ 2-fold during mid-meiosis. “Txn NC” indicates genes whose expression changes ≤ 2-fold during mid-meiosis. Numbers in parentheses indicates number of genes in each category. (D) RT-qPCR measurement of percent of intron-containing transcript at the indicated time after induction of meiosis for two meiosis-specific genes (top panel) and two constitutively expressed genes (bottom panel). See also Table S1.

Figure 2. Splicing of meiotic transcripts is more efficient during meiosis than during vegetative growth. (A) Expression and splicing of meiotic transcripts *SPO22*, *MEI4*, and *PCH2* in wild type (+) meiotic (Meio) and vegetative cells (Veg) and in *ume6*Δ (Δ) vegetative cells. Marker sizes are in base pairs. PCR products representing spliced (S) and unspliced

(U) are indicated. (B) Quantification of splicing efficiency from at least three biological replicates. Dark gray bar indicates splicing efficiency at t=5h after induction of meiosis; light gray bar indicates splicing efficiency in *ume6*Δ vegetative cells.

Figure 3. Splicing efficiency increases after treatment with rapamycin. (A) Quantification of total (exon 2) transcript levels for *RPS16A* and *RPL34A*/*B* and for unspliced *RPL34A/B* pre-mRNA by RT-qPCR relative to *SEC65*, and normalized to t=0 in *ume6*Δ vegetative cells at indicated times after treatment with rapamycin. Transcript half-lives ($t_{1/2}$) are indicated in the inset. (B) Quantification of splicing efficiency of meiotic transcripts *SPO22*, *MEI4* and *PCH2* by semi-quantitative RT-PCR in *ume6*Δ vegetative cells at indicated times after treatment with rapamycin. (C) RNA-seq measurement of global expression after rapamycin treatment. Box plot representing change in RPG (n=107 events) (left panel) and non-RPG (n=165 events) (right panel) intron reads vs exon 2 reads after 10 or 60 minutes of treatment with rapamycin, normalized to untreated wild type cells. (D) Global changes in splicing of genes whose expression does not change greater than 2-fold after 10 minutes of rapamycin treatment relative to untreated wild type cells represented by intron accumulation indexes (IAI). Black bar indicates IAI=0 or no change in splicing efficiency. Red arrow indicates splicing changes above the threshold. See also Fig S1.

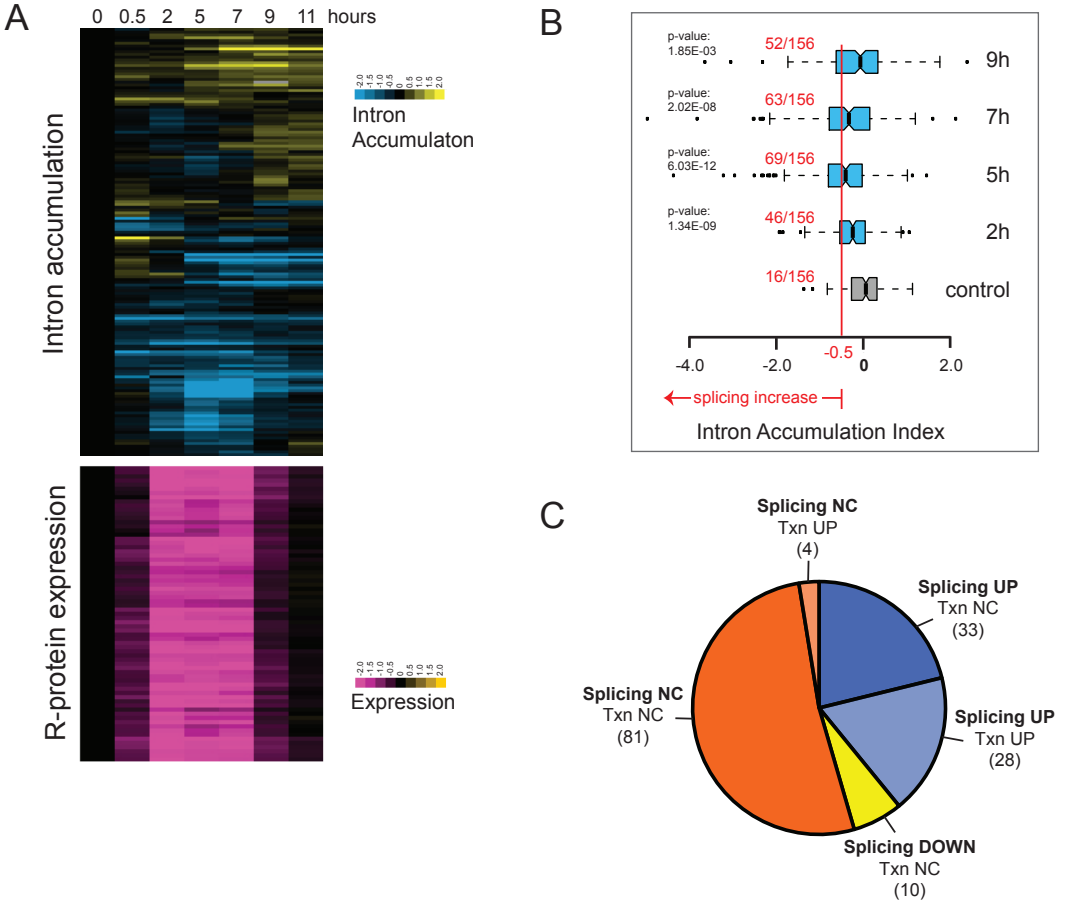
Figure 4. Splicing defects are suppressed by down-regulation of RPG transcription. (A) Growth of *IFH1* and *GAL-IFH1* strains carrying temperature sensitive splicing mutations *prp4-1* or *prp11-1* on glucose (*IFH1* down regulated) at 26ºC (permissive temperature) and 30ºC (non-permissive temperature for *prp4-1*) or 33°C (non-permissive for *prp11-1*). (B) RT-qPCR measurement of *IFH1* and RPG expression relative to *SEC65* in YPD at 26ºC in *prp4-1*; *IFH1*, *PRP4; GAL-IFH1*, and *prp4-1; GAL-IFH1* yeast normalized to WT (*PRP4; IFH1*). (C) Genome-wide changes in splicing of RPG and non-RPG transcripts in *prp4-1; GAL-IFH1* cells relative to *prp4-1; IFH1* cells. Black bar indicates IAI=0 or no change in splicing efficiency.

Red arrow indicates splicing changes above the threshold. (D) RT-qPCR validation of splicing improvement as measured by percent intron-containing transcript for *CPT1*, *HNT1*, *MOB2*, and *SEC14* in YPD at 26ºC in *prp4-1*; *IFH, PRP4; GAL-IFH1*, and *prp4-1; GAL-IFH1* yeast normalized to WT. See also Table S2.

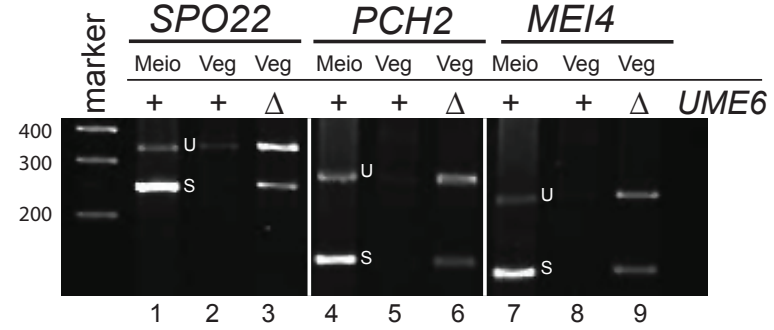
Figure 5. Competition is imposed at early steps of spliceosome assembly. (A) ACT1-CUP1 reporter pre-mRNA schematic indicating 5' splice site, branchpoint, and 3' splice site mutations used in this study. (B) Quantification of total splicing efficiency as measured by primer extension of wild type and the indicated mutant ACT1-CUP1 reporters before and after (+) treatment for 60min with rapamycin (60' rapa). Double asterisks indicate $p<0.01$ in a one-tailed t-test. (C) Quantification of 1st step (dark gray bars) and 2nd step (light gray bars) splicing efficiency as measured by primer extension of WT, C256A, and A259C ACT1-CUP1 reporters before and after (+) treatment for 60' with rapamycin (60' rapa). Single asterisk indicates $p<0.05$ and double asterisks indicate $p<0.01$ in a one-tailed t-test.

Figure 6. Trans-competition control of splicing. (A) Trans-competition control of alternative splicing. When competitor pre-mRNA levels are low, demand for the limiting factor (LF) is low resulting in efficient inclusion of the weakly competitive cassette exon. When competitor pre-mRNA levels are high, competitor pre-mRNAs titrate increased amounts of the limiting factor, resulting in much less efficient inclusion of the weakly competitive cassette exon. (B) Left Panel: Michaelis-Menten scheme showing two substrates with different affinities (S1 and S2) competing for the same enzyme, E. Formation of products P1 and P2 is determined by the concentration of each substrate and the substrate's Km when the enzyme is limiting. Right Panel: Splicing scheme of two substrates competing for a limiting splicing machinery (pink circle). In this example, both substrates are present at the same initial concentration, but the orange substrate outcompetes the blue substrate due to its higher

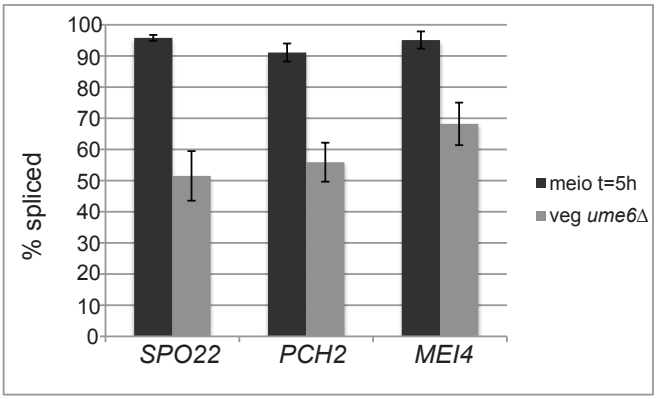
affinity (k1 >> k2). Note that rates of ES formation will also change between pre-mRNAs of equal affinity when one is at higher concentration. See also Fig S2.


