The Fission Yeast Pre-mRNA-processing Factor 18 (prp18\(^{+}\)) Has Intronspecific Splicing Functions with Links to G\(_{1}\)-S Cell Cycle Progression\(^{a,5}\)

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The fission yeast genome, which contains numerous short introns, is an apt model for studies on fungal splicing mechanisms and splicing by intron definition. Here we perform a domain analysis of the evolutionarily conserved \(\text{Schizosaccharomyces pombe}\) pre-mRNA-processing factor, SpPrp18. Our mutational and biophysical analyses of the C-terminal \(\alpha\)-helical bundle reveal critical roles for the conserved region as well as helix five. We generate a novel conditional missense mutant, \(\text{spprp18}^{-5}\). To assess the role of SpPrp18, we performed global splicing analyses on cells depleted of \(\text{prp18}^{+}\) and the conditional \(\text{spprp18}^{-5}\) mutant, which show widespread but intron-specific defects. In the absence of functional SpPrp18, primer extension analyses on a \(\text{tsfII}d^{+}\) intron 1-containing minitranscript show accumulated pre-mRNA, whereas the lariat intron-exon 2 splicing intermediate was undetectable. These phenotypes also occurred in cells lacking both SpPrp18 and SpDbp1 (lariat debranching enzyme), a genetic background suitable for detection of lariat RNAs. These data indicate a major precatalytic splicing arrest that is corroborated by the genetic interaction between \(\text{spprp18}^{-5}\) and \(\text{spprp2}^{-1}\), a mutant in the early acting U2AF59 protein. Interestingly, SpPrp18 depletion caused cell cycle arrest before S phase. The compromised splicing of transcripts coding for G\(_{1}\)-S regulators, such as Res2, a transcription factor, and Skp1, a regulated proteolysis factor, are shown. The cumulative effects of SpPrp18-dependent intron splicing partly explain the G\(_{1}\) arrest upon the loss of SpPrp18. Our study using conditional depletion of \(\text{spprp18}^{+}\) and the \(\text{spprp18}^{-5}\) mutant uncovers an intron-specific splicing function and early spliceosomai interactions and suggests links with cell cycle progression.

Pre-mRNA splicing, a fundamental step in the processing of nascent eukaryotic RNA polymerase II transcripts, achieves precise excision of introns coupled with exon ligation to generate functional mRNAs. The spliceosome, which is composed of five U snRNPs and >100 auxiliary proteins, assembles onto \(\text{cis}\) splicing signals, namely the 5' splice site (5'ss), the branch point nucleotide, the 3' splice site (3'ss), and polypyrimidine (Pyn) tracts. The spliceosomal catalytic core consists of the U2, U5, and U6 snRNPs and accessory proteins. This complex mediates the two trans-esterification reactions required for splicing to occur. First, the 5'ss is cleaved to yield the branched lariat intron-exon 2 and exon 1 intermediates, followed by the second reaction, where the 3'ss is cleaved, the exons are joined, and lariat intron is excised (1, 2).

Genetic and biochemical analyses in budding yeast and biochemical studies with mammalian cell extracts have established a network of interactions among factors that act at the second step of splicing (\(i.e.\) Prp8, Prp16, Prp17, Prp18, Slu7, and Prp22) (3–5). \(\text{PRP18}\), a non-essential budding yeast gene, encodes a U5 snRNP-associated factor. Prp18 has been analyzed extensively in budding yeast and human cell extracts for splicing of a specific intron containing pre-mRNAs, but the \(\text{Schizosaccharomyces pombe prp18}^{+}\) has not been studied in detail. Such studies have the potential to allow mechanistic insights into a genetic model system that better recapitulates splicing of short introns prevalent in several higher eukaryotes. The \(\text{PRP18}\) gene is not essential in \(\text{Saccharomyces cerevisiae}\), and \(\text{prp18}^{-}\) cells are temperature-sensitive (ts) and arrested for the second step of splicing both \(\text{in vivo}\) and \(\text{in vitro}\) (10, 11). Further analysis of \(\text{S. cerevisiae prp18}\) revealed that an N-terminally truncated Prp18 (ScPrp18A79) protein, lacking 79 residues, was functional to mediate splicing \(\text{in vitro}\). The crystal structure of this truncated protein revealed a five-helix bundle fold with a highly

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\(^{7}\) The abbreviations used are: snRNPs, small nuclear ribonucleoprotein; 5’ ss, 5’ splice site; 3’ ss, 3’ splice site; Pyn, polypyrimidine; nt, nucleotide; ts, temperature-sensitive; CR, conserved region; hPrp18, human Prp18; MBF, MluI binding factor; RP and FP, reverse primer and forward primer, respectively; PDB, Protein Data Bank; EMM, Edinburgh Minimal Medium.
Roles for SpPrp18 in Splicing and Cell Division Cycle

conserved region (CR) loop between helices 4 and 5 (9). Extensive mutational analyses of ScPrp18 identified critical functions for helices 1 and 2 in mediating interactions with the splicing factor ScSlu7. This interaction is important for ScPrp18 spliceosomal association, whereas other regions in its globular domain stabilize U5 snRNA-exonic interactions after the first catalytic reaction (7, 9, 12–15).

In vitro splicing of β-globin pre-mRNA in HeLa cell free extracts suggests that the human orthologue of Prp18, hPrp18, functions in the second step of splicing (4) but how widely or even strictly this function is conserved in other short intron-rich higher eukaryotic and fungal genomes is not yet clear. When hPrp18 is immunodepleted from HeLa cell extracts, a second step in vitro splicing arrest occurs. However, hPrp18, when expressed in budding yeast scppr18Δ cells, does not rescue their strong ts phenotype, thus suggesting some distinctions in the splicingosomal associations of these orthologs (4).

