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The SR-protein Npl3 is an essential component of the meiotic splicing regulatory network in *Saccharomyces cerevisiae*

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ABSTRACT

The meiotic gene expression program in *Saccharomyces cerevisiae* involves regulated splicing of meiosis-specific genes via multiple splicing activators (e.g. Mer1, Nam8, Tgs1). Here, we show that the SR protein Npl3 is required for meiotic splicing regulation and is essential for proper execution of the meiotic cell cycle. The loss of Npl3, though not required for viability in mitosis, caused intron retention in meiosis-specific transcripts, inefficient meiotic double strand break processing and an arrest of the meiotic cell cycle. The targets of Npl3 overlapped in some cases with other splicing regulators, while also having unique target transcripts that were not shared. In the absence of Npl3, splicing defects for three transcripts (*MER2*, *HOP2* and *SAE3*) were rescued by conversion of non-consensus splice sites to the consensus sequence. Methylation of Npl3 was further found to be required for splicing Mer1-dependent transcripts, indicating transcript-specific mechanisms by which Npl3 supports splicing. Together these data identify an essential function for the budding yeast SR protein Npl3 in meiosis as part of the meiotic splicing regulatory network.

INTRODUCTION

Meiosis is a specialized cell division required to produce haploid gametes from diploid precursor cells. Meiosis requires extensive transcriptional reprogramming, including repression of genes responsible for vegetative growth (e.g. ribosomal components) and induction of genes required to execute the multiple stages of meiosis (1–5). In *Saccharomyces cerevisiae*, Ime1, a master regulator of meiosis, promotes meiosis by inducing the transcription of early meiotic genes, as well as production of the transcription factor Ndt80, which is responsible for the expression of middle sporulation genes (6–11). The transcriptional cascade involving Ime1 and Ndt80 ensures the correct temporal expression of genes throughout meiosis. In addition to transcriptional regulation, this sequential expression of meiotic factors is controlled by mRNA splicing (12).

Pre-mRNA splicing offers many advantages in gene expression, such as increasing the repertoire of proteins produced via alternative splicing, as well as enhancing transcription rate, and mRNA export (13–20). Regulated splicing can further control the timing of gene expression to ensure production of a specific gene product occurs within the correct cellular context. Examples of regulated splicing events that contribute to proper gene expression have been detailed in differentiation, cellular stress responses, disease progression and developmental programs (21–28). In *S. cerevisiae,* meiotic genes are overrepresented within the ∼4% of intron containing genes and many meiotic genes undergo regulated splicing controlled through a meiotic splicing regulatory network (12,29–31). Exemplifying this control mechanism, while some meiotic genes are expressed at a basal level in vegetative cells, protein expression is not observed due to a lack of pre-mRNA splicing (12,29,32,33).

Cell cycle dependent splicing regulation in *S. cerevisiae* involves at least two general mechanisms. First, splicing activity is limited through pre-mRNA competition for splicing machinery (32). Consequently, splicing efficiency depends on the level of a transcript, as a part of the intron-containing transcriptome, and the affinity the pre-mRNA has for splicing factors in comparison to other competing transcripts. Ribosomal protein genes (RPGs) are one group of highly transcribed genes in mitotic cells, representing ∼50% of mRNA transcripts produced in growing yeast and ∼90% of all intron containing transcripts (4,34). Consequently, due to their high level of transcription and intron frequency, when cells enter meiosis, RPG transcription is repressed and this is accompanied by an increase in the splicing efficiency of meiotic transcripts (32). Related to this control mechanism, meiotic genes often contain non-consensus splice sites (27,29,30,33). This would be expected to reduce the ability of a transcript to compete for splicing factors and/or slow splicing when a meiotic pre-mRNA is able to engage the spliceosome, thus decreasing the probability that a meiotic gene would be spliced and expressed.

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This may be a key part of the cellular safety mechanism to prevent the inappropriate expression of meiotic genes during mitotic growth (29,33).

A second level of regulation involves the need of additional trans acting factors for efficient splicing of meiotic genes. Three key splicing activators that are part of the meiotic splicing regulatory network are Mer1, a meiosis specific RNA binding protein (12,35–38), Nam8, a subunit of the U1 snRNP (38–42), and Tgs1, a trimethyl guanosine synthase (43–46). Mer1 and Nam8 work cooperatively to promote splicing of *AMA1, ZIP4, MER2* and *MER3* comprising the Mer1 regulon (12,27,38,47), which is further regulated at the level of Mer1 expression (48). In addition, Nam8 has been shown to promote splicing of *PCH2* independently of Mer1 (49). In the case of Tgs1, which produces trimethyl guanosine caps on spliceosomal snRNAs, a loss of Tgs1 activity has been found to cause intron retention in *SAE3* and *PCH2* meiotic transcripts (43,44,46,50,51). In the absence of Tgs1, vegetative cells exhibit cold-sensitive defects in splicing due to mis-localization of U1 snRNA (50,51), but the mechanism by which Tgs1 promotes splicing of meiotic transcripts is not clear. Overall, these three activators appear to work both cooperatively and independently to promote splicing of meiotic transcripts bearing non-consensus splice sites by multiple mechanisms. However, if and how many other proteins are involved in regulated splicing of meiotic transcripts remains unknown.

In mammals, an important class of proteins that play a significant role in constitutive, as well as regulated splicing, is the SR (serine- and arginine-rich) protein family (52,53). SR proteins generally promote splicing by binding to exonic or intronic enhancer sequences, but also support other aspects of gene expression (54–56). In *S. cerevisiae*, the SR proteins Gbp2, Hrb1 and Npl3 have similarly been shown to function in multiple parts of the gene expression pathway, including splicing, 3′ processing, mRNA export and translation (57,58,67,59–66). Each individual SR-protein is non-essential in *S. cerevisiae* vegetative cells and their functions appear to be bypassed during stress conditions to favor rapid responses to changing environmental conditions (65,68).

Of the SR proteins in yeast, Npl3 is the most studied. Npl3 is recruited to transcribing genes and interacts with the C-terminal domain of RNA polymerase II in a transcription dependent manner, while Gbp2 and Hrb1 are recruited by the TREX complex (69,70). Npl3 binds to the 5′- and 3′-ends of transcripts consistent with functions in early (e.g. splicing) and late (e.g. 3′ processing) mRNA maturation events (63). Similar to mammalian counterparts, Npl3 shows genetic and physical interactions with components of the spliceosome involved in early steps of splicing (57). The loss of Npl3 results in widespread changes in splicing, which include an impact on ribosomal protein genes bearing consensus splice sites (57). Npl3 also undergoes post translational modifications such as phosphorylation and methylation (71,72). These post translational modifications alter the localization of Npl3 by regulating nuclear shuttling (73–75). In addition, phosphorylation is important for Npl3 functions in transcription termination and methylation is reported to play role in splicing and in the interaction of Npl3 with itself and other binding partners (76–78).

The known roles for *S. cerevisiae* SR proteins in gene expression have resulted from studies performed in cells undergoing mitotic growth; however, it is currently unknown if their functions are similar, or even required, for cells to enter and progress through meiosis. Here, we report that of the three SR proteins, Npl3 plays an essential role in meiotic gene expression. Our data demonstrate an essential role for Npl3 in splicing meiotic transcripts bearing non-consensus splice sites and show that loss of Npl3 activity results in an arrest in meiosis I. Npl3 further exhibits shared pre-mRNA targets with other splicing activators as part of the meiotic splicing regulatory network. Taken together, we have identified an essential requirement for an SR protein in meiosis, which furthers our understanding of splicing regulation within the *S. cerevisiae* meiotic gene expression program. In addition, these findings provide further insight into the molecular underpinnings of a complex splicing regulatory network that we expect can act as a paradigm for understanding similar networks in metazoan systems.

MATERIALS AND METHODS

Biological resources

All strains used in this study are isogenic derivatives of *S. cerevisiae* SK1 background (Supplementary Table S2). All plasmids used in this study are listed in Supplementary Table S3. The gene deletions, tagging, or promoter replacement strains were generated either by using PCR based transformation or by mating, sporulation and tetrad dissection. The gene deletions or mutations were marked with *KanMX6*, *hphNT1* or *natNT2* drug resistance cassettes. Gene deletion, tagging, and promoter replacement were confirmed by colony PCR and point mutations were confirmed by DNA sequencing. Wildtype SK1 yeast strains bearing *HIS4:LEU2* hotspot were generously provided by G. Valentin Boerner. Strains with FRB domain and RPL13A-FKBP12 anchor were kindly provided by Andreas Hochwagen. *SAE3* Plasmids bearing altered *BP*, *3′SS* and *Δhp* were generously provided by Beate Schwer. Plasmids bearing *Npl3-Myc* and *npl3-RK1–15-Myc* were gifted by Anne McBride. *spo11yf* strain and plasmid for P4 probe for Southern blot was generously provided by Neil Hunter.

Reagents

Enzymes, antibodies, kits and other reagents are listed in Supplementary Table S4. Sequences of synthetic oligonucleotides for qPCR and smiFISH are provided in Supplementary Table S5.

Meiotic time course

Meiotic time courses were performed as described in (79). Briefly, cells were patched on YPG agar plate from frozen glycerol stock and incubated at 30°C for 16 h. Cells were then streaked on YPD agar plate and incubated at 30°C for 52–56 h. Diploid single colonies were inoculated in 4 ml YPD liquid medium and incubated at 30°C roller drum for 26 h. The saturated YPD culture was inoculated in YPA liquid culture at different dilutions at 30°C for 13 h. The optical density (OD) of YPA cultures was measured after 13 h

and the cultures with the OD in the range of 1.0–1.4 were centrifuged, washed once with prewarmed meiosis medium, and were resuspended in prewarmed meiosis medium. The cultures were transferred to flask and incubated at 30°C with shaking at 290 rpm, which is considered to be the 0 h in meiosis timepoint. For anchor away system, 2 μg/ml rapamycin was added at the indicated time points in the sporulation culture. For *IME1* or *NPL3* overexpression system using pCUP1–1 promoter, 50μM CuSO4 was added to the sporulation culture at the indicated time points.