A
0 0.5 2 5 7 9 11 hours
Intron accumulation
Intron Accumulaton
R-protein expression
Expression
B
p-value: 1.85E-03
52/156
9h
p-value: 2.02E-08
63/156
7h
p-value: 6.03E-12
69/156
5h
p-value: 1.34E-09
46/156
2h
16/156
control
-4.0
-2.0
-0.5
0
2.0
splicing increase
Intron Accumulation Index
C
Splicing NC Txn UP (4)
Splicing UP Txn NC (33)
Splicing NC Txn NC (81)
Splicing UP Txn UP (28)
Splicing DOWN Txn NC (10)
D
Meiotic Genes
% Intron-containing transcript
Time after induction of meiosis (h)
MEI4
DMC1
Constitutive Genes
% Intron-containing transcript
Time after induction of meiosis (h)
SEC14
CPT1

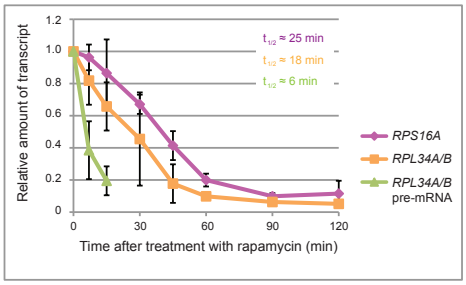
A


marker
SPO22
PCH2
MEI4
Meio Veg Veg Meio Veg Veg Meio Veg Veg
+ + Δ + + Δ + + Δ UME6
400
300
200
U
S
U
S
U
S
1 2 3 4 5 6 7 8 9

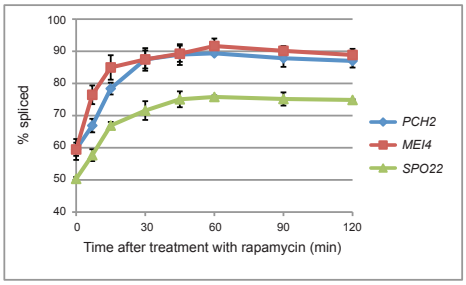
B


% spliced
100
90
80
70
60
50
40
30
20
10
0
SPO22
PCH2
MEI4
meio t=5h
veg ume6Δ

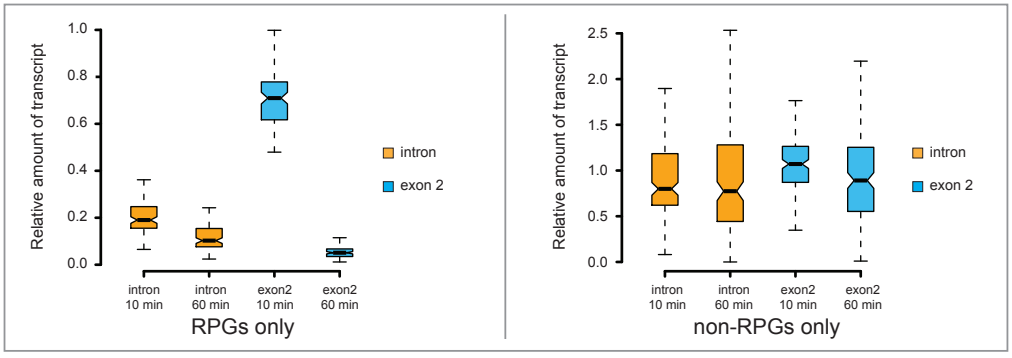
A


Relative amount of transcript
1.2
1.0
0.8
0.6
0.4
0.2
0
$t_{1/2}$ ≈ 25 min
$t_{1/2}$ ≈ 18 min
$t_{1/2}$ ≈ 6 min
0
30
60
90
120
Time after treatment with rapamycin (min)
RPS16A
RPL34A/B
RPL34A/B pre-mRNA

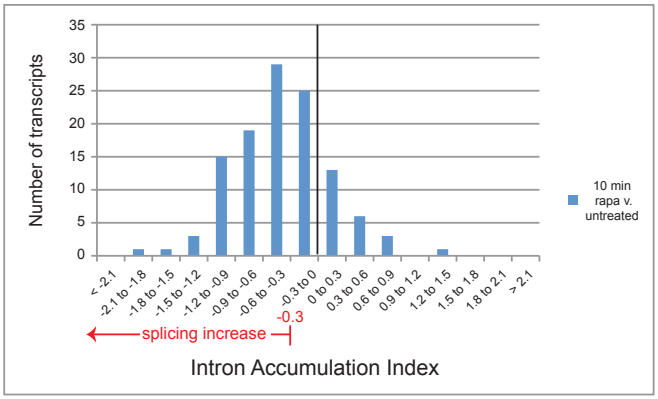
B

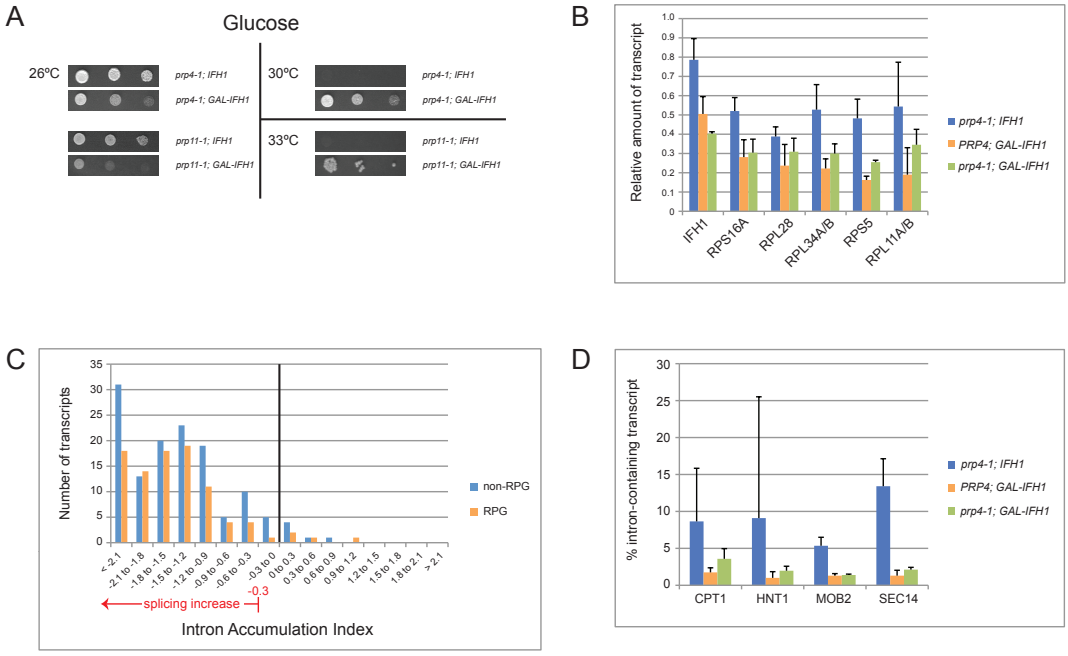

% spliced
100
90
80
70
60
50
40
0
30
60
90
120
Time after treatment with rapamycin (min)
PCH2
MEI4
SPO22

C

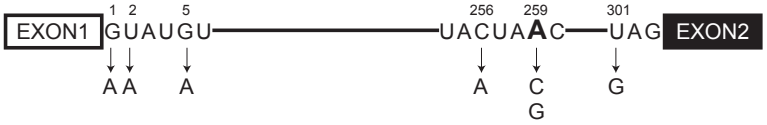

Relative amount of transcript
1.0
0.8
0.6
0.4
0.2
0.0
intron
exon 2
intron 10 min
intron 60 min
exon2 10 min
exon2 60 min
RPGs only
Relative amount of transcript
2.5
2.0
1.5
1.0
0.5
0.0
intron
exon 2
intron 10 min
intron 60 min
exon2 10 min
exon2 60 min
non-RPGs only

D

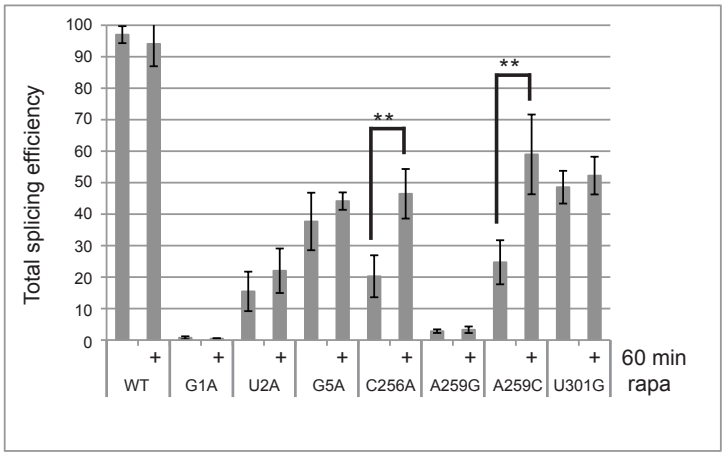

Number of transcripts
35
30
25
20
15
10
5
0
10 min rapa v. untreated
< -2.1
-2.1 to -1.8
-1.8 to -1.5
-1.5 to -1.2
-1.2 to -0.9
-0.9 to -0.6
-0.6 to -0.3
-0.3 to 0
0 to 0.3
0.3 to 0.6
0.6 to 0.9
0.9 to 1.2
1.2 to 1.5
1.5 to 1.8
1.8 to 2.1
> 2.1
-0.3
splicing increase
Intron Accumulation Index