Here we exploit the fission yeast system to further define the conserved functions of Prp18 and provide insight into how events in splicing can be coordinated. Salient features of the fission yeast genome include occurrence of multiple short introns per transcript, degenerate cis splicing signals, and unusually located Pyn tracts. These pre-mRNA features, found in many fungal genomes, make it suitable for studies on correlations between cis features and splicing factor requirements (16, 17). The splicing of short introns in fission yeast is also a model for other higher eukaryotes like plants, flies, and worms, where an intron definition model for splice site recognition is proposed (18). Interestingly, earlier studies on the role of intronic 3′ss and the Pyn tract sequences for splicing of two fission yeast introns showed that both of these cis elements are required before the first splicing reaction (19). Similar effects are seen for 3′ss mutations in a subset of mammalian introns (20). Genetic studies on predicted second step factor homologs in fission yeast are limited to only SpPrp17 and SpSlu7, yet they reveal certain differences when compared with budding yeast counterparts. Deletion of spprp17+, a non-essential gene, did not affect the splicing of introns in the tfldl, gene, including the 255-nt intron 1 (21). Genome-wide splicing analyses of transcripts in a missense mutant of spslu7-1 revealed its requirement before the first step of the splicing reaction, its widespread but intron-specific functions, and its novel interaction with another precatalytic splicing factor (22). The dependence on fission yeast factors SpPrp2 (23) and SpPrp4 (24) correlates with specific intronic features in each case. Other studies with cwf10-ΔNTE mutant of the SpCwf10 splicing factor revealed that its role in splicing is general and not transcript-specific (25). These studies lend support to the hypothesis of co-evolution of splicing factor functions with changes in gene and intron architecture. These findings warrant investigations on functions for other fission yeast splicing factors. Such studies could uncover mechanisms for splice site selection in the context of short introns.

Here, we investigated the splicing functions of the predicted fission yeast second step factor SpPrp18 through structure-driven mutational and genetic approaches. Our results reveal vital roles for the SpPrp18 conserved domain and flanking helices. Genome-wide splicing studies and genetic interaction analyses using a missense mutant show that widespread SpPrp18 functions are in pre catalytic spliceosomes, and its essential functions for early steps in splicing are intron-specific.

Links between splicing and cell cycle progression have been well established by genetic and protein interaction analyses in budding yeast, fission yeast, and mammalian cells (26–31). In S. pombe, ts mutants in several splicing factors (26–28, 32) arrest at restrictive temperatures as elongated cells. Many of these mutants (e.g. cdc28-P8 and prp12-1) affect the G1-M transition (27, 31), whereas the loss of Prp4 kinase activity derails both the G1-S and G2-M cell cycle transitions (32). But how the splicing functions of these factors and cell cycle progression are related is as yet not entirely clear. Here, using a missense mutant in prp18”, we uncover an important role in promoting G1-S cell cycle transition through intron-specific splicing effects of transcripts encoding some key regulators of this transition.

Results

Comparative Modeling and Mutational Analysis of SpPrp18—The essential S. pombe gene SPCC126.14 encodes SpPrp18, a predicted U5 snRNP-associated protein (33). Prp18 proteins from budding yeast, fission yeast, and humans share a high degree of similarity in their C-terminal halves, which adopt the five-helical bundle with a CR loop between helices 4 and 5 (Fig. 1A). S. pombe SpPrp18 has 35% identity and 58% similarity with ScPrp18 and shows a similar degree of relatedness to hPrp18. A comparison of the domain architecture across these three species revealed the splicing factor motif in the N-terminal region of SpPrp18 and hPrp18 that is absent in ScPrp18 (Fig. 1A). We generated a homology model for amino acids 160–343 of SpPrp18 (Fig. 1B), which shows a five-helix bundle nearly identical to the homologous domain in ScPrp18 (9).

Extensive mutational analysis of budding yeast ScPrp18 (7, 13, 15) showed that helices 4, 5, and the intervening conserved region have splicing functions separable from those performed by the ScPrp18-ScSlu7 complex (13). For example, a triple mutant G196A/V197A/T198A in the CR region or a double mutant G288A/R289A confers a phenotype, thus suggesting some distinct functions in the spliceosomal associations of these orthologs (4).
Roles for SpPrp18 in Splicing and Cell Division Cycle

A Missense Mutation in SpPrp18 Helix 1 Alters Its Conformation and Confers Slow Growth—Cells with a plasmid-expressed missense mutant, SpPrp18V194R (nmt81 promoter-driven), were slow growing as compared with a control strain with plasmid-expressed wild-type spprp18\(^{+}\) (data not shown). For stable expression of the WT or prp18-5 alleles, we integrated the expression cassettes at the leu1 chromosomal locus and thus created a set of strains, spprp18\(^{+}\) leu1::Pnmt:prp18\(^{+}\) (this strain is referred to as WT; Fig. 2A) and spprp18\(^{-}\) leu1::Pnmt:prp18V194R (referred to as prp18-5; Fig. 2A). In these strains, we achieved conditional expression of the wild-type and mutant spprp18 alleles from the weak nmt81 promoter derived from the nmt1 locus whose transcription is repressed on supplementation of thiamine (36). WT cells grew robustly on media lacking thiamine (Fig. 2A, left, first row) and, as expected, grow poorly upon the addition of thiamine (Fig. 2A, left, second row). However, the growth of the prp18-5 strain was slow even in medium lacking thiamine at the ambient temperature. Furthermore, this mutant was inviable when expression was repressed by the addition of thiamine (Fig. 2A, right, top two rows). Thus, wild-type protein expressed from the leu1 locus fully complemented the spprp18\(^{-}\) null allele, but the SpPrp18-5 protein only partially supported growth. As expected, the severe growth arrest of WT and prp18-5 cells on thiamine supplementation was rescued when cells were transformed with a plasmid where spprp18\(^{+}\) is driven by endogenous promoter elements (Fig. 2A, bottom two rows).

