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

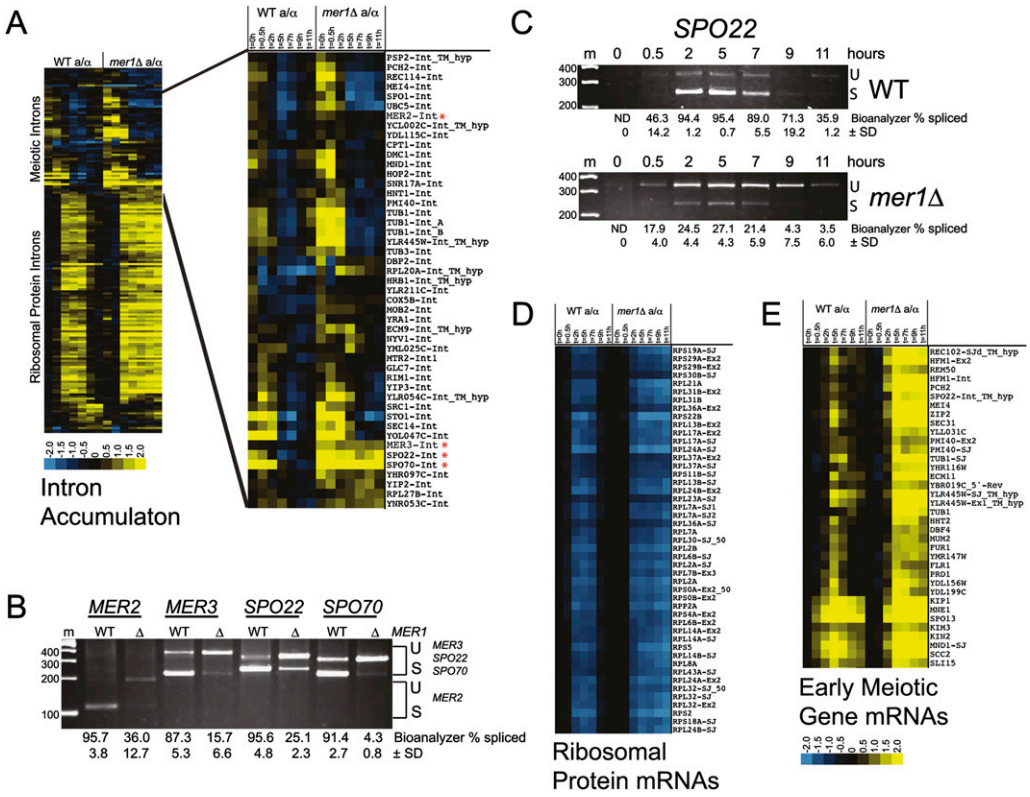

Figure 1. Meiotic gene expression in the absence of the Mer1p splicing factor. (*A*) Splicing changes as represented by intron accumulation indexes (Clark et al. 2002) during the time course of wild-type (*left* panel) and *mer1*Δ (*right* panel) meiosis. The asterisk (*) indicates introns whose splicing 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*,

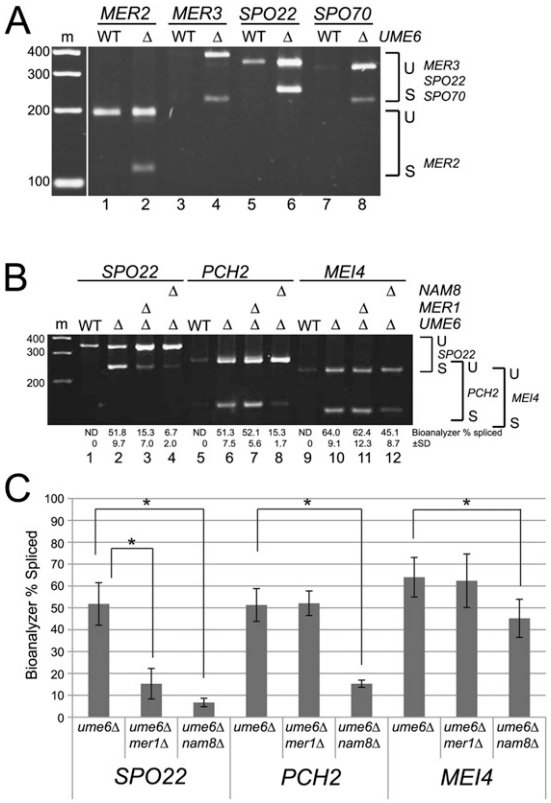

Figure 2. Derepression of meiotic genes in vegetative cells reveals splicing factor requirement for meiosis. (*A*) Expression and splicing of *MER2*, *MER3*, *SPO22*, and *SPO70* in wild-type (WT) and *ume6*Δ vegetative cells. (*B*) Expression and splicing of *SPO22*, *PCH2*, and *MEI4* in wild-type (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 *ume6Δ* 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 *mer1Δume6Δ* and *nam8Δume6Δ* 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 *mer1Δume6Δ* and *nam8Δume6Δ* 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*