A
Glucose
26ºC
30ºC
33ºC
prp4-1; IFH1
prp4-1; GAL-IFH1
prp11-1; IFH1
prp11-1; GAL-IFH1
B
Relative amount of transcript
IFH1
RPS16A
RPL28
RPL34A/B
RPS5
RPL11A/B
prp4-1; IFH1
PRP4; GAL-IFH1
prp4-1; GAL-IFH1
C
Number of transcripts
non-RPG
RPG
splicing increase
-0.3
Intron Accumulation Index
D
% intron-containing transcript
CPT1
HNT1
MOB2
SEC14
prp4-1; IFH1
PRP4; GAL-IFH1
prp4-1; GAL-IFH1

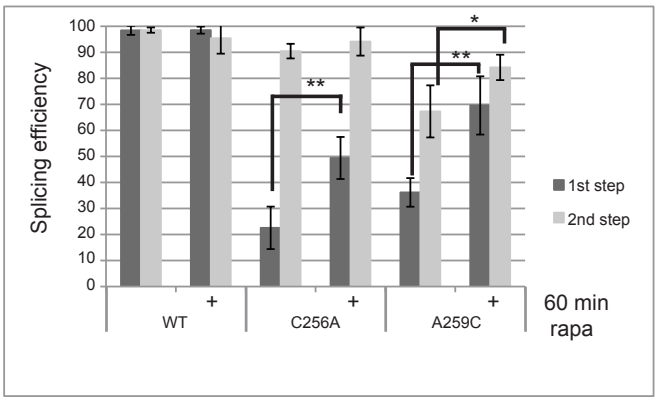
A


EXON1
1 2 5
G U A U G U
256 259 301
U A C U A A C U A G
EXON2
A A A
A
C
G
G

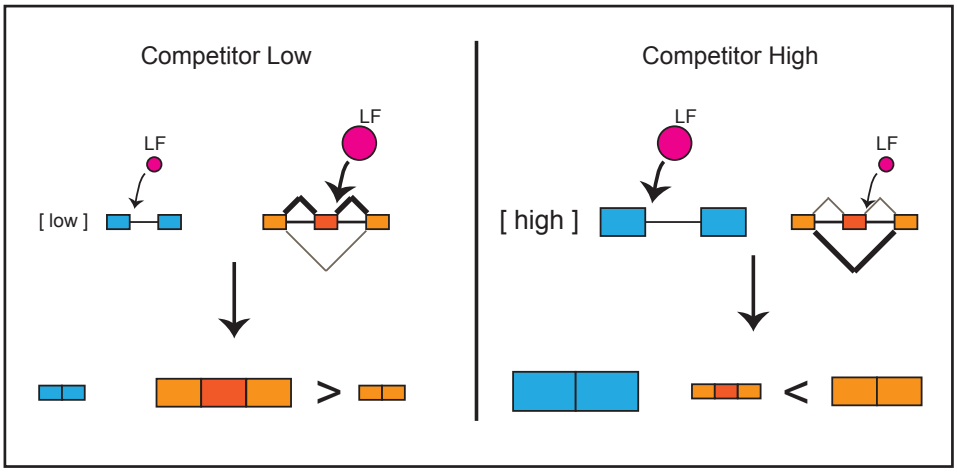
B


Total splicing efficiency
100
90
80
70
60
50
40
30
20
10
0
**
**
+ + + + + + + +
WT G1A U2A G5A C256A A259G A259C U301G
60 min rapa

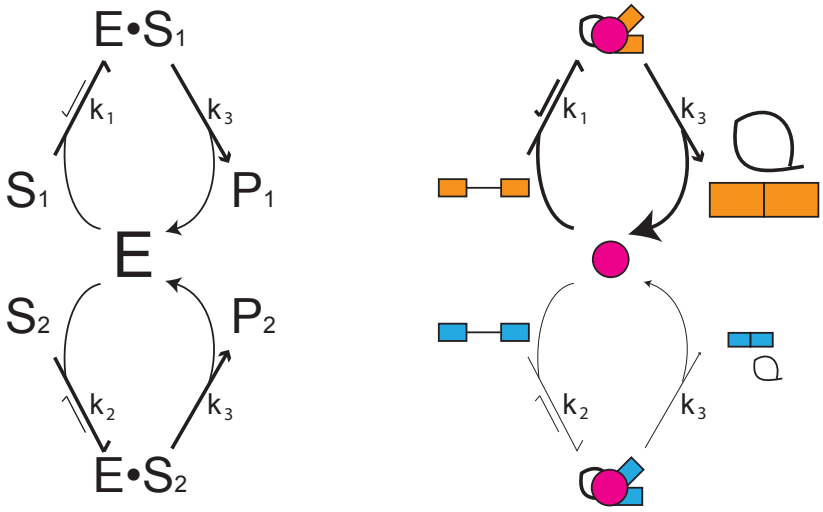
C


Splicing efficiency
100
90
80
70
60
50
40
30
20
10
0
**
**
*
1st step
2nd step
+ + +
WT C256A A259C
60 min rapa

A


Competitor Low
Competitor High
LF
LF
[low]
LF
[high]
LF
>
<

B


E•S1
k1
k3
S1
P1
E
S2
P2
k2
k3
E•S2
k1
k3
k2
k3

Supplemental Figures and Legends

FIG S1_Munding (Ares)

Figure S1. Related to Figure 3. Rapamycin-induced improvement in splicing.

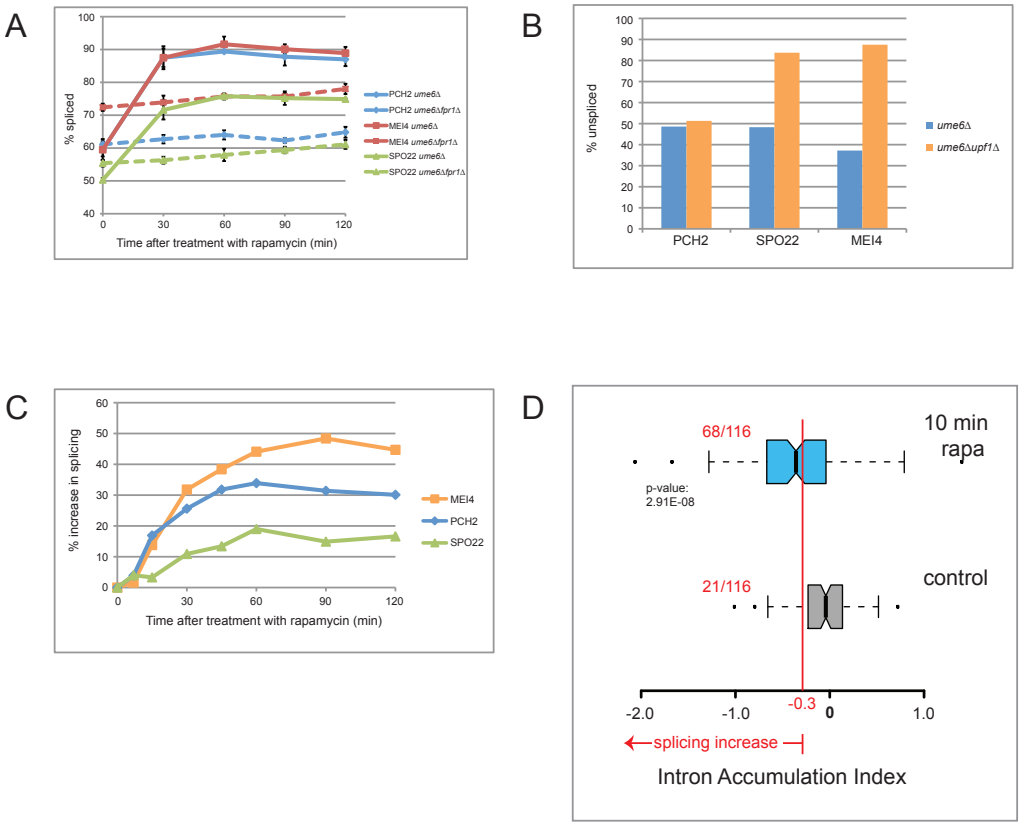


Figure S1. Related to Figure 3. Rapamycin-induced improvement in splicing. (A) Quantification of splicing efficiency of meiotic transcripts *SPO22*, *MEI4* and *PCH2* by semi-quantitative RT-PCR in *ume6*Δ and *ume6*Δ*fpr1*Δ vegetative cells at indicated times after treatment with rapamycin. The *FPR1* gene encodes the cofactor required for rapamycin binding to TOR. (B) Quantification of unspliced pre-mRNA of *SPO22*, *MEI4* and *PCH2* by semi-quantitative RT-PCR in *ume6*Δ and *ume6*Δ*upf1*Δ vegetative cells. *SPO22* and *MEI4* are

substrates of NMD while *PCH2* is a poor NMD substrate. (C) Quantification of percent increase in splicing of *SPO22*, *MEI4*, and *PCH2* by semi-quantitative RT-PCR in *ume6*Δ*upf1*Δ vegetative cells at indicated time after treatment with rapamycin. (D) IAI distributions from the average of both biological replicates at 10 minutes after rapamycin treatment relative to untreated samples (also shown in Fig 3D) and control distribution of self comparisons between biological replicates after rapamycin treatment. A t-test indicates these distributions differ significantly, reflecting a change in splicing efficiency. Red line mark 25% splicing improvement (IAI < -0.3) and numbers in red indicate the number of events in each distribution with an IAI < -0.3.

Figure S2. Related to Figure 6. Competitive inhibition.