Physical analysis for meiotic recombination

Crosslinking with psoralen, DNA extraction, restriction digestion, Southern blotting and quantitation were performed as described in (79). For hybridization, radiolabeled alpha α-32P-dGTP was used instead of α-32P-dCTP. For analysis of DSBs and COs, DNA was digested with XhoI restriction enzyme while for the detection of NCOs, double digestion with XhoI and HF-BamHI restriction enzymes was performed. Blots were probed using P4 probe (79).

Meiotic nuclear divisions

At indicated time points during the meiotic time course, the culture was collected and mixed with the DAPi fix (80% ethanol, 0.1 M sorbitol and 0.2 mM EDTA) in 1:1 ratio. The fixed culture was stored at 4°C until counted. For counting the nuclear divisions, DAPi fixed culture was mixed with 1ug/ml DAPi dye in 1:1 ratio to stain chromatin. The nuclear divisions were counted using fluorescence microscopy with 60× objective.

Sporulation and spore viability

After 24 h in meiosis medium in the meiotic time course, cells were collected and spores were counted using a light microscope. At least 200 cells were counted per sample. For determination of spore viability, tetrads from time course cultures were digested with 20 μg of zymolyase 20T in 100μl of zymolyase digestion buffer at 37°C for 20 min. At least 20 tetrads were dissected for each sample using tetrad dissection microscope. After dissection, plates were incubated at 30°C for 3 days.

RNA extraction and cDNA synthesis

For RNA extraction, ∼10 OD of the meiotic culture at indicated time points was spun down and cell pellets were frozen on dry ice, followed by storage at −80°C. Cells were disrupted using a bead beater with TBT lysis buffer (20 mM HEPES pH7.4, 110 mM potassium acetate, 2 mM magnesium chloride, 0.5% Triton, 0.1% Tween 20 and 1:5000 antifoam, as described in (80)). Nucleic acids were extracted using acidic phenol chloroform isoamyl alcohol (pH 4.3–4.7) and precipitated using 0.15 M sodium acetate and ethanol at −80°C for 1.5 h to overnight. DNase digestion was performed with TURBO™ DNase as per the manufacturer's instructions, followed by another round of phenol chloroform extraction and sodium acetate, ethanol precipitation. To generate cDNA, 1 to 2 μg of RNA was used with the Super Script III First Strand cDNA synthesis kit as per the manufacturer's protocol using an oligo dT primer. Splicing PCRs were performed with ∼5–10 ng of the cDNA.

Single molecule and poly A fluorescence *in situ* hybridization (FISH)

Single molecule and polyA-RNA FISH was performed as described in (81,82), with the exception that smiFISH probes were used to detect gene transcripts by single molecule FISH (83). At indicated time points, ∼3.5 OD of meiotic cultures was fixed with 37% formaldehyde for 20 min at room temperature followed by overnight at 4°C. Fixed cells were washed 3× with buffer B and digested in buffer B containing 40 μl of 200 mM VRC and 5 μl (for early time points) or 8 μl (for late time points) of 20 mg/ml zymolyase 20T. Zymolyase digestion was performed at 30°C for 30 min. Then cells were washed with buffer B and fixed in 70% ethanol for 4 h at room temperature. Fixed cells were spun down and washed with formamide wash buffer (15% formamide, 1× SSC). Hybridization with poly A probe and/or gene specific probes was performed in a hybridization buffer containing 10% formamide, 1× SSC, 0.34 mg/ml *Escherichia coli* tRNA, 0.2 mg/ml BSA, 11% dextran sulphate, 4 mM VRC and oligo-dT or gene specific probes as indicated at 37°C for 16 h. A final concentration of 0.4 mM was used for the labeled oligo dT LNA probe (Qiagen) and 0.625 nM for single molecule probes.

SDS PAGE and western blot

For protein extraction, ∼5 OD of meiotic culture was spun down and pellets were frozen at −80°C. Protein extraction from meiotic cultures was performed using NaOH/TCA extraction method as described in (84). Lysate was run on 12% SDS PAGE gels and proteins were transferred to nitrocellulose membrane by cold wet tank transfer. Hop2, Sae3 and Rec8 were tagged with 3xV5 epitope and were detected using mouse monoclonal anti-V5 antibody (Invitrogen) at a dilution of 1:2500. For detection of Dmc1, an endogenous goat anti-Dmc1 antibody was used (generous gift from Dr Neil Hunter) at a dilution of 1:2000. For detection of Tdh1/2, endogenous mouse anti-GAPDH antibody (Thermo Fisher, GA1R) was used at 1:10 000 dilution. Secondary antibodies were HRP labeled goat anti mouse (Invitrogen, G21040) or donkey anti goat (Life technologies, A16005) and were used at 1:10 000 dilution.

qPCR analysis

qPCR was performed using Power SYBR Green Master mix and Thermo Fisher QuantStudio PCR machine. Three technical and at least three biological replicates were used for each sample. Fraction of intron containing mRNA was calculated using sets of primers that target intronic and exonic sequences as described in (32). One primer set amplified the intron containing pre-mRNA (forward primer hybridizes to intron sequence and reverse primer hybridizes to second exon sequence) and second set amplified total mRNA (both primers hybridize to sequences in the second exon). Intron containing RNA was calculated using the formula $2^{(-\Delta\Delta Ct)}$, where $\Delta\Delta Ct = (Ct_{InF\text{-}ExR} - Ct_{ExF\text{-}ExR})$. Fold

change of intron containing RNA was calculated as ratio of the intron containing RNA in mutant to that of WT.

RESULTS

Npl3 is required for meiotic cell cycle progression in *S. cerevisiae*

To characterize the function of the *S. cerevisiae* SR proteins in meiosis, each non-essential gene encoding an individual SR-protein (i.e. *NPL3, GBP2* or *HRB1*) was deleted in a diploid yeast strain. Control and mutant strains were induced to undergo meiosis, and meiotic nuclear divisions, sporulation and spore viability were used to determine progress through the meiotic cell cycle. Of the SR proteins, *npl3Δ* cells showed a strong arrest in meiosis I, with the majority of cells having fragmented DNA masses (Supplementary Figure S1A) and only ∼20% cells undergoing nuclear divisions after 24 h in meiosis medium (Figure 1A). Consistent with a block in nuclear divisions, sporulation was also severely reduced in *npl3Δ*, with less than 5% of all cells forming spores (Figure 1D). In contrast, *gbp2Δ*, *hrb1Δ* and *gbp2Δ/hrb1Δ* double mutant cells showed meiotic progression, sporulation and spore viability similar to control cells (Figure 1B–E). Therefore, among the *S. cerevisiae* SR proteins, Npl3 has a unique and essential role in meiosis required for cell cycle progression and sporulation.

npl3Δ defects are meiosis specific and not due to defects carried over from mitotic growth

In mitotic cells, Npl3 has multiple functions in gene expression that include splicing and mRNA export (57,59,61–64,67,68). A possible cause for the meiotic defects observed in *npl3Δ* cells could be pre-existing defects in gene expression carried over from the mitotic cell cycle. Therefore, to ensure that observed *npl3Δ* defects are meiosis specific, Npl3 activity was inhibited upon meiotic entry by using an anchor away approach (85,86). Specifically, Npl3 was tagged with the FRB domain of mTOR to allow Npl3 to be depleted from the nucleus upon rapamycin addition through binding the cytoplasmic ribosomal protein Rpl13A (anchor) fused to FKBP12. The tagged version of Npl3 also carried GFP to determine the efficiency of nuclear depletion upon rapamycin addition (Figure 2A). To deplete Npl3 from nucleus at the onset of meiosis, rapamycin was added at the time of meiosis induction (0 h, when cells were suspended in meiosis medium). Consistent with Npl3 being required for meiosis, depletion of Npl3 resulted in a significant delay in nuclear divisions as compared to the mock treated culture (Figure 2B). Importantly, in these experiments the addition of rapamycin to control cells did not alter the timing of nuclear divisions (Figure 2B). After 24 h in meiosis medium, ∼40% of the cells within the Npl3 depleted culture had undergone nuclear divisions, which may be due to degradation of rapamycin over time and/or incomplete depletion of Npl3 from nucleus. Notably, when Npl3 was depleted after 180 min of meiosis, nuclear divisions occurred at the same levels as the control culture (Figure 2C). These data support an essential requirement for Npl3 in the early stages of meiosis that result from Npl3 functions within the meiotic cell cycle.

One function of Npl3 in gene expression is in mRNA export (64), so it is possible that the *npl3Δ* meiotic defects could result from a block in mRNA export. To investigate this, mRNA export status was assayed using fluorescence *in situ* hybridization (FISH) to detect polyadenylated RNA. By this measure, no defect in polyadenylated RNA export was observed in *npl3Δ* cells in meiosis (Supplementary Figure S1B). In contrast, when the essential mRNA export factor Nab2 fused to FRB and GFP was depleted by anchoring Nab2 to the ribosomal component Rpl13A, the accumulation of polyadenylated RNA in meiosis was readily apparent (Supplementary Figure S1C and D). Therefore, as in mitotic cells (87,88), Nab2 is required for mRNA export during meiosis, while Npl3 is dispensable for bulk mRNA export.

Gene expression of early meiotic genes is defective in *npl3Δ* cells

Based on our Npl3 anchor away data, Npl3 functions are required during early meiosis. Key events in the earliest stages of meiosis involve reprogramming of gene expression through Ime1, which is required to activate transcription of early meiotic genes involved in recombination including *DMC1* and *ZIP1* (8). To investigate if the transcriptional timing of *IME1,* or other early meiotic genes, was altered by *npl3Δ,* single molecule inexpensive FISH (smiFISH) was used to assay cellular *IME1, DMC1* and *ZIP1* levels at different times of meiosis (83). For quantification of transcript levels, any cell having more than 10 foci was counted as positive for expression of the target transcript. At 4 h after meiotic entry, there was a minor delay in the expression of *IME1* in *npl3Δ* cells (Supplementary Figure S2A). Similarly, transcript levels for *DMC1* and *ZIP1* were lower at early time points (Supplementary Figure S2B, C); however, in all cases at 6 h in meiosis, transcript levels were comparable in control and *npl3Δ* cells. Additionally, there was no obvious nuclear export defect of *IME1, DMC1* and *ZIP1* transcripts in *npl3Δ* cells (Supplementary Figure S2). These results suggest that in the absence of Npl3, the expression of early meiotic genes is delayed, but the number of cells producing these early meiotic genes reach levels similar to control at later time points.