	Meiotic		ume6Δ vegetative		
Gene	Induction log ratio	Peak induction	Induction log ratio	Ume6 induced?	Reference
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.

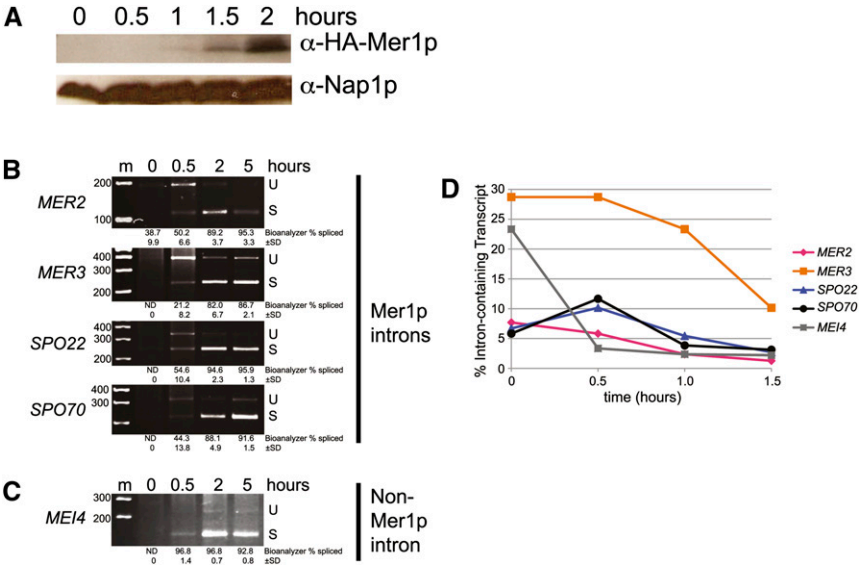

Figure 3. Accumulation of mRNA for Mer1p-responsive genes is delayed relative to other Ume6p-activated genes. (*A*) Western blot measuring Mer1p expression in wild-type cells early in meiosis. Nap1p was used as a loading control. (*B*) Measurement of expression and splicing of the Mer1p-responsive *MER2*, *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 *spo70*Δ 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 *spo70*Δ 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 *mer1*Δ strain at 9 h and found the majority (70.3%, 147 of 209) of *mer1*Δ cells resemble the *mer2*Δ phenotype and complete the meiotic gene expression program. A detectable fraction of *mer1*Δ cells arrests at positions similar to the arrest points of *mer3*Δ and *spo22*Δ (prophase) (11.0%, 23 of 209) or *spo70*Δ (segregated chromosomes but no spores) (18.7%, 39 of 209), suggesting that the phenotype of individual *mer1*Δ 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 *mer3*Δ and *spo22*Δ 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 *mer1*Δ 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 *mer3*Δ and *spo22*Δ strains, and, to a lesser extent, in *mer1*Δ (Fig. 5A, lanes 4,8,10). In wild-type, *mer2*Δ, and *spo70*Δ 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 *mer1*Δ 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 *mer1*Δ 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.