A

$$V_0 = \frac{V_{max}[S_1]}{Km_{(S_1)} + [S_1] + Km_{(S_1)}\frac{[S_2]}{Km_{(S_2)}}}$$

B

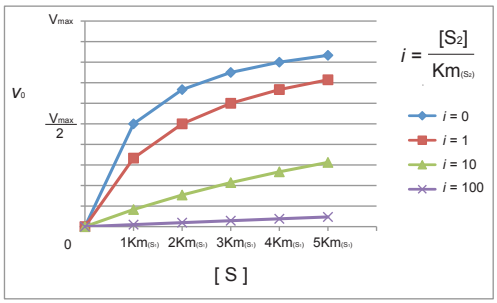


Figure S2. Related to Figure 6. Competitive inhibition. (A) Michaelis-Menten equation for competitive inhibition where the initial velocity (*vo*) of the substrate (S1) is given by presented formula and competing substrate (S2) acts as the inhibitor. (B) Plot of the initial velocity (*Vo*) of the substrate (S1) in the presence of competitor substrate (S2) that behaves as a competitive inhibitor. *i* is the inhibitory effect of the competitor represented by $\frac{[S_2]}{Km_{(S_2)}}$.

Supplemental Tables

Table S1 (related to Figure 1): Data for heatmap in Figure 1

ORF	intronID	SK1-0IAI	SK1-0.5IAI	SK1-2IAI	SK1-5IAI	SK1-7IAI	SK1-9IAI	SK1-11IAI
YPL129W	ANC1-Int	0	1.17837	1.05646	0.56289	0.52526	0.05698	0.29975
YJL024C	APS3-Int	0	0.29191	-0.1099	-0.1854	-0.042	0.5707	-0.0968
YDL137W	ARF2-Int	0	0.30568	-0.0872	0.05422	-0.1707	0.17745	0.5194
YDL029W	ARP2-Int	0	0.37494	0.1016	0.52779	0.22983	0.19919	0.08989
YMR033W	ARP9-Int	0	-0.3922	-0.5684	-0.4755	-0.1601	-0.1858	-0.1043
YER167W	BCK2-Int_TN	0	0.56524	0.62404	1.00356	0.37055	0.48657	-0.1503
YIL004C	BET1-Int	0	-0.0747	-0.4564	-0.1882	-0.118	0.20239	0.25868
YHR101C	BIG1-Int	0	-0.3012	-0.4882	-0.8968	-0.7541	-0.3339	0.08521
YDR099W	BMH2-Int_TN	0	-0.1991	-0.3264	-0.6709	-0.1733	0.29698	0.54377
YLR078C	BOS1-Int	0	-0.1741	-0.8204	-0.8762	-0.4371	-0.1666	0.10622
YPL241C	CIN2-Int	0	-1.2197	-1.1803	-1.266	-1.2179	-0.5329	-0.1606
YKL190W	CNB1-Int	0	-0.6672	-0.9203	-0.659	-1.5997	-1.3463	-0.6159
YLL050C	COF1-Int	0	0.31495	0.31539	0.37641	0.30049	0.00234	0.11405
YIL111W	COX5B-Int	0	-0.6128	-1.3282	-1.4569	-1.7462	-1.7492	-1.6617
YNL130C	CPT1-Int	0	0.50579	-0.4697	-0.1999	0.01409	-0.1866	0.19723
YNL112W	DBP2-Int	0	-1.6127	-0.1396	-1.3094	-1.3146	-1.6586	-1.1986
YER179W	DMC1-Int	0	-0.1803	-1.8534	-2.2044	-1.5651	-0.2243	-0.2415
YDR424C	DYN2-Int1	0	-0.0868	-0.3691	-0.5213	-0.2566	0.02221	0.1432
YDR424C	DYN2-Int2	0	0.00871	-0.1832	-0.4433	-0.4252	-0.0509	0.20792
YBR078W	ECM33-Int	0	-0.1229	-0.3593	-0.1397	-0.1355	0.63785	0.59265
YKR004C	ECM9-Int_TN	0	0.36506	0.33068	-0.2596	-0.5884	-1.1507	-0.8943
YHR123W	EPT1-Int	0	-0.0085	-0.1281	0.35728	0.255	0.33837	0.17767
YBL040C	ERD2-Int	0	0.09087	-0.0903	-0.1023	0.24797	0.8974	0.16154
YEL003W	GIM4-Int_TN	0	0.11927	-0.388	-0.2785	0.39166	0.47701	0.6337
YML094W	GIM5-Int	0	0.32519	0.3686	-0.1911	-0.1164	0.47891	0.61614
YER133W	GLC7-Int	0	0.48717	-0.2976	-0.3038	-0.9284	-0.5141	-0.6166
YMR292W	GOT1-Int	0	-0.3838	-0.1956	-0.5073	-0.4654	-0.3044	-0.0404
YNL038W	GPI15-Int_T	0	-0.1098	-0.1388	-0.0964	0.14272	-0.0549	-0.0901
YGL251C	HFM1-Int	0	-0.0912	-0.217	-0.9184	-0.7052	-0.4064	-0.1387
YDL125C	HNT1-Int	0	0.2661	0.41908	-1.1297	-0.5566	0.57615	0.744
YDR305C	HNT2-Int	0	-0.0235	-0.1981	-0.5026	-1.0257	-0.3831	0.23023
YGL033W	HOP2-Int	0	-0.2563	-0.8785	-1.4857	-0.7833	-0.5424	-0.4929
YBR215W	HPC2-Int_TN	0	-0.6538	-0.4248	-0.6724	-0.6987	-0.6561	-0.4791
YNL004W	HRB1-Int_TN	0	-2.1348	-1.4483	-1.495	-1.8255	-0.8243	-0.554
YML056C	IMD4-Int	0	-0.2926	0.00635	-0.1451	-0.3594	-0.5461	-0.4081
YNL265C	IST1-Int	0	-0.0732	-0.5858	-0.2325	0.84246	0.86526	0.84726
YDL108W	KIN28-Int	0	-0.4369	-0.2671	-0.3295	0.03856	-0.0679	0.08771
YBL026W	LSM2-Int	0	-0.0474	-0.1216	-0.4269	-0.4465	-0.0808	0.28589
YNL147W	LSM7-Int	0	0.2368	0.07158	0.05684	0.05852	0.94785	0.03411
YDR005C	MAF1-Int	0	-0.7419	-1.185	-1.3112	-0.9692	-0.7629	-0.7621
YCR097W	MATA1-Int1	0	-0.0953	-0.7929	-0.5123	-0.4402	0.25668	0.61701
YCR097W	MATA1-Int2	0	0.3473	0.03024	0.17815	0.38617	0.51405	0.66411
YDR318W	MCM21-Int_	0	-0.2546	-0.1454	-0.2653	-0.2758	-0.1178	0.16511
YER044C-A	MEI4-Int	0	-0.2921	-1.19	-1.8311	-1.4207	-0.4676	-0.5277
YGL087C	MMS2-Int	0	-0.6803	-0.4528	-0.3652	0.24439	0.75008	0.78975
YGL183C	MND1-Int	0	-0.9319	-1.9376	-2.3106	-1.8776	-1.1056	-1.0421
YIL106W	MOB1-Int	0	-0.4568	0.0566	-0.5968	-0.8099	-0.5125	0.04545

YFL035C-A	MOB2-Int	0	-0.5081	-1.1742	-1.2495	-1.5743	-1.0321	-1.0379
YGL178W	MPT5-Int	0	0.20107	0.18817	0.25045	0.36327	0.0042	0.03997
YDL079C	MRK1-Int	0	-0.1595	0.31031	0.2495	-0.2342	-0.0515	-0.2707
YKL186C	MTR2-Int1	0	0.91744	0.52887	0.14668	-0.3538	-1.0585	-0.7915
YKL186C	MTR2-Int2	0	0.24194	-0.1916	-0.2859	-0.624	-0.6068	-0.3745
YKL186C	MTR2-Int3_4	0	0.34251	-0.1321	-0.3558	-0.7345	-0.8791	-0.4849
YBR119W	MUD1-Int	0	0.02108	0.01095	-0.6071	0.07843	0.37904	0.34398
YDR397C	NCB2-Int	0	0.11869	-0.2737	0.35794	0.54809	0.57804	0.98541
YJL206C-A	NCE101-Int	0	0.23206	0.03635	-0.0257	-0.0777	0.09174	0.06244
YBR089C-A	NHP6B-Int	0	-0.0861	-0.2768	-0.4454	-0.2626	0.11405	0.34683
YHR077C	NMD2-Int	0	-0.267	-0.2863	-0.6977	-0.541	-0.6822	-0.5806
YJL041W	NSP1-Int	0	-0.3518	-0.5658	-0.4826	-0.4495	-0.2292	-0.1632
YLR093C	NYV1-Int	0	-0.6057	-0.4904	-0.4231	-1.2623	-1.5531	-1.0969
YGL226C-A	OST5-Int	0	-0.0682	-0.4614	-0.7894	-0.6567	-0.4308	0.17642
YBR186W	PCH2-Int	0	-0.2857	-0.9543	-1.7005	-1.2698	-0.6687	-0.3864
YOR122C	PFY1-Int	0	-0.1383	-0.2957	-0.5705	0.16885	0.38219	0.18938
YPL031C	PHO85-Int	0	0.27388	0.05291	0.21168	0.38036	1.14999	0.81221
YER003C	PMI40-Int	0	0.04173	-0.6119	-1.0674	-1.214	-0.3109	-0.0873
YBL018C	POP8-Int	0	-0.0334	0.52612	1.12928	1.1864	0.53505	0.10105
YJL001W	PRE3-Int	0	-0.3074	-0.2207	-0.8095	-0.3873	0.17561	0.64635
YML017W	PSP2-Int_TM	0	-0.1898	-0.2938	-1.3646	-0.8786	-0.4616	-0.5507
YGL063W	PUS2-Int_TM	0	-0.6755	-0.506	-0.5498	-0.6648	-0.9651	-0.5168
YMR201C	RAD14-Int	0	-0.0019	-0.2158	0.22203	0.28834	-0.064	0.07782
YJR021C	REC107-Int	0	-0.3575	-1.2988	-2.1603	-1.2915	-0.6278	-0.4507
YMR133W	REC114-Int	0	-0.0593	-1.2735	-2.5141	-2.168	-1.6905	-1.0732
YNL312W	RFA2-Int	0	-0.4323	-1.0981	-1.5016	-0.575	-0.4584	-0.2562
YDR139C	RUB1-Int	0	0.16998	0.20786	0.93111	0.9692	0.80547	0.65741
YDR129C	SAC6-Int	0	0.19629	-0.3546	0.63262	0.89214	1.26606	0.45031
YPL218W	SAR1-Int	0	0.42018	0.3915	0.30774	0.63089	0.52733	0.45696
YMR079W	SEC14-Int	0	0.46742	0.8584	-0.7069	-1.6709	-1.3783	-1.0054
YBL050W	SEC17-Int	0	0.17196	0.22267	0.10059	0.03727	0.35565	0.63623
YGL137W	SEC27-Int	0	0.62898	0.52111	1.45579	1.59934	1.75132	0.9302
YKL006C-A	SFT1-Int	0	-0.2698	-0.5123	-0.7052	-0.4566	-0.4934	-0.1901
YIL123W	SIM1-Int_TM	0	-0.0944	0.21059	0.20677	0.13108	0.30759	-0.0042
YLR275W	SMD2-Int	0	-0.0459	-0.1913	-0.2762	-0.6037	-0.6479	0.13609
YAL030W	SNC1-Int	0	-0.2763	-0.4698	-0.8231	-0.7132	-0.1462	-0.0482
SNR17A	SNR17A-Int	0	-0.2358	-0.018	-0.6949	-0.5198	0.05232	-0.4192
SNR17B	SNR17B-Int	0	0.00431	-0.2194	-0.5844	-0.476	0.59868	-0.1166
YNL012W	SPO1-Int	0	-0.106	-1.1376	-2.0631	-1.5428	-0.5053	-0.2165
YIL073C	SPO22-Int_T	0	-0.3112	-1.361	-2.9663	-2.5212	-1.5293	-1.4335
YGR225W	SPO70-Int	0	-0.1272	-0.2465	-1.6371	-2.5356	-3.0542	-2.8604
YPL175W	SPT14-Int	0	0.03574	-0.3509	-0.2558	0.0583	0.64981	0.15457
YHR041C	SRB2-Int	0	-0.2101	-0.3883	-0.1012	0.34536	0.67586	0.32861
YML034W	SRC1-Int	0	0.47226	0.05415	-0.7867	-1.5375	-1.3582	-0.9508
YMR125W	STO1-Int	0	0.65267	-0.0753	-3.2255	-3.8298	-2.3217	-1.8606
YNL066W	SUN4-Int_TM	0	-0.1585	-0.0647	0.60665	0.57273	#VALUE!	0.58476
YAL001C	TFC3-Int	0	-0.5207	-0.5494	-0.616	-0.7398	-0.274	-0.4556
YML085C	TUB1-Int	0	0.14768	-0.847	-2.0152	-2.336	-0.8392	-0.0294
YML085C	TUB1-Int_A	0	0.32033	-0.7865	-2.1587	-2.3777	-0.7459	-0.2186