Immunoblotting was performed to assess the levels of SpPrp18 wild-type and mutant protein expressed in these strains (WT and prp18-5). These strains were grown in the presence and absence of thiamine. Protein levels were compared with the endogenous level of SpPrp18 generated from its native locus (wild type). We detect approximately equivalent protein levels in these strains (Fig. 2B, lanes 1, 3, and 5). As expected, the growth retardation of WT and arrest of prp18-5 cells upon the addition of thiamine can be attributed to severely depleted SpPrp18 protein levels (Fig. 2B, lanes 2 and 4). Interestingly, immunoblotting analyses of wild-type SpPrp18 protein reveals an additional slower migrating species with ~3–4 kDa increased size (Fig. 2B, asterisk). This species is also drastically depleted upon supplementation of thiamine to repress expression of the leu1 locus-integrated wild-type allele. We ruled out the possibility that the slower migrating band represents a nonspecific cross-reacting protein because epitope-tagged wild-type SpPrp18, expressed from a plasmid, also generates two protein species of approximately the same molecular weights (Fig. 2C). This led us to speculate that a post-translational modification or altered conformational form of wild-type SpPrp18 could exist. Interestingly, this slow migrating species is absent in prp18-5 cells.

To explore the potential structural consequences of substitution of the non-polar (Val) to a charged (Arg) residue for the secondary and tertiary structure of SpPrp18, we examined the biophysical attributes of the bacterially expressed wild-type and mutant proteins. Far UV CD spectra showed negative ellipticity with sharp bands at 208 and 222 nm, indicative of \(\alpha\)-helical content of the protein. These are discernable for wild-type SpPrp18 over a range of temperatures from 20 to 90 °C (Fig. 2D).
Roles for SpPrp18 in Splicing and Cell Division Cycle

**Table 1**

| Residue | Position | Mutation | S. pombe | S. cerevisiae |
|---------|----------|----------|----------|--------------|
| Val-194 | Helix 1  | V194R    | ts/cs    | ~            |
| 294(GVT) | CR       | G288A/V289A/T290A | Null     | ts           |
| 322(KR) | Helix 5  | K325A/R326E | Null     | ts           |

**Table 2**

| Complementation profile of SpPrp18 C- and N-terminal mutants |
|---------------------------------------------------------------|
| Diplodipoles analyzed | No. of diploids analyzed | No. of spores selected on EMM leu-25 °C | No. of spores selected on EMM ura-25 °C | No. of “leak-through” diploids growing on EMM ade-25 °C | No. of “leak-through” diploids growing on EMM his-25 °C | No. of ade2-5M spores growing on EMM ade-25 °C |
|-----------------------|--------------------------|----------------------------------------|----------------------------------------|--------------------------------|--------------------------------|--------------------------------|
| spprp18::his3::spprp18 | 2                        | 48X2 (96)                             | 0                                      |                               |                               | 55                                    |
| spprp18::his3::spprp18 | 2                        | 48X2 (96)                             | 0                                      |                               |                               | 57                                    |
| spprp18::his3::spprp18 | 4                        | 48X2 (96)                             | 0                                      |                               |                               | 0                                     |
| spprp18::his3::spprp18 | 4                        | 48X2 (96)                             | 0                                      |                               |                               | 0                                     |
| spprp18::his3::spprp18 | 2                        | 96X2 (192)                            | 0                                      |                               |                               | 0                                     |
| spprp18::his3::spprp18 | 2                        | 96X2 (192)                            | 0                                      |                               |                               | 116                                   |
| spprp18::his3::spprp18 | 2                        | 96X2 (192)                            | 0                                      |                               |                               | 98                                    |

In contrast, the SpPrp18-5 (V194R) protein showed significant change in secondary structure starting at temperatures >40 °C (Fig. 2D, top right), indicating thermal instability probably due to protein unfolding. The native SpPrp18 protein has three tryptophan residues; Trp-32 (N-terminal), Trp-205 (helix 1), and Trp-285 (CR loop). We exploited these tryptophans to measure the steady-state intrinsic fluorescence of wild-type SpPrp18 and found that the emission maximum was at ~350 nm (Fig. 2E, bottom left). A red shift toward 365 nm was observed for the SpPrp18V194R protein (Fig. 2E, bottom right), which also suggested unfolding. We also observed that increased temperatures had little or no effect on the intrinsic fluorescence intensity for wild-type SpPrp18. In contrast, the SpPrp18V194R protein showed a gradual quenching of fluorescence intensity with increasing temperatures, indicating a loss of tertiary structure (Fig. 2E, bottom right). Thus, both genetic and biophysical data indicate that the SpPrp18V194R is a partially functional protein that is likely to be unstable in vivo.