The delay seen in expression of *DMC1* and *ZIP1* transcripts could stem from a delay in Ime1 production, since the expression of many meiotic genes depends on Ime1 activity either directly or indirectly (7–9). To determine if increased *IME1* expression could alter meiotic progression in *npl3Δ* cells, *IME1* expression was induced using a copper inducible promoter (*pCUP1–1*) (89). In *npl3Δ* cells, *IME1* expression was strongly induced and detectable in majority of cells when under the *pCUP1–1* promoter with the addition of $CuSO_4$ (Supplementary Figure S3A). However, no improvement in meiotic progression was observed upon *IME1* overexpression in a *npl3Δ* strain based on the number of cells undergoing meiotic nuclear divisions at 24 h, while in control cells overexpressing *IME1* nuclear divisions were >90% (Supplementary Figure S3B). Together, while these data show that early meiotic gene expression may be less efficient or delayed, it does not provide a strong indication

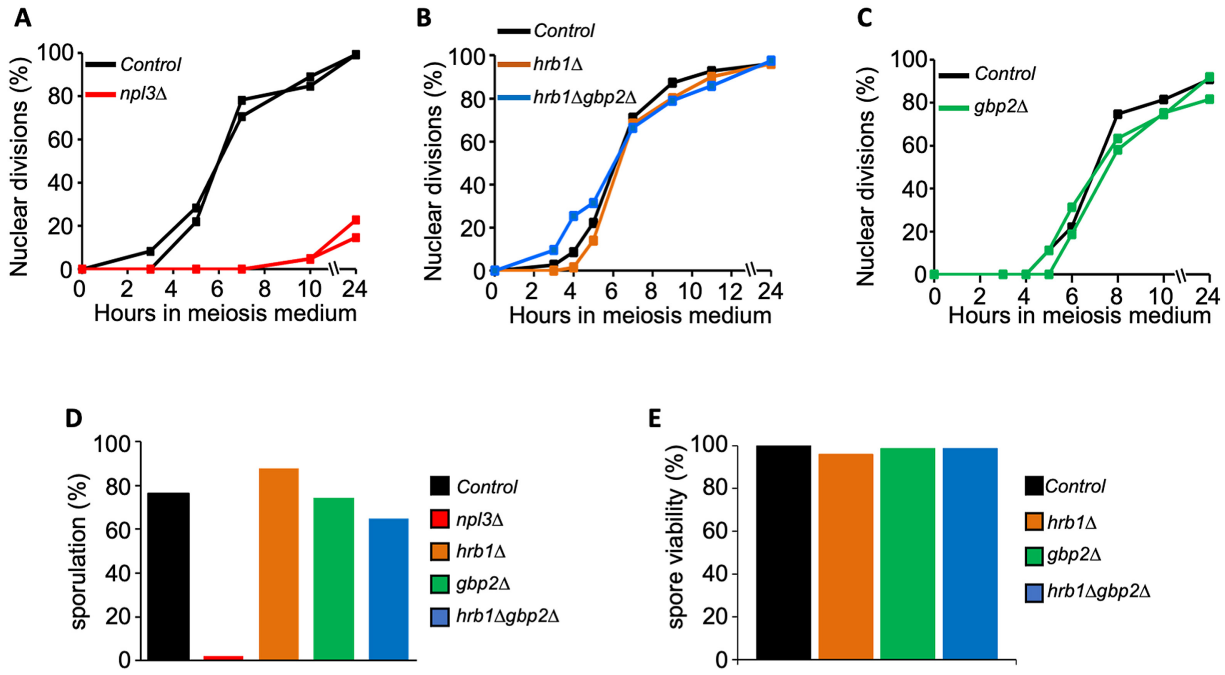

Figure 1. Npl3 is essential for meiosis. (**A–C**) Graphs show the % of cells having undergone the first and/or second meiotic division at the indicated time points of meiosis in two independent cultures of control and SR protein deletion strains. Meiotic divisions were determined by counting the number of DAPi stained DNA masses in ≥100 cells. (**D**) Graph shows percent of tetrads formed after 24 h in sporulation medium based on visual observation. (**E**) Graph shows percent of viable spores formed after 24 h in sporulation medium. For each genotype, 20 tetrads were dissected.

that this is the major cause of the meiotic arrest phenotype in *npl3Δ* cells.

Splicing of meiosis specific gene transcripts is altered in the absence of Npl3

Npl3 is known to promote splicing of mitotic transcripts and interacts with spliceosome components (57). Since many essential meiotic genes contain introns and need to be spliced for meiotic progression (29–31,33), splicing of meiotic genes was next considered as a reason for the meiotic defects seen in *npl3Δ* cells. To analyze splicing, RNA was extracted at various time points after meiosis induction, cDNA was generated and intron containing genes were assayed by PCR using primers flanking the intron in control and *npl3Δ* cells. From these analyses (Figure 3A), accumulation of intron containing pre-mRNAs can be observed in 8 of 16 targets (*MER2*, *HOP2*, *SAE3, REC102, REC114, SRC1, ZIP4* and *ECM9)*, indicating splicing defects in *npl3Δ* cells that range from strong (e.g. *MER2*, *HOP2* and *SAE3)* to none (e.g. *GMC2*, *PCH2*, *MND1* and *DMC1*). To quantitate the splicing defect of *MER2*, *HOP2* and *SAE3* transcripts, RT-qPCR was performed using RNA extracted from control and *npl3Δ* cultures after 5 h in meiosis. This analysis supported the splicing defects seen by gel-based analyses, indicating that splicing of these transcripts was defective in *npl3Δ* cells (Figure 3B). The majority of meiotic transcripts have non-consensus splice sites (12,29,30,32,33), but a comparison of splice site sequences (5′, 3′ or branch point) indicated no clear correlation between *npl3Δ* mediated splicing defects and a discrete non-consensus sequence feature.

Loss of Npl3 results in the defective splicing of many meiotic transcripts, but splicing was not completely blocked for any transcript. To determine how such defects relate to protein production, protein levels were determined by western blot for proteins produced from *HOP2* and *SAE3* (strong splicing defect in *npl3Δ* cells) and *DMC1* (splicing does not depend on Npl3). As a control, protein produced from *REC8* was also assayed, as a meiotic gene that does not contain an intron. The data show that protein levels of Hop2 and Sae3 were severely reduced at all time points tested in *npl3Δ* cells (Figure 3C), which correlated with the strength of the splicing defect (Figure 3A). In contrast, Dmc1 reached levels comparable to control at later timepoints, which is in line with *DMC1* transcriptional delays seen by smiFISH (Supplementary Figure S2B). Rec8 protein level also appeared low at early time points consistent with potential delays early in meiosis, becoming comparable to control at 5 h, and was maintained in *npl3Δ* cells at 8 h of meiosis (Figure 3C). The presence of Rec8 in *npl3Δ* cells at timepoints later than control are consistent with the failure of *npl3Δ* cells to progress in meiosis I, as most Rec8 is cleaved during chromosome segregation during meiosis I (90). These data suggest that the splicing defects observed in *npl3Δ* result in a significant decrease in Hop2 and Sae3 protein levels.

Of the genes impacted by loss of *npl3Δ*, only deletion of *HOP2* or *SAE3* causes a strong arrest in meiosis I that is similar to *npl3Δ* cells (91,92). This raises the possibility that the meiotic arrest observed in *npl3Δ* is due to defects in *HOP2* or *SAE3* splicing. To test this hypothesis, cDNA versions of *HOP2* and *SAE3* were integrated at both gene loci for *HOP2* and *SAE3* in control and *npl3Δ* cells. An

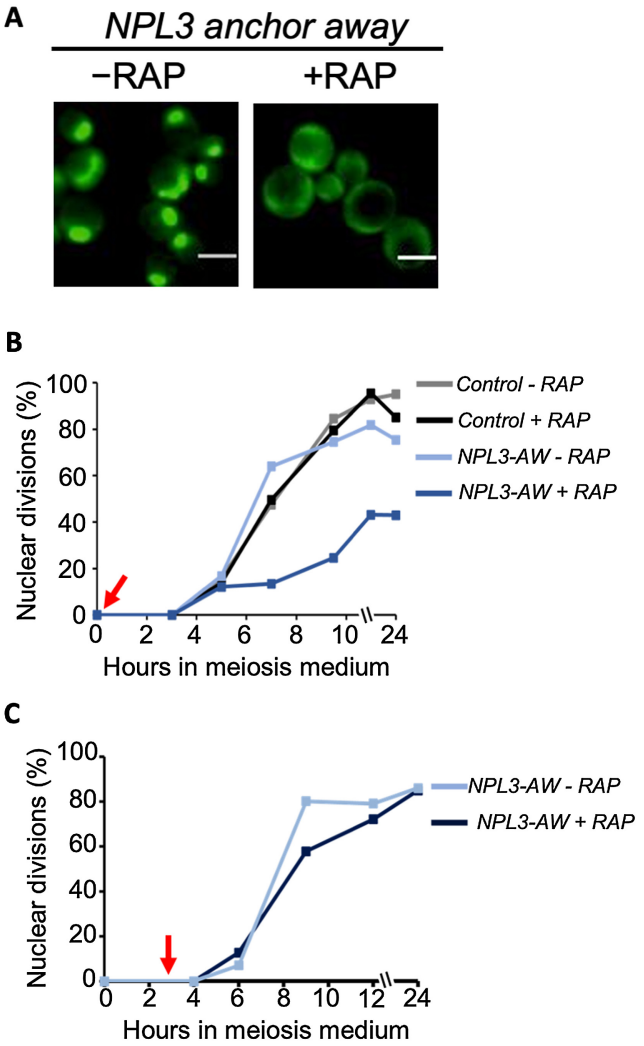

Figure 2. *npl3Δ* defects are meiosis specific. (**A**) Localization of Npl3-FRB-GFP without (-RAP) and with (+RAP) addition of rapamycin to the culture at 0hr of meiosis. Image was taken 5 h after rapamycin addition. Scale bar is 2μm. (**B**, **C**) Graph shows % of cells having undergone the first and/or second meiotic division at the indicated time points of meiosis. Meiotic divisions were determined by counting the number of DAPi stained DNA masses in ≥100 cells. Red arrow indicated time of rapamycin addition at 0hr (in graph B) or at 3 h (in graph C) after meiosis induction. For graph B, one of the three independent experiments are shown.