YML085C	TUB1-Int_B	0	0.13164	-0.8252	-2.0709	-2.3222	-1.2527	-0.7878
YML124C	TUB3-Int	0	0.30463	-0.1655	-1.1033	-1.1734	0.00296	0.70932
YLR306W	UBC12-Int	0	-0.1689	-0.4662	-0.0993	-0.159	0.11754	0.2533
YDR092W	UBC13-Int	0	-1.2822	-1.2212	-0.6503	-0.2629	0.30925	-0.0029
YDR059C	UBC5-Int	0	-0.5085	-1.3096	-2.3434	-2.3061	-1.2841	-0.2845
YEL012W	UBC8-Int	0	-0.1262	-0.5318	-0.1837	0.00869	0.34162	0.46607
YDL064W	UBC9-Int	0	0.22011	0.09413	-0.1933	-0.3341	-0.0661	0.05113
YJL130C	URA2-Int_TN	0	-2.317	-0.8186	-0.041	0.56626	0.41707	0.34162
YHR039C-A	VMA10-Int	0	-0.2034	-0.516	-0.1132	0.17568	0.56851	0.49086
YHR012W	VPS29-Int	0	0.11517	0.20195	-0.0356	0.56743	0.6107	0.29086
YBL059W	YBL059W-In	0	0.13733	-0.2273	-0.7004	-0.2946	0.19684	0.51176
YBL091C-A	YBL091C-A-I	0	-0.1443	-0.0935	0.0492	0.00504	0.30373	0.44172
YBR062C	YBR062C-Int	0	-0.1049	-0.8065	-0.3922	-0.049	0.2445	0.49309
YBR101C	YBR101C-Int	0	-0.1093	-0.0228	0.00673	0.12262	0.67118	0.58488
YBR255C-A	YBR255C-A-I	0	0.37358	-0.0106	-0.2584	-0.1716	0.19642	0.58994
YCL002C	YCL002C-Int	0	0.10752	-0.4663	-1.3113	-0.7169	-0.3803	-0.1433
YDL012C	YDL012C-Int	0	0.41512	0.1533	-0.4144	-0.4636	-0.2841	-0.1524
YDL115C	YDL115C-Int	0	-0.4921	-1.0042	-1.5418	-2.0534	-0.8382	-0.6765
YDL189W	YDL189W-In	0	-0.7141	-0.5042	-0.2377	-0.3033	-0.4381	-0.5298
YDL219W	YDL219W-In	0	0.0076	-0.2566	-0.5445	0.58634	0.4732	0.58877
YDR367W	YDR367W-In	0	0.14932	0.03327	-0.2426	-0.1124	0.34789	0.24395
YDR381C-A	YDR381C-A-	0	-0.4934	-0.3791	-0.9049	-0.6609	-0.3152	-0.5822
YER074W-A	YER074W-A-	0	0.0223	-0.1782	-0.5587	-0.1858	-0.6929	-0.6055
YER074W-A	YER074W-A-	0	0.25129	0.07985	-0.2547	0.27274	-0.5377	0.03207
YER093C-A	YER093C-A-I	0	0.19919	-0.2389	-0.6171	-0.9257	-0.022	0.20309
YFR045W	YFR045W-In	0	-0.6152	-0.5251	-0.7522	-1.0078	-1.1503	-0.4779
YGL232W	YGL232W-In	0	-0.1331	-0.3673	-0.3622	-0.3624	0.0694	0.16143
YGR001C	YGR001C-Int	0	-1.4167	-1.0656	-0.4868	-0.6708	-0.5764	-0.1149
YGR001C	YGR001C-Int	0	-1.3413	-0.8987	-0.2166	-0.5272	-0.7095	0.17063
YHR079C-A	YHR079C-A-	0	-0.0946	-0.1054	0.08849	0.42784	0.20129	0.15844
YHR097C	YHR097C-Int	0	0.81885	-0.2797	0.28418	0.05398	-0.5067	0.13133
YHR199C-A	YHR199C-A-	0	0.35232	0.43644	0.62483	1.12859	1.14239	0.67403
YIL156W-A	YIL156W-A-I	0	-0.1802	-0.1122	-0.3165	-0.0721	-0.0196	-0.1545
YPR028W	YIP2-Int	0	0.18745	-0.8453	-0.386	-0.395	-0.6697	-0.822
YNL044W	YIP3-Int	0	0.71806	0.17264	-0.8924	-1.4307	-0.8212	-0.2889
YKL158W	YKL158W-In	0	-0.0685	-0.0679	0.88854	2.12684	2.38953	1.91992
YKR095W-A	YKR095W-A-	0	-0.4553	-0.3984	-0.1173	-0.1609	-0.2485	0.1546
YLR054C	YLR054C-Int	0	2.40296	0.91075	-0.5795	-1.3661	-1.136	-0.3192
YLR128W	YLR128W-In	0	0.28048	0.12267	0.39665	0.51284	0.29343	0.3641
YLR199C	YLR199C-Int	0	-0.2913	-0.3158	-0.4551	-0.6474	0.01841	0.04013
YLR211C	YLR211C-Int	0	-1.9208	-1.9146	-1.4295	-1.4806	-1.0587	-1.4282
YLR426W	YLR426W-In	0	-1.0853	-1.2108	-0.8991	-0.9225	-0.9861	-0.6593
YLR445W	YLR445W-In	0	0.26072	0.08405	-1.3205	-1.3008	-1.1272	-0.5356
YML036W	YML036W-In	0	-0.3104	-0.4097	-0.672	-0.2569	-0.5134	-0.1786
YML067C	YML067C-Int	0	0.06152	0.29784	0.3855	0.33225	0.49514	0.56973
YMR147W	YMR147W-In	0	-0.0877	-0.475	-1.0068	-0.5084	-0.3185	-0.4934
YNL050C	YNL050C-Int	0	-0.4961	-0.5959	-0.7006	-0.6254	-0.5476	-0.4745
YNL138W-A	YNL138W-A-	0	0.08104	0.18128	0.28046	0.90574	1.00567	0.59271
YNL246W	YNL246W-In	0	0.41444	0.01934	0.11838	0.30967	0.23035	0.20399

YNR053C	YNR053C-Int	0	0.75419	0.58081	0.8706	-0.0822	-0.6916	-0.9816
YOL047C	YOL047C-Int	0	-0.028	-0.0359	-4.3753	-4.9773	-3.6511	-3.4113
YOL048C	YOL048C-Int	0	0.06689	-0.0617	-0.101	-0.0326	-0.1675	-0.5609
YPL109C	YPL109C-Int	0	0.7877	0.26404	0.35403	0.61542	0.84053	0.80067
YPL230W	YPL230W-Int	0	-0.2669	-0.5579	-0.1538	0.11773	0.26259	0.02205
YPR063C	YPR063C-Int	0	-0.1891	-0.2534	-0.4126	-0.2827	0.10865	-0.0419
YPR098C	YPR098C-Int	0	0.26389	0.25703	0.25584	0.53491	0.69943	0.55501
YPR153W	YPR153W-In	0	-0.4346	0.054	0.48022	0.42556	0.49461	0.20685
YPR171W-A	YPR171W-A-	0	0.04523	0.5581	0.42512	0.46388	0.24341	0.39176
YDR381W	YRA1-Int	0	-0.9276	-0.6392	-0.6057	-0.4461	-0.7091	-0.6122
YHR016C	YSC84-Int	0	-0.5343	-1.0123	-0.4756	-0.6562	-0.006	-0.0049

Table S2 (related to Figure 1, 3, 4): Expression of spliceosomal components during meiosis, rapamycin treatment, and *IFH1* down-regulation.