Global Splicing Profiling of SpPrp18 Reveals Varying Splicing Defects—To probe the cellular functions of spprp18+, we used RNA from WT and prp18-5 cells, each grown with and without thiamine supplementation (36 h), to analyze the splicing status of a selection of cellular transcripts. Introns studied had diverse features (Fig. 3 and Table 3). We first analyzed an abundant cellular transcript, tfld+. Whereas intron 1 and intron 2 in tfld+ are efficiently spliced in wild-type cells, a ~3-fold increase in unspliced pre-mRNA was detected when spprp18+ was transcriptionally repressed (Fig. 3A, panel P, lane 2). Under these same conditions, any splicing defects were marginal for tfld+ intron 3. Cells expressing the SpPrp18-5 mutant protein were defective for splicing of all tfld+ introns, largely recapitulating the effect of wild-type SpPrp18 protein depletion (Fig. 3A, lane 3). These defects persisted in cultures where expression of spprp18-5 was repressed by the addition of thiamine (Fig. 3A, panel P, lane 4). As observed for the introns in tfld+, the two introns in the ade2+ transcript have differing splicing efficiencies even in wild-type cells. Repression of the WT or the prp18-5 allele caused a ~2.5-fold decrease in levels of spliced mRNA across the short ade2+ intron 1 (Fig. 3B, panel M). In contrast, splicing of ade2+ intron 2 in both strains was unaffected (data not shown). These data suggest essential but not ubiquitous splicing functions for spprp18+ and also provide evidence that the mutant Prp18-5 protein is only partially functional. To assess the genome-wide splicing role of fission yeast SpPrp18, we utilized splicing-sensitive microarrays as used to uncover substrate-specific functions for the S. pombe splicing factor SpStu7 (22). RNA from cultures of WT and prp18-5 cells, each grown in the presence or absence of 15 μM thiamine, was used for these analyses. These experiments are designed to reveal the effects of deleting either wild-type or mutant SpPrp18 and also to assess the function of the SpPrp18-5 protein. A very stringent data set of 253 introns with statistically significant and biologically correlated -fold changes for various probes across several transcripts upon depletion of wild-type SpPrp18 was selected for this analysis. In the absence of wild-type SpPrp18, this set of 253 introns showed a range of splicing defects (Fig. 4, A–E, WT- and WT+ T panels). Interestingly, similar splicing phenotypes, although in some instances with differing intensities, were observed in cells that express the
Roles for SpPrp18 in Splicing and Cell Division Cycle

mutant SpPrp18-5 protein (Fig. 4; prp18-5 – T, probes P and M). Overall, splicing defects could be categorized as introns that accumulate as unspliced pre-mRNA (Fig. 4B), those that accumulate unspliced pre-mRNA with a corresponding decrease in spliced mRNA levels (Fig. 4D), and those with reduced spliced mRNA levels together with lowered gene expression (Fig. 4E). A small fraction of introns were spliced efficiently even upon metabolic depletion of SpPrp18 (Fig. 4C). The readout from the intron probe (P) for accumulation of unspliced pre-mRNA was further validated by the signals from intron-exon junction probes (IE) (data not shown). These data confirm that the splicing functions for SpPrp18 are substrate-specific, and the variant protein is only partly functional for genome-wide splicing, as was suggested by the results presented above on splicing of introns in the tfldA+ and ade2+ transcripts.

Semiquantitative RT-PCR Analyses Validate Genome-wide Spectrum of Splicing Defects—The splicing phenotypes inferred from microarrays were validated by semiquantitative RT-PCR analyses of some representative introns. The class with heightened splicing defects manifested as both pre-mRNA accumulation and mRNA reduction was represented by mdm35+ intron 1. In agreement with the microarray data, depletion of the wild-type protein caused a ~3-fold increase in the pre-mRNA/mRNA index, a combined effect of accumulated pre-mRNA and reduced spliced mRNA levels. The same phenotype was seen in prp18-5 cells (Fig. 5A, lanes 2 – 4). To validate the predominant class of splicing defects (i.e. introns with pre-mRNA accumulation alone), we selected sfc9+ intron 2 and spf38+ intron 5 for analysis. Intron 5 in spf38+ was strongly dependent on SpPrp18, as assessed by these semiquantitative RT-PCR analyses (Fig. 5B, lanes 2 – 4). Similarly, sfc9+ intron 2 splicing was SpPrp18-dependent because increased unspliced pre-mRNA was detected upon metabolic depletion of either wild-type or mutant protein (Fig. 5B, lanes 2 – 4). Finally, we confirmed that splicing of ubc4+ intron 1 and cwf2+ intron 1 can occur independent of SpPrp18 because depletion of wild-type or SpPrp18V194R mutant protein had no effect on the
Roles for SpPrp18 in Splicing and Cell Division Cycle

We investigated the roles for fission yeast SpPrp18 (4, 10). We assessed the splicing status of three introns in tflld1 and the ade21 intron 1. Schematic representations show each intron together with its flanking exons. Intron length is given within brackets. RNA from WT and prp18-5 cells grown at 30 °C for 36 h in the presence (+T) and absence (−T) of 15 μM thiamine was taken for limiting cycle, tracer-labeled (α-32P)dATP semiquantitative RT-PCR. For each intron, the pre-mRNA (P) or mRNA (M) levels were normalized to that of the intronless act1 ("A") mRNA. The normalized pre-mRNA or mRNA levels, from 3–4 biological replicates, are plotted with the WT (black bar, lane 1), WT + T (gray bar, lane 2), prp18-5 + T (black bar, lane 3), and prp18-5 + T (dark gray bar, lane 4). Error bars, S.D.; *, p < 0.05 as determined by unpaired Student’s t test. ns, non-significant change with p > 0.05.