analysis of nuclear divisions showed that control cells were able to undergo meiosis normally when *HOP2* and *SAE3* were produced from a cDNA (Figure 3D). RT-PCR analysis further showed that cDNA versions of *HOP2* and *SAE3* were expressed at similar level in control and *npl3Δ* cells and other *npl3Δ* splicing phenotypes, such as defective splicing of *MER2* and normal splicing of *PCH2* were present (Figure 3E), yet the meiotic arrest of *npl3Δ* was not altered (Figure 3D). As such, we expect that the observed *npl3Δ* cell cycle arrest in meiosis I is due to gene expression changes involving a large number of gene products (i.e. beyond that of just *HOP2* and *SAE3*). The data as a whole provide evidence for a role of Npl3 in meiotic splicing, which significantly impacts at least half of the meiosis-specific genes that contain introns.

npl3Δ defects are linked to meiotic recombination defects

Meiotic recombination is initiated by ∼150–200 double strand breaks (DSBs) throughout the genome, which are repaired to generate crossovers (COs) and noncrossovers (NCOs). Defects in meiotic DSB processing result in a checkpoint mediated arrest of meiotic cell cycle progression (93), similar to the arrest observed in cells that lack Npl3. Since the majority of the intron containing meiotic genes function in recombination, we reasoned that the reduced expression of these genes due to defective splicing in *npl3Δ* cells may result in meiotic recombination defects and cell cycle defects. To determine if the meiotic arrest in *npl3Δ* cells is dependent upon aberrant DSB processing, DSB formation was blocked using a catalytic null mutant of Spo11, *spo11-yf* (94). The *npl3Δ/spo11-yf* double mutant showed moderate suppression of *npl3Δ* mediated meiotic arrest, increasing the number of nuclear divisions from ∼20% in *npl3Δ* to ∼50% in *npl3Δ/spo11-yf cells* (Figure 4A). Although the absence of DSBs partially bypassed the meiotic arrest phenotype of *npl3Δ*, spore formation was still defective (Figure 4B). These data suggest that Npl3 likely supports multiple steps within the meiotic program and that defective DSB processing is one aspect of the meiotic program that is altered in *npl3Δ* mutant.

To better understand the nature of DSB processing defect in *npl3Δ* cells, meiotic recombination was analyzed using the well-studied *HIS4:LEU2* locus (95). This recombination hotspot is flanked by polymorphic XhoI sites on the two homologs of chromosome III providing for restriction fragment length polymorphism to be used to follow DSBs, COs and NCOs by Southern blotting. In control cells, it was observed that DSBs peaked at 3 h and were mostly resolved by 6 h after entry into meiosis. In *npl3Δ* cells, DSBs did not peak until 6 h and at least half of the DSBs persisted through the end of the 24 h time course (Figure 4C, E (i)). Consistent with a defect in DSB processing, COs and NCOs were also significantly delayed and were not generated at the same level as in control (Figure 4C, D, E). The delay in DSB formation and resolution is likely attributed to reduced levels of the gene products synthesized in *npl3Δ* due to defective splicing. Indeed, three out of four intron containing genes involved in DSB formation show a defect in splicing in *npl3Δ* (*MER2*, *REC102* and *REC114*). Similarly, three out of eight intron containing genes involved in DSB resolution and/or synapsis have a splicing defect in *npl3Δ* (*HOP2*, *SAE3* and *ZIP4*). Collectively, these results suggest that due to defective splicing of several transcripts involved in meiotic recombination, *npl3Δ* cells show asynchronous and/or faulty progression through meiotic recombination. Since Npl3 is a multifunctional protein, the meiotic recombination defects observed in *npl3Δ* could further arise from alterations in other aspects of meiotic gene expression program (e.g. translation), in addition to defective splicing.

Npl3 promotes splicing of suboptimal introns

Many of the transcripts with altered splicing in *npl3Δ* cells, including *MER2*, *HOP2* and *SAE3*, have introns bearing non-consensus splice sites (35,43,96), suggesting that Npl3 may promote splicing of such meiotic transcripts. To investigate this possibility, the splice sites of *MER2, HOP2* and

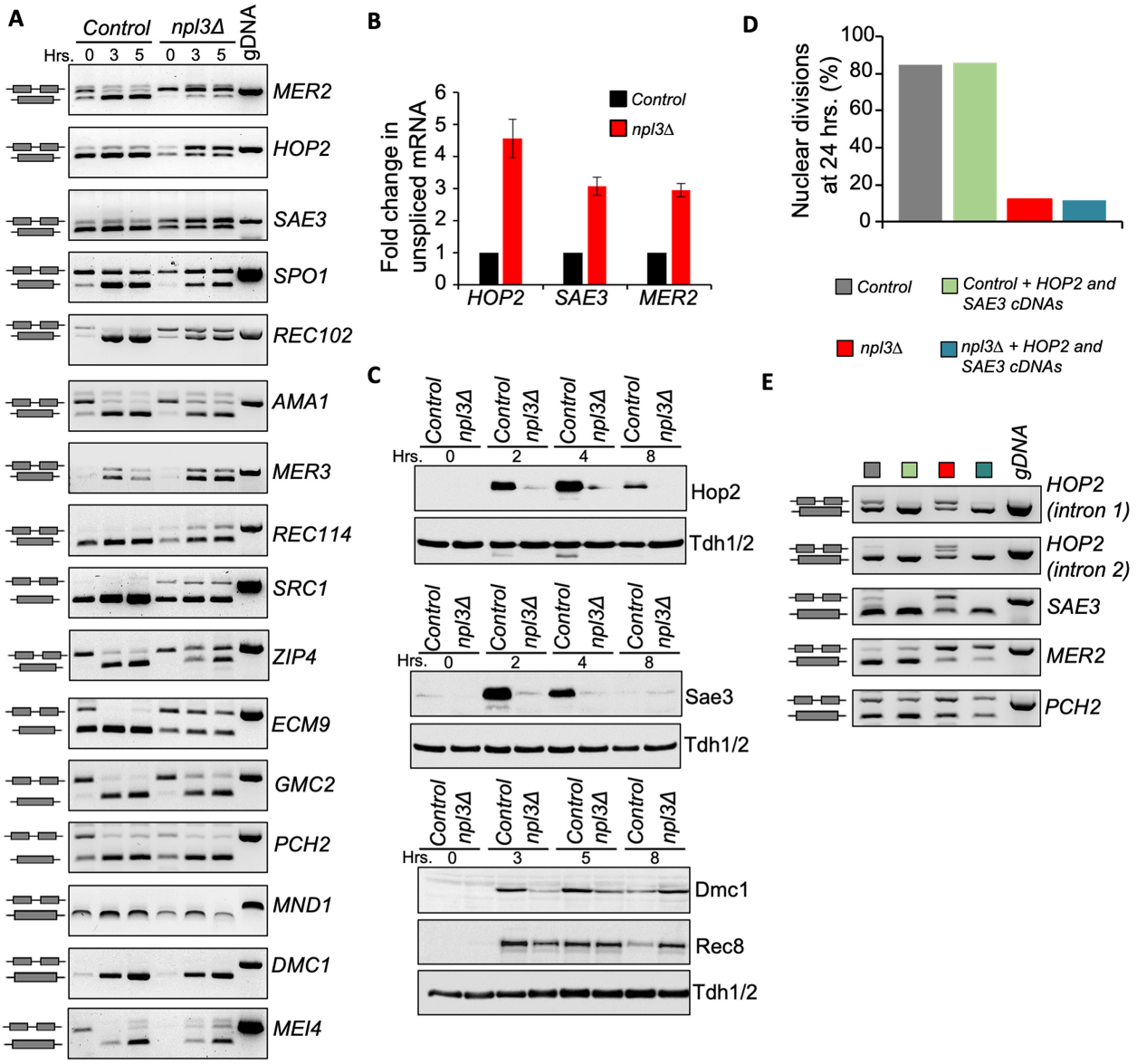

Figure 3. Npl3 is required for splicing and expression of meiotic transcripts. (**A**) Agarose gel showing PCR products generated by primers flanking the intron of the indicated gene from control and *npl3Δ* cells at indicated time points in meiosis. The pre-mRNA and spliced RNA are indicated and the PCR product from genomic DNA (gDNA) is included for comparison. (**B**) RT-qPCR analysis quantifying fold enrichment in intron containing transcripts in select meiotic transcripts in control and *npl3Δ* cells. Error bars represent standard deviation, $n = 3$. (**C**) Western blot analysis showing protein expression levels from meiosis specific genes in control and *npl3Δ* cells at indicated time points in meiosis. Tdh1/2 is included as a loading control. (**D**) Graph shows % of cells having undergone the first and/or second meiotic division after 24 h of meiosis induction. Meiotic divisions were determined by counting the number of DAPi stained DNA masses in ≥100 cells. (**E**) Agarose gel of PCR products showing expression of endogenous and cDNA versions of *HOP2* and *SAE3* transcripts in control and *npl3Δ* cells. *MER2* and *PCH2* were also assayed as controls.