Gene Symbol	meio_2h	meio_5h	meio_7h	meio_9h	rapa_10 min	rapa_60 min	prp4-1_IFH1
BRR1	-0.19	-0.80	-0.72	0.03	-0.13	0.72	-0.01
BRR2	0.46	0.70	0.38	0.42	-0.53	-0.75	0.44
BUD13	-0.46	-0.36	-0.95	-0.99	0.20	-0.06	0.45
BUD31	0.12	-0.13	-0.12	0.27	-0.68	-0.06	-0.31
CBC2	-0.28	1.43	1.78	1.32	-0.24	-0.93	0.99
CBF2	0.69	0.83	0.32	0.17	-0.67	-0.66	0.16
CDC40	0.17	0.38	0.40	0.19	-0.80	-0.50	0.50
CEF1	-0.14	-0.05	-0.24	-0.16	-0.76	-0.58	0.50
CLF1	-0.04	-0.05	-0.62	-0.65	-0.18	0.34	0.27
CUS1	0.54	0.50	0.00	0.02	0.18	0.57	0.04
CUS2	0.00	-0.22	-0.48	-0.74	-0.31	-0.35	0.07
CWC15	0.46	0.00	0.18	0.06	0.02	0.39	0.18
CWC2	0.13	0.09	0.03	-0.03	-0.98	-0.71	0.99
CWC21	0.02	-0.46	-0.52	-0.46	-0.37	0.08	0.70
CWC22	-0.02	-0.06	0.15	-0.13	0.03	0.73	0.39
CWC23	-0.25	-0.16	0.18	-0.11	0.02	0.00	-0.51
CWC24	0.06	0.50	0.30	0.03	-0.80	-0.63	-0.08
CWC25	0.44	0.13	0.18	0.22	-0.49	0.15	0.09
CWC27	-0.06	-0.29	-0.24	0.55	-0.21	-0.04	-0.26
DBP1	0.61	0.47	0.27	0.50	1.31	1.53	-1.21
DBP2	0.33	0.37	0.26	0.03	-2.32	-6.15	1.65
DBP5	-0.22	0.32	0.03	0.35	-0.42	-1.17	0.52
DED1	-0.06	-0.51	-0.42	-0.97	-0.68	-1.06	0.32
DHH1	0.35	0.27	0.30	0.33	0.11	0.05	-0.62
DIB1	-0.36	-0.62	-0.69	-0.90	-0.06	0.36	-0.40
DIS3	0.42	0.13	0.09	0.00	-0.80	-1.15	0.88
DRS1	-0.55	0.77	1.54	1.64	-3.06	-3.59	1.46
ECM2	0.42	0.05	0.25	0.38	0.12	1.32	0.20
FAL1	0.43	-0.02	-0.46	-0.07	-3.54	-2.51	1.54
HSH155	0.13	0.31	0.27	0.12	-0.69	0.01	0.59
HSH49	0.75	0.01	-0.15	0.03	-0.33	0.04	0.33
IST3	0.59	0.53	0.54	0.11	0.30	1.29	0.29
ISY1	0.05	-0.45	-0.55	-0.08	-0.08	0.19	0.27
LEA1	0.14	-0.12	0.04	-0.24	0.79	1.28	0.22
LIN1	0.66	1.04	0.58	0.11	-0.09	0.84	-0.27
LSM2	-0.60	-0.03	0.12	0.12	-0.10	-0.71	0.55
LSM3	1.02	0.98	0.81	0.84	-0.66	-0.76	0.76
LSM4	0.03	0.34	0.08	0.53	-0.08	-1.44	0.39
LSM5	0.67	1.02	0.91	0.48	-0.21	-1.51	0.38
LSM6	-0.05	-0.34	-0.11	-0.02	0.00	-0.41	0.17
LSM7	-0.37	-0.37	-0.59	-0.68	0.17	-1.16	0.43
LSM8	0.24	0.11	-0.47	-0.74	0.42	-0.14	-0.19
LSR1	0.39	2.10	1.32	1.40	-1.40	-0.66	0.08
LUC7	0.76	0.79	1.06	1.00	0.80	0.33	-0.03

MLP1	0.25	1.11	1.18	0.99	-0.41	-0.34	-0.38
MSL1	0.24	0.25	0.21	0.04	-0.01	0.66	0.37
MSL5	-0.27	-0.20	0.16	0.06	-0.67	-0.64	0.35
MUD1	0.14	0.54	0.07	0.07	-0.09	-0.57	-0.08
MUD2	-0.02	0.13	-0.18	-0.29	-0.89	-1.04	0.28
NAM8	-0.30	0.21	0.41	-0.09	0.40	0.31	0.06
NPL3	-0.36	-0.19	0.13	0.41	-0.22	0.08	0.32
NTC20	0.05	0.22	-0.20	-0.15	-0.31	0.13	-0.56
NTR2	0.13	0.04	-0.46	-0.45	-0.02	0.26	-0.24
PAB1	-0.59	0.13	0.74	0.62	-0.71	-1.53	0.22
PML1	0.04	-0.04	0.25	0.08	-0.30	-0.28	0.03
PRP11	-0.04	-0.33	-0.31	-0.23	-0.74	-0.93	0.46
PRP16	0.23	0.33	0.17	0.31	-0.69	0.00	0.52
PRP18	-0.31	-0.13	-0.20	-0.48	0.34	0.06	-0.07
PRP19	-0.08	-0.28	-0.46	-0.23	-0.77	-0.88	0.74
PRP2	0.62	0.82	1.19	1.11	-0.61	-0.10	0.34
PRP21	0.36	0.26	-0.07	0.17	-0.03	-0.13	0.04
PRP22	0.31	0.63	0.24	0.20	-0.06	0.18	0.09
PRP24	0.20	-0.23	-0.78	-0.39	-1.32	-0.81	0.99
PRP28	0.34	-0.04	-0.06	0.47	-0.48	-0.82	0.24
PRP3	0.17	0.07	-0.19	-0.02	-0.42	0.47	0.03
PRP31	0.19	0.67	0.46	0.07	-0.41	-0.15	0.35
PRP38	-0.03	0.25	0.40	0.46	0.09	0.36	0.13
PRP39	0.30	0.41	0.74	0.98	-0.43	-1.00	0.47
PRP4	-0.13	0.59	0.63	0.31	0.35	0.31	-0.20
PRP40	-0.02	0.52	0.08	-0.24	-0.04	0.90	0.24
PRP42	-0.35	-0.07	0.17	-0.04	0.02	-0.44	0.33
PRP43	-0.89	-0.53	-0.35	-0.55	-1.78	-3.40	0.97
PRP45	-0.16	-0.09	-0.22	-0.48	-0.19	-0.29	0.47
PRP46	0.05	0.01	0.01	0.00	-0.45	-0.60	0.26
PRP5	0.71	1.20	0.74	0.50	0.10	1.16	-0.17
PRP6	-0.24	-0.53	-0.42	-0.37	-0.55	-0.73	0.16
PRP8	0.17	0.73	0.14	0.33	-0.53	-0.50	0.02
PRP9	-0.12	0.08	0.61	0.52	-0.65	-0.29	0.54
RAT1	0.77	0.26	-0.22	0.09	-0.60	-0.31	0.69
RDS3	-0.51	0.26	0.93	0.36	-0.16	-0.45	0.24
RSE1	0.76	0.59	-0.17	-0.29	0.02	-0.21	-0.52
SAD1	-0.05	0.72	2.10	1.89	-0.39	-0.35	0.54
SKI6	-0.39	-0.03	0.24	0.16	-1.02	-1.32	0.84
SLU7	-0.15	-0.15	0.35	-0.05	-0.48	-0.25	-0.04
SMB1	-0.30	-0.09	0.28	-0.10	-0.53	-0.92	1.19
SMD1	-0.47	-0.79	-0.30	0.13	-0.12	-0.68	0.37
SMD2	0.25	1.19	0.80	0.46	-0.57	-0.08	1.06
SMD3	-0.13	-0.47	-0.82	-0.74	-0.28	-0.39	0.49
SME1	-0.85	-0.96	-0.74	-0.50	-0.69	-0.73	0.82
SMX2	-0.12	0.36	1.02	0.92	-0.05	-0.25	1.91

SMX3	-0.19	-0.23	-0.14	-0.39	-0.38	-0.52	-0.11
SNP1	0.46	0.12	0.06	0.11	-0.53	0.49	0.11
SNR14	0.24	0.37	-0.21	0.19	0.64	-0.68	3.33
SNR19	0.20	0.71	0.44	0.24	-0.34	0.29	0.74
SNR6	-0.06	0.01	0.04	0.24	-2.92	-1.22	0.00
SNR7-L	0.22	0.96	0.31	0.49	-0.27	0.24	-1.23
SNT309	-0.41	-0.61	-0.20	0.32	-0.21	-0.28	0.16
SNU114	-0.02	0.38	0.73	0.64	-0.68	-0.64	0.50
SNU13	-1.31	-0.62	-0.11	-0.20	-0.99	-3.20	0.68
SNU23	-0.07	0.05	-0.41	-0.56	-0.17	-0.86	-0.13
SNU56	-0.56	1.79	3.58	4.39	-0.59	-0.29	0.48
SNU66	0.02	0.01	0.14	0.08	-0.68	0.14	0.08
SNU71	0.61	0.91	0.97	0.58	0.05	0.93	0.00
SPP2	0.76	0.38	0.40	0.45	-0.19	0.46	0.02
SPP381	-0.12	0.05	0.33	0.19	-0.30	-0.36	0.27
SPP382	0.48	0.62	0.04	0.02	0.06	0.01	-0.09
SSA4	-1.19	0.13	0.73	0.34	0.42	0.48	-2.24
STO1	-0.06	0.31	0.38	0.57	-0.21	-1.17	0.04
SUB2	-0.82	-0.55	-0.78	0.00	-0.20	-1.06	0.35
SYF1	0.24	0.92	1.60	1.47	-0.48	-0.48	0.17
SYF2	0.02	-0.31	0.11	0.65	-1.24	-0.47	0.31
YHC1	-0.93	-1.03	-1.25	-1.12	-0.02	0.28	0.59
YJU2	0.01	-0.13	-0.42	-0.22	0.16	0.74	0.16
YRA1	0.08	-0.05	0.01	0.48	0.14	-2.35	0.71
YSF3	-0.12	-0.21	-0.56	-0.60	0.09	0.10	0.61

Table S3. Yeast Strains.