FIGURE 3. Loss of functional SpPrp18 results in intron-specific splicing defects. A and B, investigations of the splicing status of three introns in tflld1 and the ade21 intron 1. Schematic representations show each intron together with its flanking exons. Intron length is given within brackets. RNA from WT and prp18-5 cells grown at 30 °C for 36 h in the presence (+T) and absence (−T) of 15 μM thiamine was taken for limiting cycle, tracer-labeled (α-32P)dATP semiquantitative RT-PCR. For each intron, the pre-mRNA (P) or mRNA (M) levels were normalized to that of the intronless act1 ("A") mRNA. The normalized pre-mRNA or mRNA levels, from 3–4 biological replicates, are plotted with the WT (black bar, lane 1), WT + T (gray bar, lane 2), prp18-5 + T (black bar, lane 3), and prp18-5 + T (dark gray bar, lane 4). Error bars, S.D.; *, p < 0.05 as determined by unpaired Student’s t test. ns, non-significant change with p > 0.05.

Using tflld1 intron 1 as a substrate because it is a spprpr18+-dependent intron. Primer extension assays were done to detect the cDNAs of distinct lengths that reflect levels of tflld1 intron 1 lariat intermediate (intron-3′-exon), the unspliced precursor (E1-I1-E2), and the spliced message (E1-E2) from plasmid-expressed tflld1 E1-I1-E2-GFP minitranscripts (Fig. 6A, schematic). In WT cells, unspliced pre-mRNA accumulated upon metabolic depletion of SpPrp18 (Fig. 6A, left, lanes 1 and 2). Similarly, upon thiamine repression of prp18-5, high levels of unspliced RNA were observed (Fig. 6A, left, lanes 3 and 4). Interestingly, no cDNA species corresponding to lariat intron-exon 2, which is expected to accumulate in mutants with a slow and/or arrested second step reaction. For this analysis, we employed cells lacking the debranching enzyme, Dbr1. We generated the double mutant to inactivate lariat RNA debranching activity in a strain that expresses the mutant SpPrp18-5 protein. We also made the corresponding WT dbr1Δ control strain. Strikingly, the prp18-5 dbr1Δ double mutant showed synthetic sickness at 28 °C when compared with the single mutant parents or the control WT dbr1Δ strain (Fig. 6B, left).

We assessed the splicing status of tflld1 intron 1-containing minitranscript using primer extension assays in these strains (prp18-5 dbr1Δ and WT dbr1Δ). In WTdbr1Δ cells, abundant spliced mRNAs and low levels of pre-mRNA were detected. In contrast, elevated unspliced pre-mRNA levels were found upon metabolic depletion of SpPrp18 in the dbr1Δ-sensitized background (Fig. 6A, right, lanes 1 and 2). Similarly, in cells with SpPrp18-5 mutant protein and lacking Dbr1, increased levels of unspliced RNA were observed (Fig. 6A, right, lanes 3 and 4) upon metabolic depletion, indicating an early splicing arrest, yet in cells lacking functional SpPrp18, only a very faint primer extension cDNA product around the expected size of lariat RNA debranching activity in a strain that expresses the mutant SpPrp18-5 protein. We also made the corresponding WT dbr1Δ control strain. Strikingly, the prp18-5 dbr1Δ double mutant showed synthetic sickness at 28 °C when compared with the single mutant parents or the control WT dbr1Δ strain (Fig. 6B, left).

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| Intron name | Intron length | BrP to 3′ss distance |
|-------------|---------------|---------------------|
| tflld1      | 255           | nt                  |
| tflld2      | 52            | nt                  |
| tflld3      | 70            | 14                  |
| mtn35 I1    | 62            | 15                  |
| sfc9 I2     | 58            | 12                  |
| spf38 I5    | 61            | 7                   |
| ubc4 I1     | 79            | 12                  |
| cvo2 I1     | 49            | 8                   |
| res1 I1     | 127           | 11                  |
| res2 I1     | 164           | 10                  |
| skp I1      | 53            | 14                  |
| cdc2 I1     | 68            | 10                  |
| cdc2 I2     | 72            | 12                  |
| cdc2 I3     | 101           | 8                   |
| cdc2 I4     | 54            | 13                  |
| ade2 I1     | 39            | NA*                 |
| ade2 I2     | 383           | 11                  |

* Not applicable.

SpPrp18 Depletion Arrests Splicing before Catalysis—In budding yeast and human cell-free in vitro splicing reactions, Prp18 strongly associates with Slu7 and is required for a second step reaction (4, 10). We investigated roles for fission yeast SpPrp18 before the first catalytic reaction and for second step splicing using tflld1 intron 1 as a substrate because it is a spprpr18+-dependent intron. Primer extension assays were done to detect the cDNAs of distinct lengths that reflect levels of tflld1 intron 1 lariat intermediate (intron-3′-exon), the unspliced precursor (E1-I1-E2), and the spliced message (E1-E2) from plasmid-expressed tflld1 E1-I1-E2-GFP minitranscripts (Fig. 6A, schematic). In WT cells, unspliced pre-mRNA accumulated upon metabolic depletion of SpPrp18 (Fig. 6A, left, lanes 1 and 2). Similarly, upon thiamine repression of prp18-5, high levels of unspliced RNA were observed (Fig. 6A, left, lanes 3 and 4). Interestingly, no cDNA species corresponding to lariat intron-exon 2, which is expected to accumulate in mutants with a slow and/or arrested second step reaction. For this analysis, we employed cells lacking the debranching enzyme, Dbr1. We generated the double mutant to inactivate lariat RNA debranching activity in a strain that expresses the mutant SpPrp18-5 protein. We also made the corresponding WT dbr1Δ control strain. Strikingly, the prp18-5 dbr1Δ double mutant showed synthetic sickness at 28 °C when compared with the single mutant parents or the control WT dbr1Δ strain (Fig. 6B, left).