SAE3 were made consensus and assayed for splicing in *npl3Δ* cells. For *MER2*, changing the non-consensus 5′ splice site from -GUUCGU- to -GUACGU- largely abolished the requirement of Npl3 for *MER2* splicing (Figure 5A, left panel). In the case of *HOP2*, the transcript contains two introns, with the first intron having a non-consensus 5′ splice site -GUUAAG- that deviates from consensus -GUAPyGU-, while the second intron contains consensus splices sites. Changing the non-consensus 5′ splice site sequence of the first intron in *HOP2* to -GUAUGU- rescued the splicing defect in *npl3Δ* cells (Figure 5A, right panel). Finally, *SAE3* contains a non-canonical branchpoint, 3′ splice site, and a hairpin structure near its branch point that inhibits splicing of *SAE3* (43), (see Figure 5B, left panel). To determine if Npl3 is required for splicing of *SAE3* due to these intron features, we tested splicing of *SAE3* ectopically transcribed in a *npl3Δ/ sae3Δ* double mutant strain from plasmids containing *SAE3* with a canonical 3′ splice site, the hairpin deleted, or a canonical branchpoint (43). The requirement of Npl3 for *SAE3* splicing was abolished when the hairpin was deleted or when the branchpoint was canonical, but not when the 3′splice site was consensus (Figure 5B,

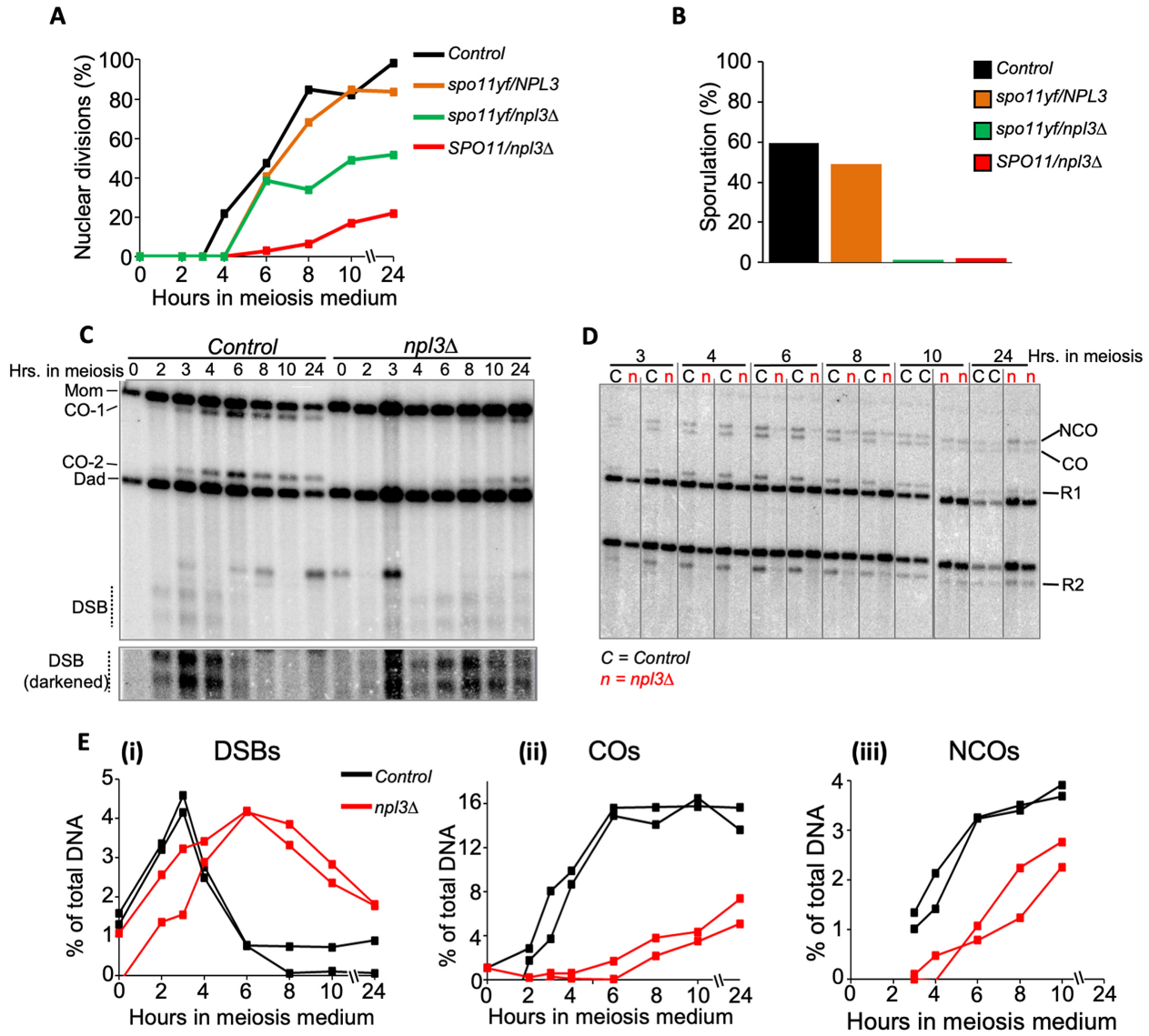

Figure 4. Npl3 is required for efficient meiotic recombination. (**A**) Graph shows % of cells having undergone the first and/or second meiotic division at the indicated time points of meiosis. Meiotic divisions were determined by counting the number of DAPi stained DNA masses in ≥100 cells. (**B**) Graph shows percent of tetrads formed after 24 h in sporulation medium based on visual observation. (**C**, **D**) Southern blot analyses of DNA isolated from control and *npl3Δ* cells showing double strand breaks (DSBs), crossovers (COs) and Non crossovers (NCOs) at indicated time points in meiosis. Images are shown from one (C) or both (D) independent analyses performed. In panel D, the C represents control and *n* is used for *npl3Δ*. (**E**) Quantitation of independent Southern blot analyses for DSBs, COs and NCOs.

right panel). Collectively, these results suggest that in the context of the tested genes, Npl3 functions to promote splicing when non-consensus sites or secondary structures prevent efficient splicing. Although changing non-consensus splice sites of *MER2*, *HOP2* and *SAE3* individually rescued their respective splicing defects, each failed to rescue the cell cycle defect observed in *npl3Δ*, again suggesting that the meiotic arrest of *npl3Δ* is not due to a splicing defect involving just one of these genes (Figure 5C).

In mitotic cells, Npl3 promotes splicing of ribosomal protein genes, yet Npl3 is non-essential in this cell cycle. The essential requirement of Npl3 in meiosis, but not mitosis, raises the question of whether the splicing function of Npl3 differs in the meiotic and mitotic cell cycles. To address this issue, the splicing of a meiotic (e.g. *SAE3*) and mitotic (e.g. *SCS22* and *RPL18B*) targets of Npl3 were analyzed in both mitotic and meiotic cells (Supplementary Figure S4). To express the meiotic gene *SAE3* in mitosis, the gene encoding the transcriptional repressor *UME6* was deleted, which allows *SAE3* to be expressed (12). Consistent with a previous report (57), the splicing of *SCS22* and *RPL18B* were altered in *npl3Δ* cells in mitosis, which was also observed in *npl3Δ* meiotic culture (Supplementary Figure S4). The splicing of *SAE3* also depended on Npl3 in both mitotic and meiotic cultures. Hence, the function and requirement of Npl3 for splicing does not appear to be altered between the mitotic and meiotic cell cycles.