STRAIN	GENOTYPE	SOURCE NOTES
SK1-K8409	*MATa/MATalpha HO/HO URA3-tetR-GFP/URA3-tetR-GFP URA3:tetO224/URA3:tetO224 REC8-HA3/REC8-HA3 his3::hisG/his3::hisG trp1 /trp1*	ATCC
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
EMY1	*MATalpha ume6::KANMX6 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Spore from heterozygous diploid knockout collection; Open Biosystems
EMY2	*BY4741, k-HIS3:GAL1-IFH1*	Integration
SRY4-1b	*MATalpha prp4-1 ade2- leu2-3,112 ura3-52 his3-Δ200*	S. Ruby
EMY3	*prp4-1, k-HIS3:GAL1-IFH1*	spore from EMY2 X SRY4-1b
SRY11-1d	*MATalpha prp11-1 ade2- his- his4- leu2- tyr1- ura3-52*	S. Ruby
EMY4	*prp11-1, k-HIS3:GAL1-IFH1*	spore from EMY2 X SRY11-1d

Table S4. RT-PCR and RT-qPCR primers.

PRIMER NAME	SEQUENCE
qPCR MEI4-inF	5' acgtgaaattgtcacatcctt 3'
qPCR MEI4-exF	5' ccaggaatcctacgttgtgg 3'
qPCR MEI4-exR	5' aggcgcaacccatttgtat 3'
qPCR DMC1-inF	5' gaggttctttccccctttctt 3'
qPCR DMC1-exF	5' gttttgtcaacaacaagaagacat 3'
qPCR DMC1-exR	5' tgataaggagtacacacgctgtc 3'
qPCR SEC14-inF	5' agttctgtctatatgaagcaaaaatga 3'
qPCR SEC14-exF	5' agaaaaggaatttttagaatcctaccc 3'
qPCR SEC14-exR	5' gttcaatgaaaccagcgtctt 3'
qPCR CPT1-inF	5' tgcaccctaaatcttctgtgg 3'
qPCR CPT1-exF	5' tgatgaccgctctttccttt 3'
qPCR CPT1-exR	5' ctggtcaaaatacgggtcgt 3'
qPCR HNT1-inF	5' cacaccaatgatggcgatag 3'
qPCR HNT1-exF	5' gcgaaattccatccttcaaa 3'
qPCR HNT1-exR	5' ggcatagcatcggtaaggaa 3'
qPCR MOB2-inF	5' tctggacctgcgttatcattt 3'
qPCR MOB2-exF	5' aaaaccagccccttaatgttg 3'
qPCR MOB2-exR	5' cggggaaacttgtttgagaa 3'
qPCR RPL34A/B-inF	5’ gaagtgattactaacattaatgggaaa 3’
qPCR RPL34A/B-exF	5' aggttgttaagaccccaggtg 3'
qPCR RPL34A/B-exR	5' gaaccaccgtaagctctgga 3'
qPCR RPS16A-exF	5' cgatgaacaatccaagaacg 3'
qPCR RPS16A-exR	5' tctggaacgagcacccttac 3'
qPCR RPL28-exF	5' ggtggtcaacatcaccacag 3'
qPCR RPL28-exR	5' ggcttccagaaatgagcttg 3'
qPCR RPS5-F	5' actgaccaaaacccaatcca 3'
qPCR RPS5-R	5’ ttgacgtctagcagcaccac 3’
qPCR RPL11A/B-F	5’ cagaggtccaaaggctgaag 3’
qPCR RPL11A/B-R	5’ taccgaaaccgaagttaccg 3’
qPCR IFH1-F	5’ ttctggtaaactgccagcaaa 3’
qPCR IFH1-R	5’ ggctaaatcttcttggcctcg 3’
qPCR SEC65-F	5' catatggccctgatttcgac 3'
qPCR SEC65-R	5' ggcttgaacgacttttctgc 3'
SPO22-F1	5' tcagaccacaacgttaactc 3'
SPO22-R1	5' tccatagacttgatgctgca 3'
MEI4-F1	5' gaggcaaactggaagatatg 3'
MEI4-R1	5' agagcacctacatcttcgac 3'
PCH2-F1	5' caagatcaactggagtcaag 3'
PCH2-R1	5' tcgtctacaggaaatgtccg 3'

Supplemental Experimental Procedures

Transcriptome Profiling

The microarray data in Fig1 is from four independent meiotic time courses where each meiotic time point was compared to a reference pool RNA comprised of 50% time zero RNA plus 10% each of time 2 hours, 5, hours, 7 hours, 9 hours and 11 hours was used as an arbitrary reference pool (Munding et al., 2010). To evaluate splicing changes the Intron Accumulation Index (IAI) (IAI= $\log_2$ ratio of intron probe - $\log_2$ ratio of exon2 probe) (Clark et al., 2002) was calculated for each intron/time point. The t=0 IAI was then subtracted from each time point IAI to give the change in IAI.

To estimate the magnitude of a change in IAI that would constitute a true splicing change we developed a control distribution of IAIs as a background model that would capture noise in the IAI measurement. To do this we compared IAIs derived from biological replicate samples that should show no change in IAI. We calculated the apparent change in IAI for each of the 156 genes by comparing the two samples from 2 hours of meiosis, the two from 5 hours and the two from 7 hours and averaged these IAIs to create the control distribution. We determined that only 10 of 156 genes showed a change in IAI of >40% (1.4 fold) in the control distribution, suggesting that this threshold is associated with an FDR of less than 0.1.

To generate the image in Fig 1A, we used Gene Cluster 3.0 (de Hoon et al., 2004) and Java Treeview (Saldanha, 2004). The pie chart in Fig1C includes 156 intron-containing genes whose expression does not decrease more than 2-fold (Log_2 Ratio ≥ -1.0) during the meiotic time course. Introns with a zero-subtracted IAI < -0.5 (indicating at least a 40% improvement in splicing) at two out of three mid-meiotic time points (t=2, 5, 7h after induction of meiosis) are called as “increased splicing”; similarly introns with a zero-subtracted IAI ≥ 0.5 at two out of three mid-meiotic time points are called “decreased splicing”, while no change in splicing is signified by 0.5 > IAI > -0.5.

The data described in Fig 3 and Fig 4 was collected using RNA-Seq. RNA from the respective strains was isolated and DNased using Turbo DNase (Life Technologies) and

RNA quality was assayed using the 2100 Bioanalyzer (Agilent). Poly(A) RNA was selected from 20µg total RNA using oligo-(dT) Dynabeads (Life Technologies). Strand-specific cDNA sequencing libraries were prepared as described in (Yassour et al., 2010) and paired-end sequenced on the HiSeq2000 (Illumina). Reads were mapped using TopHat (Trapnell et al., 2009) which aligns reads using Bowtie2 (Langmead and Salzberg, 2012). Changes in gene expression were estimated by comparing the $\log_2$ ratios of the exon 2 reads. Splicing changes were estimated by calculating an IAI using counts of intron-containing reads relative to exon 2 reads in treated samples relative to control. To produce the box plots in 3C, intron-containing events with junction reads and at least 50 exon 2 reads were used. To produce the histogram in Fig 3D, only introns with splice junction reads and at least 50 exon 2 reads whose gene expression did not change by 2-fold or greater were used. The IAIs of the biological replicates were averaged. To produce the histogram in Fig 4C, introns with splice junction reads and at least 50 exon 2 reads whose gene expression did not change by 2-fold or greater were evaluated.

To call splicing changes using RNA-seq data, we created a control distribution of IAI changes observed in replicate samples where no splicing change should occur, as described above for the array-derived IAIs. In this case the control distribution indicated that an IAI with absolute value >0.3 (or ±25%) could serve as a threshold for splicing change with an FDR of about 0.2.

RT-PCR and qPCR

Relative transcript expression was measured using RT-qPCR of RNA extracted from at least three biological replicates. The graphs shown in Fig 3A and Fig 4B is a measure of expression of a given transcript relative to *SEC65*, a gene whose expression remains constant under all conditions used in this study. For this analysis, two primer pairs were used, one set (within the second exon for intron-containing genes) to measure total RNA for a given

gene and one set to measure *SEC65* expression. Relative amount of transcript = $2^{(-\Delta\Delta Ct)}$ where $\Delta\Delta Ct=(Ct_{geneX} - Ct_{SEC65})$.

Splicing efficiency measured by RT-qPCR (such as in Fig 1C and Fig 4D) was calculating using the percent intron-containing transcript from RNA extracted from at least three biological replicates. This analysis employed two primer sets for each gene: one pair for intron-containing pre-mRNA (spanning the 3' splice site), and one set for total RNA (within the second exon). Threshold cycles were determined using reactions containing the same amount of cDNA and the % intron-containing RNA = $2^{(-\Delta\Delta Ct)} * 100$, where $\Delta\Delta Ct=(Ct_{inF\text{-}exR} - Ct_{exF\text{-}exR})_{geneX}$.