We assessed the splicing status of tflld1 intron 1-containing minitranscript using primer extension assays in these strains (prp18-5 dbr1Δ and WT dbr1Δ). In WTdbr1Δ cells, abundant spliced mRNAs and low levels of pre-mRNA were detected. In contrast, elevated unspliced pre-mRNA levels were found upon metabolic depletion of SpPrp18 in the dbr1Δ-sensitized background (Fig. 6A, right, lanes 1 and 2). Similarly, in cells with SpPrp18-5 mutant protein and lacking Dbr1, increased levels of unspliced RNA were observed (Fig. 6A, right, lanes 3 and 4) upon metabolic depletion, indicating an early splicing arrest, yet in cells lacking functional SpPrp18, only a very faint primer extension cDNA product around the expected size of lariat intermediate was detected, indicating a major block before the first catalytic step. A positive control fission yeast strain with a second step splicing defect, either a mutant in a splicing factor or a cis splice site mutant, is as yet lacking in fission yeast; hence, comparative analyses are not possible. Thus, we propose that a
strong and early splicing arrest occurs before catalysis when SpPrp18 is compromised.

With these data pointing toward a precata lytic arrest in these cells, we probed for genetic interactions of prp18-5 with the splicing factor SpPrp2/U2AF59, which functions at the very early step of U2 snRNP recruitment to the intronic branch point (37, 38). Interestingly, we observed a strong positive genetic interaction, at 28 °C, in the double mutant prp18-5 prp2-1, which had better growth as compared with the parent prp2-1. At the permissive temperature for prp2-1, the growth retardation triggered by metabolic depletion of prp18-5 is suppressed (Fig. 6C, right, rows 2 and 3). Further, we assessed whether the near normal growth of prp18-5 prp2-1 double mutant in +T conditions relates to a rescue of splicing defects of SpPrp18-dependent introns when prp2-1 is at permissive conditions. Semiquantitative RT-PCR assays showed that the double mutant had only basal levels of unspliced pre-mRNA for sp38+ intron 5 (Fig. 6D, left, lanes 5 and 6), an intron that was strongly dependent on SpPrp18 for splicing (Fig. 6D, left, lanes 1 and 2). Thus, the data indicate a rescue of its splicing defect in the double mutant. This intron was efficiently spliced in prp2-1 mutant grown at the permissive temperature (28 °C) regardless of the presence or absence of thiamine (Fig. 6D, left, lanes 3 and 4). However, interestingly, for tfld+ intron 1, we observe persistent accumulation of unspliced pre-mRNA in the double mutant (Fig. 6D, right, lanes 5 and 6). This intron was inefficiently spliced even in parent prp2-1 single mutant at semipermissive 28 °C (Fig. 6D, left, lanes 3 and 4). Thus, rescue of splicing defects is observed only for an intron that is spliced in the presence of Prp2-1 protein. These data suggested that splicing suppression in the double mutant is intron-specific and that SpPrp2 acts upstream to SpPrp18.

Efficient Splicing of G2-S Regulators Suggests a Role of sppr18+ in Cell Cycle—Several fission yeast pre-mRNA splicing factor mutants, in addition to impaired splicing, also show striking cell cycle defects, indicating a strong link between splicing and cell cycle regulation (28). The cells depleted of the wild-type SpPrp18 arrested as elongated cells, reminiscent of cdc mutants. Microscopy and fluorescence-assisted cell sorting (FACS) of WT and prp18-5 cells grown under conditions when the wild-type or mutant proteins are expressed were compared with cultures treated for 36 h with thiamine. In the absence of thiamine, cells of both genotypes had equivalent cell lengths and a single nucleus (Fig. 7A, left panels). Upon depletion of the wild-type protein, elongated cells, nearly twice the normal length, formed the majority of the population (Fig. 7A, top right). The prp18-5 cells became ~20% longer (p < 0.001) when the mutant protein was depleted by growth in thiamine (Fig. 7A, bottom right). The FACS analyses of propidium iodide-stained cells show, as expected, that WT cultures have largely cells with 2C nuclear DNA content (Fig. 7B, white peak). Strikingly, upon metabolic depletion of SpPrp18, a majority of cells accumulate with 1C nuclear content, indicating an arrest largely before DNA replication (Fig. 7B, light gray peak). In fact, most prp18-5 cells had 1C nuclear content even when the mutant protein was expressed (Fig. 7B, black peak).

We examined the splicing efficiency of transcripts that encode cell division regulators because several of these transcripts are intron-containing. All introns in cdc2+ encoding the central cyclin-dependent kinase Cdc2 were studied in WT and prp18-5 strains. Reduced splicing across the cdc2+ intron 4 was seen when the sppr18+ allele was transcriptionally repressed. The same splicing defect is seen in prp18-5 cells (Fig. 7C, top left, M). Other cdc2+ introns were unaffected (data not shown).
The G1-S phase Cdc2 kinase functions are controlled by regulated proteolysis of its inhibitor Rum1 and by the E3-ubiquitin ligase SCF complex-dependent degradation of the S-phase regulator Cdc18 (39–41). Because rum1/H11001 and cdc18/H11001 are non-intron-containing genes, we investigated the splicing status of intron 1 in skp1/H11001, encoding an essential adaptor factor of the SCF ubiquitin ligase. Elevated pre-mRNA levels for skp1/H11001 were observed both upon depletion of spprp18 and in prp18-5 cells (Fig. 7C, top right, P). This defect was enhanced when expression of the mutant protein was reduced (Fig. 7C, top right, P).