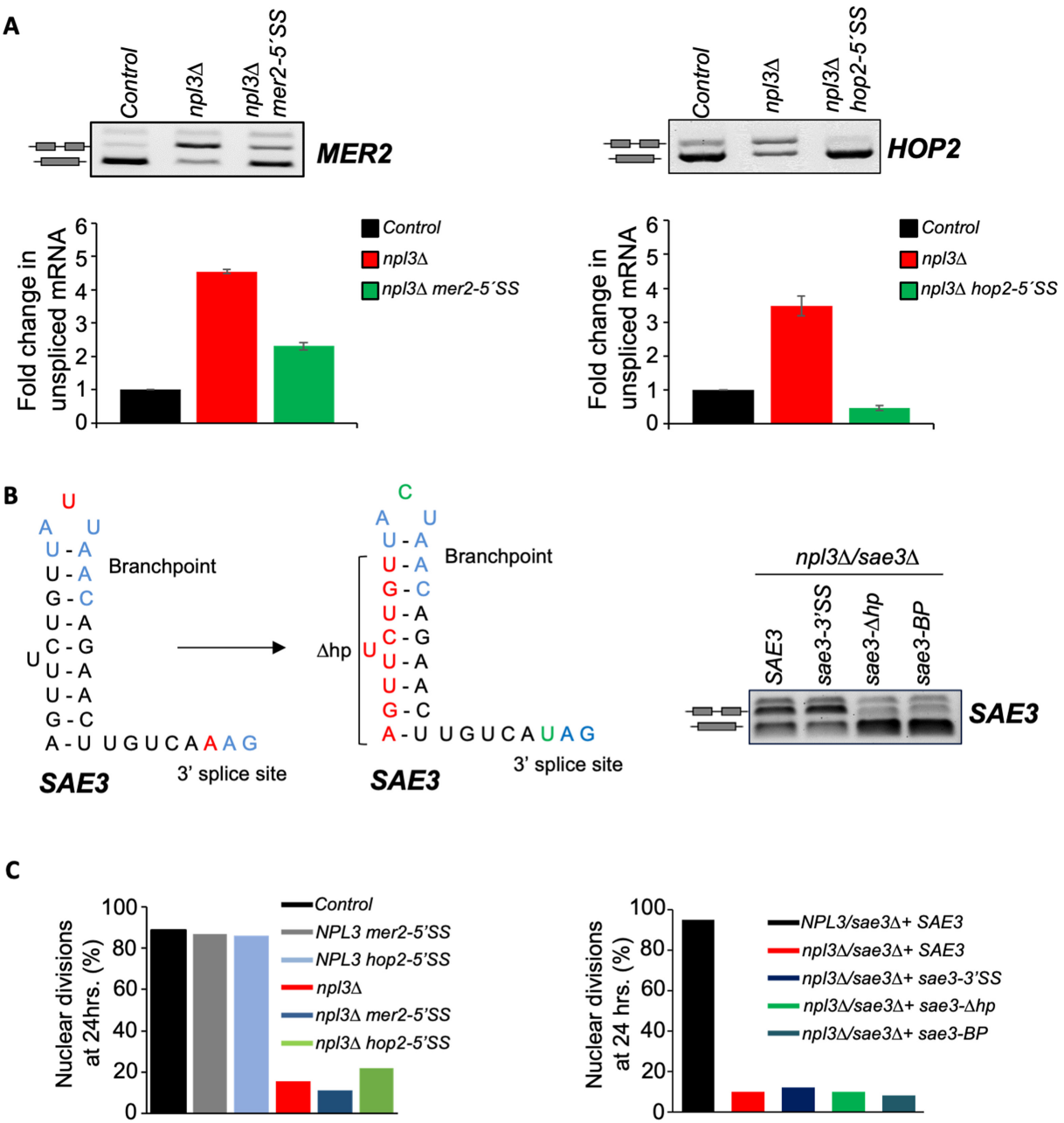

Figure 5. Npl3 is required to promote splicing of non-consensus introns. (**A**) Agarose gels at top show PCR products generated by primers flanking the intron of the indicated gene from control and *npl3Δ* cells at 5 h of meiosis. The pre-mRNA and spliced RNA are indicated. Graphs below show RT-qPCR analysis quantifying fold enrichment in intron containing transcripts in control and *npl3Δ* cells at 5 h of meiosis. Error bars represent standard deviation, $n = 3$. (**B**) Schematic showing non-consensus 3′splice site, branch point and hairpin in *SAE3* and the corresponding mutations introduced to individually alter each feature to make it consensus. Agarose gel on right show PCR products generated by primers flanking the intron of ectopically expressed *SAE3* in *npl3Δ/sae3Δ* cells at 5 h of meiosis. The pre-mRNA and spliced RNA are indicated. (**C**) Graph shows % of cells having undergone the first and/or second meiotic division after 24 h of meiosis induction. Meiotic divisions were determined by counting the number of DAPi stained DNA masses in ≥100 cells. 5′SS is 5′ splice site, 3′SS is 3′ splice site, BP is branchpoint, and Δhp is a deletion of the hairpin.

Npl3 is part of an overlapping splicing regulatory network

As described above, some meiotic transcripts require additional activators to promote their splicing due to non-consensus sites or other transcript features (29,30,33). Meiotic splicing activators include Mer1, Nam8 and Tgs1, which in some cases share pre-mRNA targets and in other instances function independently of each other (27,35,38,43,47). In addition, *npl3Δ/nam8Δ* and *npl3Δ/tgs1Δ* double mutants have reported synthetic sick interactions (57,97), suggesting Npl3 could function with these proteins in splicing. To test this hypothesis, the Npl3 anchor away system was employed in *tgs1Δ* or *nam8Δ* backgrounds to bypass the synthetic sick interactions that occur in mitotic cells between double mutants. Importantly, adding rapamycin to a culture in which Npl3 is not

tagged with FRB had no impact on splicing (Figure 6A). In contrast, a defect in splicing was reproduced for *HOP2*, *SAE3* and *MER2* when Npl3 was anchored, most prominently for *SAE3* and *MER2* after 2 h of meiosis (Figure 6A). These results indicate that Npl3-dependent splicing can be altered using the anchor away system and further demonstrates that the splicing defects observed in *npl3Δ* are meiosis specific (i.e. the defects do not stem from pre-existing issues in gene expression carried over from the mitotic cell cycle in *npl3Δ* cells).

To determine the functional relationship between Npl3 and Tgs1 or Nam8 in terms of splicing, Npl3 was depleted in a *tgs1Δ* or *nam8Δ* background at the start of meiosis and splicing of *HOP2*, *SAE3* and *MER2* was assayed. For Tgs1, previous reports showed that *HOP2* and *MER2* do not rely on Tgs1 for splicing, but *SAE3* does (43). In the anchor away strain with *tgs1Δ* and no rapamycin addition, splicing of *SAE3 was* defective as expected (43), but unexpectedly *HOP2* and *MER2* also displayed strong splicing defects and this was not further changed with Npl3 depletion (Figure 6A). Our data (Supplementary Figure S5B, see *tgs1Δ* panel), reconfirm that *HOP2* and *MER2* do not have a detectable splicing defect in *tgs1Δ* cells, indicating that tagged Npl3 is likely hypomorphic and has a synthetic genetic interaction with *tgs1Δ*. These data suggest that Tgs1, either directly or indirectly, acts to support splicing of *HOP2* and *MER2* when Npl3 activity is reduced, which is revealed here by Npl3 tagging in a *tgs1Δ* mutant.

For Nam8, previous reports indicate that splicing of Mer1-dependent targets strongly depends on Nam8, including the *MER2* pre-mRNA (38,49). In the *nam8Δ* mutant, the splicing of *MER2* was completely blocked with no spliced transcript signal visible and this was not altered by Npl3 depletion. The strength of this phenotype precludes interpretation of the double mutant, but data from *npl3Δ* cells and the Npl3 anchor away system show that *MER2* splicing also depends on Npl3 (Figures 3 and 6A). In the case of *HOP2* and *SAE3,* splicing in the anchor away strain with *nam8Δ* was comparable to the anchor away control strain without rapamycin, with the pre-mRNA increasing upon Npl3 depletion (Figure 6A). These results show that among these targets only the splicing of *MER2* depends on both Nam8 and Npl3.

The third protein reported to promote splicing of meiotic transcripts is Mer1. *MER1* is a meiosis specific gene with no known function in mitotic cells (37), as such the *mer1Δ/npl3Δ* strain was viable and found to behave similarly to the *npl3Δ* single mutant during mitotic growth. Previous reports have shown that Mer1 promotes splicing of *MER2, ZIP4, AMA1* and *MER3* (36,38,47). To determine the functional relationship between Npl3 and Mer1 on these four transcripts, splicing patterns were analyzed in *mer1Δ*, *npl3Δ* and *mer1Δ/npl3Δ* double mutants. As before, a clear impact on splicing can be seen in *npl3Δ* cells for *MER2* and *ZIP4* (Figures 3A and 6B), while *AMA1* and *MER3* were marginally impacted. In case of *mer1Δ*, splicing of *MER2* was impacted to a similar level as *npl3Δ*, while splicing of *ZIP4, AMA1* and *MER3* was completely blocked. The low level of *MER2* splicing occurring in either single mutant was abolished in a *mer1Δ/npl3Δ* double mutant (Figure 6B). Intriguingly, there was a slight improvement in *MER3* splicing in *mer1Δ/npl3Δ* double mutant as compared to the *mer1Δ* single mutant, which was repeatably observed. We expect this could be due to changes in the double mutant that could differentially alter splicing of individual transcripts due to the availability of spliceosome machinery (e.g. competition).

To further probe potential functional interactions between these proteins, Npl3 was overexpressed using the copper inducible *pCUP1–1* promoter to test if overexpression would alter splicing or meiotic progression defects in *mer1Δ, nam8Δ* or *tgs1Δ* cells. *NPL3* overexpression at the transcriptional level was confirmed using RT-PCR in each mutant background (Supplementary Figure S5A). Under these conditions, there was no obvious change in splicing for the majority of meiotic transcripts tested, with the potential exception of *AMA1* in a *tgs1Δ* background and *SAE3* in the *nam8Δ* background (Supplementary Figure S5B). Consistent with the failure of Npl3 overexpression to alter splicing defects in these mutants, the meiotic progression of *mer1Δ, nam8Δ* and *tgs1Δ* cells were also unaffected by Npl3 overexpression (Supplementary Figure S5C). These results suggest that excess Npl3 cannot bypass the functions provided by these other splicing activators. Moreover, these data indicate that Npl3, Mer1, Nam8 and Tgs1 have both shared and unique pre-mRNA targets as part of an overlapping splicing regulatory network in meiosis (Figure 7E).

Methylation of Npl3 is required for the splicing of Mer1 dependent transcripts

Npl3 is subject to both phosphorylation and methylation, which are known to alter the subcellular localization and functions of Npl3 (75). Loss of phosphorylation at serine residue 411, has been shown to impact Npl3 nuclear import and transcription termination without altering splicing in mitotic cells (76,77,97). Therefore, we used the *npl3-S411A* mutant to further dissect specific Npl3 functions contributing to the meiotic cell cycle. Using this mutant, we did not detect any defect in the kinetics or overall level of meiotic nuclear divisions as compared to control cultures (Figure 7B). Moreover, the three meiotic targets with strong splicing defects in *npl3Δ* cells, *MER2*, *SAE3* and *HOP2*, were not impacted in the phosphorylation deficient mutant (Figure 7A). These results indicate that phosphorylation of Npl3 at S411 is not required for splicing in meiosis or meiotic cell cycle progression. In addition, since phosphorylation of S411 is linked to transcript termination, these data further suggest that the splicing defects observed in *npl3Δ* cells are not solely arising from global changes in transcript termination

Another post-translational modification, methylation, is reported to promote Npl3 nuclear export and splicing function in mitotic cells (72,74,75,97). To test the role of methylation in meiotic splicing, a *npl3-RK1–15-myc* mutant was used in which 15 arginine residues at the C-terminus of Npl3 are mutated to lysine to block methylation (72). Control and methylation mutant cultures were induced to undergo meiosis and nuclear divisions were counted throughout the meiotic time course. A substantial delay in meiotic progression and reduced meiotic nuclear divisions were observed in the methylation mutant as compared to the control (Figure 7C). To analyze the splicing phenotype of the methylation mu-