Splicing efficiency measured by semi-quantitative RT-PCR (such as in Fig 2B and Fig 3) was determined using the Agilent 2100 Bioanalyzer using RNA extracted from at least three biological replicates. Molar amounts of each PCR product were used to estimate splicing efficiency as follows: %spliced= ((molarity of spliced peak)/(molarity of unspliced peak+ molarity of spliced peak)) *100. Bioanalyzer % spliced values from triplicate biological replicates were averaged and standard deviations are shown.

All RT-PCR and RT-qPCR primers are described in Table S4.

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CHAPTER 4

CONCLUSION

Gene expression programs are controlled by regulatory factors that orchestrate the timely and coordinated expression of specific genes that contribute to cell identity (reviewed in Ben-Tabou de-Leon and Davidson, 2007; Braunschweig et al., 2013). Regulatory factors may affect various aspects of gene expression, such as transcription or splicing, at different stages of the program but they work coherently to promote a particular cell state. The studies presented in this thesis were aimed at understanding how pre-mRNA splicing regulation is integrated with developmental gene expression programs. The meiotic developmental program in *Saccharomyces cerevisiae* was chosen as a model system for these studies as it is known to require both transcription and splicing regulation (Chu et al., 1998; Cooper et al., 2000; Davis et al., 2000; Engebrecht and Roeder, 1989; Engebrecht et al., 1991; Nakagawa and Ogawa, 1999; Primig et al., 2000).

Work described here examined splicing regulation conferred by a specific splicing factor as well as through a general regulatory mechanism. The studies reported in Chapter 2 profiled the contribution of the Mer1 splicing factor to progression of the meiotic gene expression program (Chapter 2). The Mer1 splicing factor is known to be required for meiosis due to its role in splicing activation of target transcripts (Cooper et al., 2000; Davis et al., 2000; Engebrecht et al., 1991; Nakagawa and Ogawa, 1999). The Mer1 splicing network was first characterized and found to contain Mer1 and four Mer1-target transcripts (*MER2/REC107, MER3/HFM1, SPO22/ZIP4,* and *SPO70/AMA1*). Next the contribution of Mer1 to the meiotic gene expression program was studied. The Mer1 splicing network is found to be integrated between two transcriptional networks (the Ume6 and Ime1 transcriptional network and the Ndt80 transcriptional network) that function sequentially during meiosis. The Ume6 and Ime1 transcriptional network promotes transcription of the Mer1 splicing network while expression of two Mer1-responsive genes (promoted by Mer1) is required for induction of the Ndt80 transcriptional network and continued progression through meiosis. Additionally, simultaneous induction of Mer1 and Mer1-activated transcripts is shown

to create an expression delay of the Mer1-activated genes that depends on accumulation of the Mer1 protein. This novel splicing-dependent timing mechanism appears to contribute to productive meiosis. Qiu and colleagues expressed intronless cDNA versions of the four target transcripts from their native promoters to try to bypass the requirement for Mer1 (Qiu et al., 2011). Consistent with the importance of the splicing-dependent delay mechanism, this resulted in a 30% decrease in sporulation efficiency that was attributed to premature expression of *SPO70/AMA1*. The study presented in Chapter 2 is the first to dissect direct effects (splicing targets of Mer1) from indirect effects (delayed progression through meiosis due to failure to express two Mer1-target transcripts) and assess the global contribution of both direct and indirect effects to a gene expression program.

The microarray studies performed to analyze the genome-wide contribution of the Mer1 to the meiotic developmental program revealed a general increase in splicing that occurs during meiosis in wild type cells. The splicing increase involves both meiotically-induced genes as well as genes constitutively expressed during vegetative growth and meiosis. In Chapter 3 the molecular basis for this global splicing improvement was studied and found to occur due to a relief in competition among intron-containing genes for the splicing machinery. Pre-mRNAs appear to compete with each other for a limiting splicing machinery and competition is relieved by repression of ribosomal protein genes (RPGs). Since RPG pre-mRNAs account for about 90% of all pre-mRNA splicing events in vegetatively growing cells (Ares et al., 1999; Holstege et al., 1998; Lopez and Seraphin, 1999; Warner, 1999), their repression during meiosis results in a reduction of the pre-mRNA pool. Repression of RPGs occurs as part of the meiotic gene expression program and may be required for efficient splicing of meiotic transcripts, most of which contain non-canonical splice signals (Table 1-2). Furthermore, the poor competitive ability of the meiotic transcripts likely contributes to their repression during vegetative growth when RPGs are highly expressed. This study is the first to show that changes to the pre-mRNA pool can globally

alter splicing by either decreasing or increasing demand for the spliceosome. We call this novel mechanism of splicing regulation trans-competition control.

At what step in splicing do transcripts compete?

Repression of RPGs specifically improves the first step of splicing in reporter substrates that contain mutations at the intron branchpoint. Based on this, we hypothesize that transcripts are competing for a factor that involves intron branchpoint which functions early in the splicesome assembly pathway. A possible step at which transcripts compete is the formation of the prespliceosome, a step that stabilizes the interaction between the pre-mRNA and the U2 snRNP. Candidate factors that might be limiting and contribute to prespliceosome formation include those associated with the pre-mRNA branchpoint early in the process as well as factors required to prepare the U2 snRNP for spliceosome assembly. For example, Mud2 (human U2AF65) and BBP (human SF1) promote recognition of the pre-mRNA branchpoint (Rutz and Seraphin, 2000; Wang et al., 2008), and Sub2 (human UAP56) may be needed to remove Mud2/BBP from the branchpoint so that U2 snRNA can base pair with the intron (Fleckner et al., 1997; Kistler and Guthrie, 2001; Wang et al., 2008). Factors that promote U2 snRNA rearrangements in anticipation of spliceosome assembly, such as the formation of stem IIa (Yan et al., 1998; Zavanelli and Ares, 1991) and the BSL (Perriman and Ares, 2010) mediated by Prp5 and Cus2 (Perriman and Ares, 2000; Perriman et al., 2003; Perriman and Ares, 2007) may also be limiting. The pre-mRNA reporter used in Chapter 3 is derived from the actin intron however, different pre-mRNAs clearly have distinct dependencies on conserved components of the splicing machinery (Burckin et al., 2005; Clark et al., 2002; Park et al., 2004; Pleiss et al., 2007b), suggesting transcripts may compete for different limiting factors depending on the context, such that the rate-limiting step in vivo may be different between transcripts.

Systems biology of gene expression

Systems biology of gene expression is the integration of cellular processes that work coherently to drive cellular processes (reviewed in Fischbach and Krogan, 2010). The experiments in Chapter 3 indicate that changes in gene expression must be assessed globally because unrelated genes become connected if their expression depends on a common regulatory factor. In a parallel post-translational example, Cookson et al showed that activity of unrelated proteins is coupled due to dependence on a common protease for degradation (Cookson et al., 2011). A number of recent studies have reported changes in regulation of splicing under certain conditions (in yeast these include: Bergkessel et al., 2011; Munding et al., 2010; Plass et al., 2012; Pleiss et al., 2007a; Yassour et al., 2009). However, its important to consider that changes in splicing may occur indirectly due to changes in demand for the spliceosome.

Regulatory factors may act as scaffolds to promote specificity of the enzyme to a set of substrates. Schrogelbauer and colleagues show that specificity of the kinase IKKb for IkB is directed by NEMO (Schrofelbauer et al., 2012). In the absense of NEMO, IKKb hyperphosphorylates alternative substrates. Similarly, a splicing factor may direct the splicing machinery to a subset of pre-mRNAs containing the binding site for the splicing factor. However, in the absence of the splicing factor, splicing of other transcripts not dependent on the splicing factor should increase due to their increased competitive ability. A limiting factor may also be a splicing factor that regulates only a few transcripts. For example, a network of pre-mRNAs that depend on the same splicing activator may be in competition with one another under conditions where concentration of pre-mRNAs dependent on the factor outnumbers the splicing activator molecules.

Perspectives for studying regulation of gene expression

Gene expression processes are coupled together mechanistically (i.e. transcription is coupled to RNA processing and processing is coupled to translation), therefore future studies exploring the contribution of a regulatory factor to a gene expression program must try to

integrate the network of the factor with other networks that promote the program. Comprehensive genome-wide studies that dissect and explore both the direct and the indirect effects of a regulatory factor will aid towards understanding how the factor contributes to the gene expression program as a whole rather than through individual direct targets. This task will be especially complex in higher eukaryotes where RNA binding proteins often function in multiple aspects of gene expression (such as the SR proteins which promote splice site recognition, mRNA export, non-sense mediated decay of premature termination codon-containing transcripts, and translation of substrates containing the enhancer sequence (Sanford et al., 2005)). Additionally, one regulatory factor may target tens to hundreds or even thousands of substrates, which further complicates dissection of direct and indirect effects.

A commonly ignored aspect of regulation of gene expression is an accounting of regulatory factors required along the gene expression pathway relative to the total number of substrates. When studying an enzymatic reaction, one should consider the concentration of substrate, enzyme and competitive “inhibitors” of a reaction to understand the rate and concentration of product formation. The experiments presented in this thesis display the importance of understanding these principles towards splicing regulation and should be considered for other steps of the gene expression pathway. Conditions that change the effective load of substrates on a limiting regulatory factor could have global effects on regulation of gene expression. Quantitative measurements of both substrates and regulatory factors at steady state versus during transitions between cell state will need to be performed to address the capacity of gene expression processes. This information will be useful in understanding the underlying mechanisms driving transitions between cell states. Furthermore, deregulation of the balance of substrate to regulatory factor ratios is likely to be important for both directly and indirectly effecting changes in gene expression that lead to transformation of normal cells to disease or cancer cells. Thus to obtain a comprehensive picture of a gene expression program we will need to understand how transcriptional and

post-transcriptional networks are integrated together as well as the capacity of the gene expression machinery to carry out the induced networks.

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