In fission yeast, the gene expression peak during G1-S phase is regulated by the MluI binding factor (MBF) transcription regulatory complex composed of Res1 and Res2, two zinc finger proteins, and Cdc10 (42, 43). We detected elevated pre-mRNA for res2/H11001 intron 1 upon transcriptional repression of spprp18/H11001 (Fig. 7C, bottom left, P), which is recapitulated in prp18-5 cells. The effects on res1/H11001 splicing were moderate (data not shown). To assess whether the splicing defects in transcripts for G1-S regulators correlate with changes in the protein levels, we examined endogenous SpCdc2 protein levels by immunoblotting because specific antibodies were available (see “Experimental Procedures”). Lysates from wild-type and
prp18-5 mutant cells show a moderate decrease in the steady-state SpCdc2 protein levels in the thiamine-supplemented prp18-5 mutant cells (Fig. 7D, lane 4). These data hint that cumulative splicing defects in multiple transcripts and the ensuing changes to their protein levels may partly contribute to the cell cycle defects caused by loss of SpPrp18.

Discussion

In this study, we deduced critical functions for both the conserved region of SpPrp18 and the flanking helices. Transcriptome profiling of cells lacking functional SpPrp18 revealed intron-specific roles for this protein. Splicing analysis in a dbr1Δ-sensitized background for a SpPrp18-dependent intron and genetic interaction with early acting U2AF59 show that loss of SpPrp18 arrests spliceosomes before catalysis. Reduced splicing efficiency for some transcripts encoding key regulators of the G1-S transition indicate a role for SpPrp18 in mediating the splicing of key regulators of cell division cycle progression.

Although second step splicing functions have been demonstrated for budding yeast and human Prp18 using in vitro and in vivo approaches, there have been very limited studies on functionally characterizing Prp18 orthologs from other species (4, 9, 10). sppr18 is an essential gene in S. pombe, and in a recent genetic interaction mapping study, a DAmP allele of SppPr18...
Roles for SpPrp18 in Splicing and Cell Division Cycle

was also found to have extremely poor growth (33, 44). Apart from this phenotype, no functional studies on this fission yeast splicing factor have been reported thus far. Here, we undertook detailed analysis of the function of SpPrp18 in fission yeast intron recognition and splicing using strains with depleted levels of wild-type protein and those expressing a recessive missense mutant. Extensive mutational analysis of budding yeast ScPrp18 revealed that the highly conserved CR loop and its flanking helices have splicing functions distinct from regions that interact with ScSlu7 and influence spliceosome recruitment (13). Mutants in the CR of ScPrp18 are dominant negative and ts. On the other hand, whereas the null phenotypes of the G196A/V197A/T198A triple mutant in the CR region and the K325A/R326A mutant in helix 5 in fission yeast SpPrp18 con-
Roles for SpPrp18 in Splicing and Cell Division Cycle

firm a vital functional role for these regions, these alleles are recessive loss-of-function mutants in fission yeast. These data hint at a failure in vital spliceosomal interactions of these non-functional variants. In budding yeast ScPrp18, the CR loop is proposed, in a context-dependent manner, to improve interactions between exonic residues and budding yeast U5 snRNA loop 1 (13–15, 45). We speculate that the critical roles for SpPrp18 helix 5 and CR domain may also be to improve U5 loop 1 with exonic sequence interactions.

Interestingly, our missense mutant in helix 1 (V194R), prp18-5, which is also a recessive mutant, supports viability, albeit with compromised growth properties probably due to altered protein stability. We show that compromised functions of SpPrp18V194R may be a consequence of its altered conformation, triggered by the positively charged residue at the protein core. Immunoblotting experiments to probe wild-type SpPrp18 protein levels detected an additional slower migrating species, a form that is intriguingly absent in the SpPrp18 V194R mutant. We speculate that either a modification or altered conformation of wild-type SpPrp18 may cause its altered mobility. A recent study of the proteome and phosphoproteome of S. pombe during various cell cycle stages hints at phosphorylated forms of SpPrp18 (Ser-115 residue) (46).

The splicing defects that we discern from the global splicing analysis for S. pombe introns suggest that its functions are essential but substrate-specific. Cis intrinsic features, such as intron length, branch point nucleotide to 3’ss distance, 3’ss sequence, and the adenosine and uridine content (AU%) did not differ between SpPrp18-dependent and -independent introns. However, an analysis of the 5’ss hexamer for the occurrence of consensus versus non-consensus nucleotides (log odd score analysis) suggests that a statistically significant proportion of N3 nucleotides are non-consensus in our SpPrp18-dependent intron data set (data not shown). Introns with weak/abnormal splice sites can have greater dependence on splicing factors, as reported even in budding yeast (47, 48). Also, it is suggested that the splicing apparatus is limiting, and thus different cellular transcripts encounter inherent competition for these splicing factors (49). Thus, it is plausible that multiple variables in fission yeast transcripts create dependence on this low abundance (50) but essential factor SpPrp18.