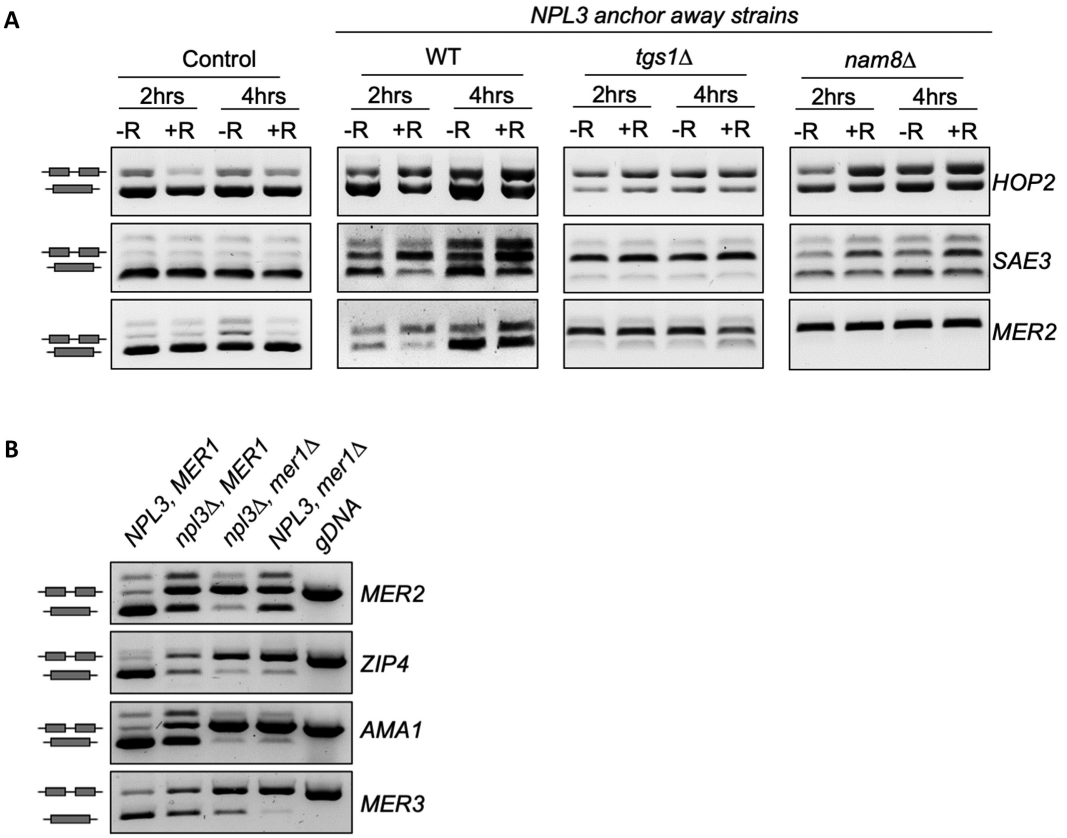

Figure 6. Npl3 promotes splicing of an overlapping set of meiotic transcripts with Tgs1, Nam8 and Mer1. (**A**) Agarose gels showing PCR products generated by primers flanking the intron of the indicated gene from cultures with (+R) and without (−R) rapamycin addition at the start of meiosis (0 h) to deplete Npl3 in anchor away strains. The pre-mRNA and spliced RNA are indicated. Strain genotypes are indicated for each. (**B**) Agarose gel showing PCR products generated by primers flanking the intron of the indicated gene from control, *mer1Δ*, *npl3Δ* and *mer1Δ/npl3Δ* mutants at 5 h of meiosis.

tant, meiotic transcript splicing was determined in two independent cultures of *Npl3-myc* and *npl3-RK1–15-myc* mutant, with *NPL3* and *npl3Δ* cultures as controls. The splicing of *HOP2* and *SAE3* was similar in control cells and the methylation mutant, but altered by *npl3Δ,* suggesting that methylation of Npl3 is not required for splicing *HOP2* or *SAE3* transcripts (Figure 7D). Intriguingly, the splicing of both *MER2* and *ZIP4* were impacted in the *npl3-RK1–15* mutant to a level that was comparable to *npl3Δ* (Figure 7D). These findings demonstrate a specific requirement for Npl3 methylation in splicing Mer1-dependent transcripts *MER2* and *ZIP4*, while splicing of transcripts that otherwise depend on Npl3 do not require methylation of Npl3. Overall, our findings indicate an important role for Npl3 in the meiotic splicing regulatory network by promoting splicing of select meiotic transcripts, which is further distinguished by Npl3 methylation.

DISCUSSION

The meiotic cell cycle in *S. cerevisiae* requires expression of meiosis specific genes, many of which have introns with non-consensus splice sites. Consequently, meiotic gene expression, and completion of meiosis, relies on various proteins to promote pre-mRNA splicing, including Mer1, Nam8 and Tgs1. Here, we show that the SR protein Npl3 plays a critical role in the meiotic cell cycle as part of the meiotic splicing regulatory network (Figure 7E). As a protein family in *S. cerevisiae*, the three SR-like protein members (i.e. Npl3, Gbp2 and Hrb1) have been shown to play partially overlapping non-essential roles in gene expression within the mitotic cell cycle (58,60,66,68), yet we find that Npl3 is required for meiosis, demonstrating a unique and essential function for the budding yeast SR protein Npl3.

Npl3 is required for efficient splicing of meiotic transcripts

Our data demonstrate an important role for Npl3 in the splicing of select meiotic transcripts. Npl3 depletion assays show that Npl3 is required only at the initial stages of the meiotic program, consistent with a critical function of Npl3 in splicing and expression of early meiotic genes. The genes impacted are enriched for transcripts that encode proteins with functions in meiotic recombination (e.g. Rec102, Rec114, Mer2, Hop2, Sae3 and Zip4), which in a few tested cases (e.g. Hop2 and Sae3) have a corresponding reduction in protein levels. We expect lowered production of proteins functioning in both DSB formation and resolution is the cause for the observed delays in processing DSBs, and the generally asynchronous progression through meiotic recombination. Such defects in meiotic recombination would be expected to activate the pachytene checkpoint resulting in strong meiotic cell cycle arrest, as observed in *npl3Δ*. This is further supported by our data showing that a *npl3Δ* me-

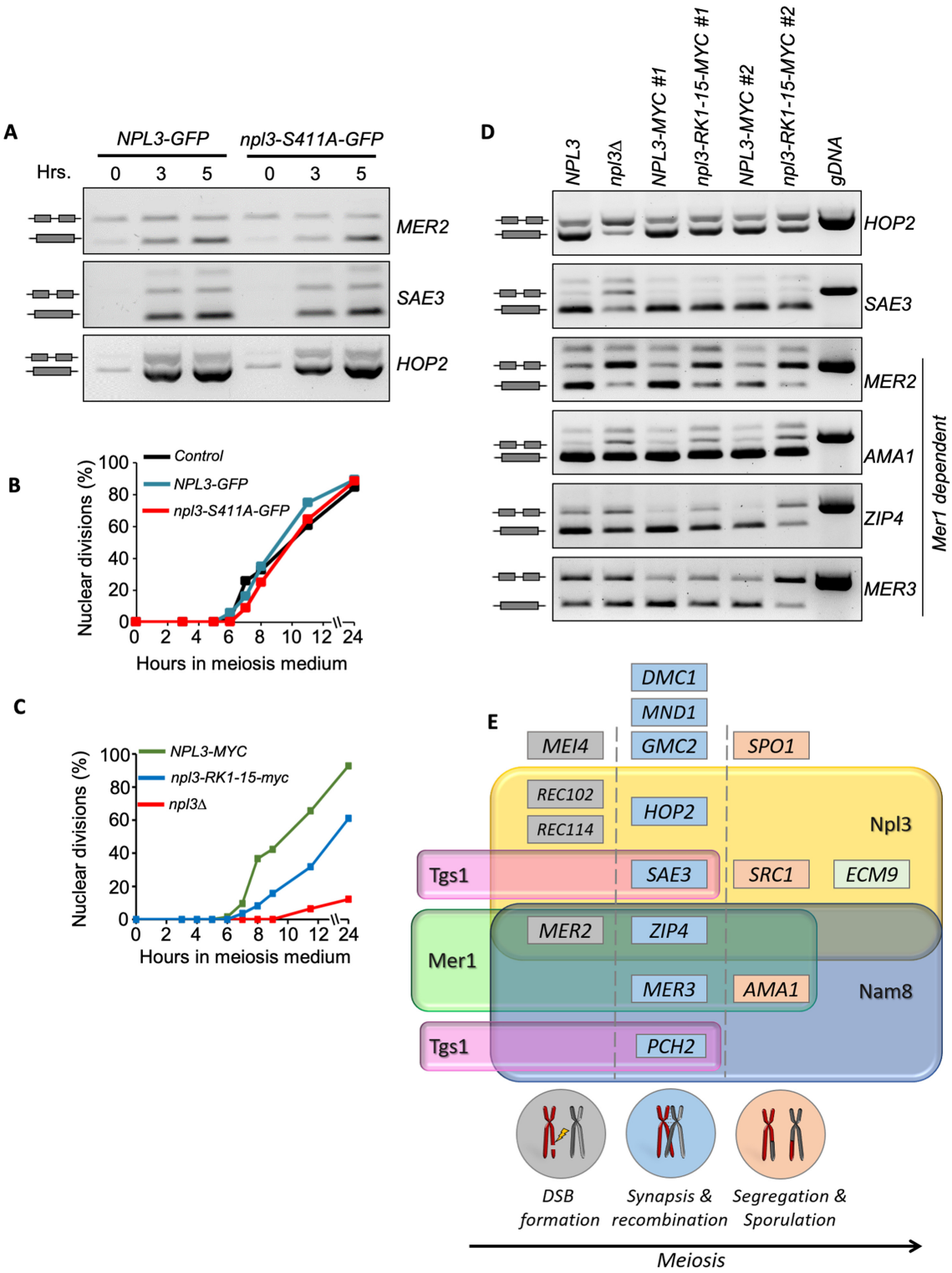

Figure 7. Methylation, not phosphorylation, of Npl3 supports meiotic progression. (**A**) Agarose gel showing PCR products generated by primers flanking the intron of the indicated gene from *NPL3-GFP* and *npl3-S411A-GFP* cells at indicated time points in meiosis. The pre-mRNA and spliced RNA are indicated. (**B**, **C**) Graphs show % of cells having undergone the first and/or second meiotic division at the indicated time points of meiosis. Meiotic divisions were determined by counting the number of DAPi stained DNA masses in ≥100 cells. For C, one of the two independent cultures of *NPL3-MYC* and *npl3-RK1–15-MYC* is shown. (**D**) Agarose gel showing PCR products generated by primers flanking the intron of the indicated gene. The pre-mRNA and spliced RNA are indicated. Two independent cultures of *NPL3-MYC* and *npl3-RK1–15-MYC* are shown. Time point shown is 5 h. **(E)** Model highlighting the unique and shared targets of each splicing activator within the meiotic splicing regulatory network. Genes are further organized based on the known function of the expressed protein product in meiosis.

diated arrest is partially rescued by the absence of meiotic DSBs (e.g. in *spo11-yf* background), suggesting that the requirement of Npl3 is diminished in the absence of meiotic recombination. Notably, only a partial bypass of the *npl3Δ* mediated arrest was observed in the absence of DSBs, indicating that other aspects of the meiotic cell cycle are supported by Npl3.