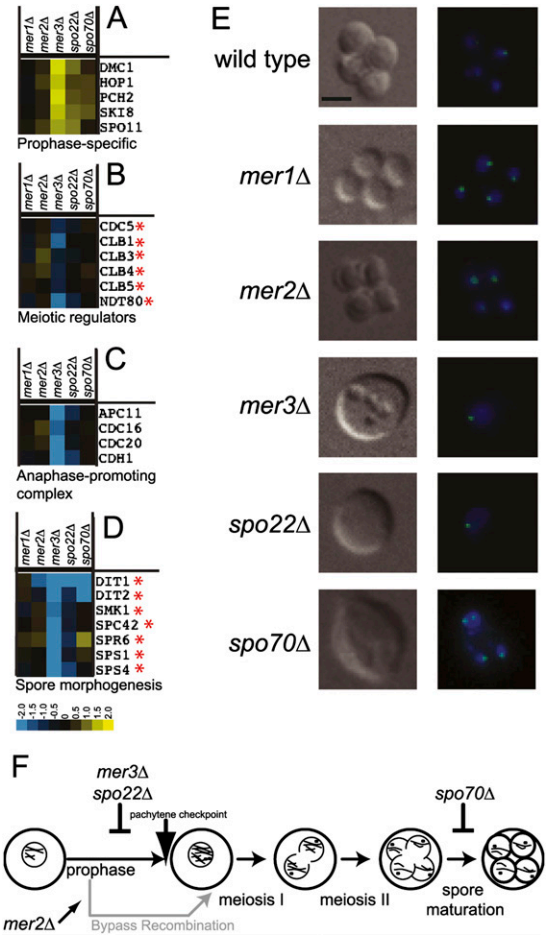

Figure 4. *MER1* regulon expression is required for induction of *NDT80* and Ndt80p-regulated genes. (*A*) Expression of genes whose products function during prophase in *mer1Δ*, *mer2Δ*, *mer3Δ*, *spo22*Δ, and *spo70Δ* compared with wild type 9 h after induction of meiosis. (*B*) Same as *A* for genes 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*, *spo22*Δ, 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 *spo22*Δ with *mer1*Δ showed reduced activation of the checkpoint (Fig. 5B, lanes 2,6), the same as the *mer1*Δ single mutant (Fig. 5A, lane 4), rather than the strong checkpoint activation observed in the *mer3Δ* or *spo22*Δ single mutants. Double mutants of *mer3Δ* or *spo22*Δ with *mer2*Δ showed little or no detectable pachytene checkpoint activation (Fig. 5B, lanes 4,8), the same as the *mer2*Δ single mutant (Fig. 5A, lane 6). The meiotic arrest points of the *mer1*Δ strain resemble the *mer2*Δ mutant (Fig. 4E). Likewise, the arrest points of the *mer3*Δ*mer1*Δ and *mer3*Δ*mer2*Δ resemble the *mer2*Δ single mutant, rather than *mer3*Δ (Fig. 5C). These results indicate that both *mer1*Δ and *mer2*Δ are epistatic to *mer3Δ* and *spo22*Δ 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

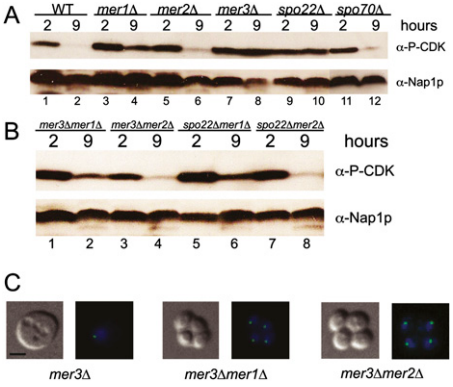

Figure 5. Pachytene checkpoint activation persists in *mer3*Δ and *spo22*Δ, and, to a lesser degree, in the *mer1*Δ strain. (*A*) Western blot measuring phosphorylation state of CDK on Y19 in wild-type (WT), *mer1Δ*, *mer2Δ*, *mer3Δ*, *spo22*Δ, and *spo70Δ* strains 2 h and 9 h after induction of meiosis. (*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.

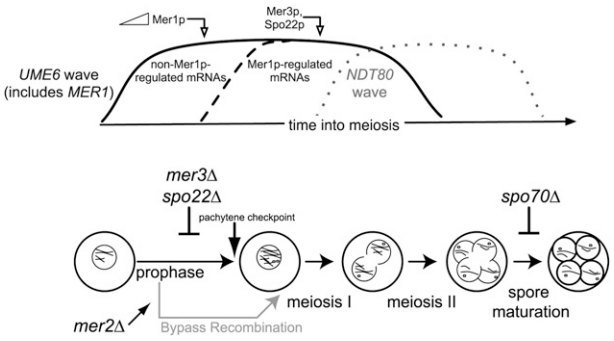

Figure 6. The *MER1* regulatory network and the meiotic gene expression program. The *UME6* expression wave is divided into two temporal components: an early component including non-Mer1p-regulated mRNAs (including Mer1p mRNA itself), and a later component including the mRNAs whose splicing is dependent 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; 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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