In mitotically dividing cells, Npl3 has also been shown to function in transcription termination and 3′ processing, mRNA export, and translation (57,59–62,64,67). Indeed, our data indicate that transcription of certain meiotic genes required for key steps in meiosis I (such as *IME1*, *DMC1* and *ZIP1*) may be delayed in *npl3Δ* cells, which could be linked to Npl3 functions in transcription and 3′ processing. Alternatively, delayed appearance of these transcripts could be due to a slowed initiation of the meiotic program and/or premeiotic DNA replication, as loss of Npl3 has been linked to genome-wide impairment of DNA replication in mitosis (98). This delay in expression of early meiotic genes is not likely causative of the strong meiotic arrest in *npl3Δ* cells since the level of these meiotic transcripts reaches near control levels at later time points (∼6 h after initiation of meiosis). Of these transcripts, *IME1* encodes a master regulator of meiosis that if not properly expressed in *npl3Δ* cells would be expected to severely impact meiotic progression; however, when *IME1* was overexpressed it did not alter meiotic arrest in *npl3Δ* cells. Overall, while Npl3 functions at multiple steps in gene expression pathway, our data suggest that enhanced splicing of a subset of meiotic transcripts constitutes a critical activity of Npl3 in meiosis, although other aspects of meiotic gene expression program may also be impacted by the absence of Npl3.

Npl3 is known to be regulated by post-translational modifications, including phosphorylation and methylation. Our data further indicate that phosphorylation at S411 is not required for meiosis, while methylation is important for splicing and meiotic progression. Interestingly, methylation of Npl3 is not required for splicing *HOP2* and *SAE3* that have strong defects in *npl3Δ* cells, but is required for *MER2* and *ZIP4*. These results suggest that Npl3 may promote splicing via distinct mechanisms if the meiotic transcript is independent (*HOP2* and *SAE3*) or dependent on (*MER2* and *ZIP4*) Mer1. Methylation of Npl3 increases its interaction with Snu56, which is a component of the U1 SnRNP and is reported to be required for splicing of Mer1 dependent transcripts (99), raising the possibility that methylation of Npl3 promotes splicing of Mer1 dependent transcripts via Snu56. It is also possible that in the absence of Npl3, defects in splicing are more widespread due to a disruption to nuclear homeostasis (e.g. sequestration of the spliceosome and other proteins on a subset of transcripts) (100,101). These system-wide defects may differentiate the meiotic splicing phenotypes that are observed in *npl3Δ* versus a Npl3 methylation mutant. In other words, those transcripts showing altered splicing in the methylation mutant may represent more direct targets of Npl3, while defects observed in the *npl3Δ* strain may encompass both direct and systems-level mechanisms.

Still, the viability of a *npl3Δ* strain indicates mitotic cells are functional in the absence of Npl3, while Npl3 is essential for meiosis. One likely reason for the observed difference in the essential nature of Npl3 across the two cell cycles is that many of the mitotic splicing targets of Npl3 consist of ribosomal protein genes (57). With paralogs present for RPGs, that are transcribed at high levels, the amount of mature transcript and resulting protein may be sufficient for cell viability even when splicing is less efficient. In addition, transcriptome splicing data show *npl3Δ* cells display weaker splicing defect that often varies between RPG paralogs, in contrast to mutants in the spliceosome ((102), see Supplemental Table S1). This likely results in the required level of ribosomal proteins to be produced from one or both of the paralogs in the absence of Npl3. Additionally, our data demonstrate that Npl3 is required for splicing of meiotic transcripts bearing non-consensus splice sites (see discussion below). As many RPGs are highly expressed and bear consensus introns, the functional requirement of Npl3 for their splicing may be less than that of meiotic gene targets. It is also possible that the actual splicing activity enhanced by Npl3 varies in different cell cycle stages, but our data do not support this idea, as the requirement for Npl3 mediated splicing was not altered when meiotic transcripts were expressed in mitosis or vice versa. Therefore, while Npl3 is required for splicing in mitosis and meiosis, meiotic splicing is likely more dependent on Npl3 due to the nature of the specific pre-mRNA targets, including an enrichment of intron containing genes in meiosis with suboptimal splice sites.

Npl3 promotes splicing of introns with non-consensus splice sites in meiosis

SR proteins in other eukaryotes are known to function in regulated splicing, such as alternative splicing and splicing of introns bearing suboptimal splice sites (52). However, in *S. cerevisiae*, it was previously demonstrated that Npl3 promotes splicing of RPGs, which bear strong introns with consensus splice sites. This raised the possibility that Npl3 may promote splicing via alternative mechanism as compared to SR proteins in other organisms (57). In our work, mutation of a suboptimal 5′ splice site in *MER2* and *HOP2* improved splicing in *npl3Δ* cells, as did mutation of a branchpoint sequence or hairpin forming sequence in *SAE3*. These data indicate that Npl3 supports splicing of transcripts bearing non-consensus splice sites, at least in meiosis, aligning Npl3 function with other metazoan counterparts. Yet, the loss of Npl3 function does not alter splicing of all meiotic transcripts bearing suboptimal splice sites, suggesting Npl3 is only required for efficient splicing of a subset of transcripts. Many possibilities can be considered for the mechanism by which this occurs. First, it is possible that Npl3 mediated splicing is directed by regulated recruitment of Npl3 to a transcribed gene. Indeed, SR proteins exhibit differential recruitment to transcripts due to varying protein-protein and protein-RNA interactions (103). In the case of Npl3, it is known to be recruited co-transcriptionally to most transcripts being produced in mitotic cells and to bind to the CTD of RNA Pol II (69), but this is altered in response to stress (68). Given the regulated recruitment of Npl3 during stress, and that entry into meiosis involves nutrient limitation, it is possible that Npl3 is differentially recruited to transcripts in meiosis, but this remains to be investigated. It

is also possible that the timing of Npl3 recruitment directs different functions on a transcript. Npl3 has been shown to be enriched at the 5′- and 3′end of genes in mitotic cells through RNA crosslinking experiments that is consistent with a role in splicing and transcriptional termination (63). Whether this distribution of Npl3 is altered in meiosis, or on individual spliced transcripts, is not currently known.

As a multifunctional protein, Npl3 has a diverse set of interaction partners that function in pre-mRNA processing, mRNA export, chromatin modification, and DNA damage response (97), which allows regulated recruitment of Npl3 to pre-mRNAs to be easily envisioned. One such interaction partner that could alter Npl3 recruitment to transcripts is Bre1, an E3 ubiquitin ligase involved in ubiquitination of histone H2B that interacts genetically and physically with Npl3 (97). Given that *bre1Δ* cells exhibit a weak defect in splicing and loss of Bre1 exacerbates splicing defects in *npl3Δ* cells (97), the interaction of Npl3 with Bre1 could impart specificity to Npl3 to promote splicing of target transcripts. Interestingly, Bre1 is also required for meiotic cell cycle progression, as *bre1Δ* cells exhibit a delayed and overall lower level of meiotic nuclear divisions (104). Other variables that could impart specificity to Npl3 mediated splicing could involve the overall rate of splicing, mode of splicing (co-transcriptional or post-transcriptional), nucleotide sequences outside of the splice site, and the presence of secondary structure within an intron.

Mer1, Nam8 and Tgs1 are similarly reported to promote splicing of meiosis specific transcripts as part of a meiotic splicing regulatory network (27,38,43,47). These proteins are non-essential for the mitotic cell cycle, but are indispensable for sporulation (38,43). The synthetic sickness between *npl3Δ* and *nam8Δ* or *tgs1Δ* further suggests that these proteins function in similar processes (57,97). Indeed, through use of double mutants and Npl3 depletion strains, we characterized both shared and unique pre-mRNA targets for Npl3 with other splicing activators (Figure 7E). The mechanism by which Npl3 supports splicing efficiency during meiosis is unknown, but in mitotic cells it has been shown that Npl3 directly engages splicing factors, including an RNase insensitive interaction with Nam8 (57). Given that Npl3 is recruited co-transcriptionally to transcripts, one role of Npl3 could be to increase the ability of pre-mRNAs with weak introns to compete for splicing machinery through reinforcing transcript – spliceosome interactions. This mode of action would be consistent with our findings that mutation of non-consensus splice sites to the consensus sequence bypasses the requirement for Npl3 in most cases.

Overall, the discovery of a role for Npl3 within the meiotic splicing regulatory network provides for a more complete understanding of this process (Figure 7E). A major challenge going forward is to understand how Mer1, Nam8, Tgs1 and Npl3 contribute to the splicing of individual pre-mRNAs in a shared or independent manner and how this ultimately is used to coordinate proper gene expression across the meiotic cell cycle. This will be made more challenging by the fact that Npl3 functions in multiple aspects of gene expression, which may allow Npl3 functions in other parts of the gene expression pathway (e.g. 3′ processing and translation) to indirectly feedback on splicing. Notably, this may make Npl3 an ideal candidate to coordinate gene expression at a system level by functioning in multiple steps of the process to coordinate efficient progression through the meiotic cell cycle via waves of gene expression that include early, middle and late meiotic events (1–3,7). Moreover, these findings provide further molecular-level details of a rather complex splicing regulatory network that we expect can be used as a foundation for understanding metazoan splicing networks.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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