Mutation of the 3’ss, which leads to lariat intermediate accumulation in budding yeast, shows complete splicing arrest before the first step in S. pombe (19). Furthermore, an expanded Pyn tract was unable to compensate for the 3’ss mutation in fission yeast (19). Mutations in the nucleotides of the 5’ss consensus hexamer motif (G5-A and U2-A/G) in budding yeast cause lariat intermediate accumulation and second step arrest, whereas surprisingly, mutations in all nucleotides in the 5’ss hexamer sequence cause a block before any catalysis in fission yeast (51). Among the trans-acting factors, studies from our laboratory on SpSlu7, the ortholog of budding yeast second step factor, ScSlu7, also clearly show splicing arrest before catalysis (23). Fission yeast cells deleted for lariat debranching gene (dbr1Δ) have more severe growth defects than the corresponding budding yeast mutant. This has been attributed to the higher intronic content of the S. pombe transcriptome that demands rapid turnover of excised lariat introns that are held in stable U2.U5.U6 post-splicing complexes (52). Regardless, because prp18-5 dbr1Δ cells were viable, we used them to search for accumulation of lariat intron-exon 2 intermediates. Because we detected predominantly accumulated pre-mRNAs in the prp18-5 single mutant and in the prp18-5 dbr1Δ double mutant, our data point to a major arrest before the first catalytic step in the mutant of SpPrp18. We cannot rule out a continued subsequent role in the second step that again could be substrate-context-specific. These studies await additional partial loss of function allelic variants of fission yeast Prp18. Allele-specific splicing functions have been noted for the budding yeast splicing factor, Prp8, wherein mutant alleles inhibiting either first step or second step splicing have been reported (53, 54).

In budding yeast, Prp18 acts as a bridging molecule between Slu7 and U5 snRNP to aid 3’ss selection after catalytic step 1 (9, 12). The strong association between Prp18 and Slu7 aids their spliceosome assembly (55). Our prior studies suggested weak or no interactions between fission yeast SpPrp18 and SpSlu7 (22). Thus, other domains in SpPrp18 are probably more relevant for its roles in the spliceosome. Remarkably, we found a strong genetic suppression interaction between prp18-5 and spprp2-1, a ts mutant in U2AF59/SpPrp2, because we found that prp2-1 functionally rescued the growth retardation of prp18-5 cells triggered by thiamine supplementation. We also found the suppression is not mutual because the growth arrest of prp2-1 cells at 37 °C was not rescued in the double mutant. In the double mutant, the suppression of splicing defects in prp18-5 cells was intron-specific and apparently related to dependence of the intron on prp2-1. These findings indicate that SpPrp2 that assembles in the early precatalytic spliceosomes containing SF1-U2AF59-U2AF23 (38) acts upstream of SpPrp18. In budding yeast, two categories of alleles have been identified in Prp8, Cef1, U6 snRNA, and Prp16, based on their functions in suppressing either the first step or the second step splicing arrest caused by specific intronic cis mutations. Such suppressor alleles have opposing effects on the two catalytic steps (53, 54, 56). These first step or second step alleles stabilize one catalytic site conformation over the other; thus, combining two mutants in opposing steps can lead to overall improved splicing efficiency as captured in the two-state model (53, 56). Fission yeast prp2-1 (U2AF59) is a well studied mutant with an arrest before the first catalytic step. We speculate that the requirement of SpPrp18, possibly for the 3’ss recognition, may be bypassed by a favorable first step conformation when prp2-1 is active; the intermediates thus formed proceed through the second catalytic step.

Several genetic and proteomic studies, in model organisms, establish a relationship between cell cycle regulatory proteins and splicing factors (26–31, 57). Strikingly, we demonstrate that repression of WT or of prp18-5 generated a G1→S transition arrest, yet we do not exclude a role for SpPrp18 at later cell cycle transitions or in other pathways that control cell size and cell cycle progression. Mutations in budding yeast PRP17 or PRP8 that impair splicing cause G1→S and G1→M cell cycle defects, linking splicing with multiple regulatory checkpoints in the cell cycle (57, 58). We correlate the cell cycle arrest observed in the

*S. Banerjee and U. Vijayraghavan, unpublished data.
Roles for SpPrp18 in Splicing and Cell Division Cycle

| Strain | Genotype | Source |
|--------|----------|--------|
| FY527  | h<sup>+</sup> ura4-D18 leu1-32 his3-D1 ade6-M216 | S. Forsburg |
| FY528  | h<sup>+</sup> ura4-D18 leu1-32 his3-D1 ade6-M210 | S. Forsburg |
| prp2-1 | h<sup>+</sup> prp2-1 leu2-1 | K. Gould |
| prp2-1-9c | h<sup>+</sup> prp2-1 leu2-1 his3D1 ura4-D18 | This study |
| dbr1Δ | h<sup>+</sup> dbr1::KANMX4 leu1-32 ura4-D18 ade6-M210 | Bioneer |
| spprp18Δ::his3<sup>+</sup>/spprp18<sup>+</sup> | h<sup>+</sup> 1h::spprp18::his3<sup>+</sup>/spprp18<sup>+</sup> ade6-M210/ade6M216 leu1-32/leu1-32 his3-D1/his3-D1 ura4-D18/ura4-D18 | This study |
| spprp18Δ::pREP4X::spprp18<sup>+</sup> | h<sup>+</sup> spprp18::his3<sup>+</sup> ade6 leu1-32 his3-D1 ura4-D18 pREP4X::spprp18<sup>+</sup> cDNA (ura4<sup>+</sup>) | This study |
| FY528 pREP42HA::spprp18mut GVT-A  | h<sup>+</sup> ura4-D18 leu1-32 his3-D1 ade6-M210 pREP42HA::spprp18mut GVT-A  | This study |
| FY528 pREP42HA::spprp18mut KR-AE | h<sup>+</sup> ura4-D18 leu1-32 his3-D1 ade6-M210 pREP42HA::spprp18mut GVT-A  | This study |
| WT     | h<sup>+</sup> spprp18::his3<sup>+</sup> leu1::pJK148-nmt81spprp18<sup>+</sup> ade6<sup>+</sup> his3-D1 ura4-D18 | This study |
| prp18-5 | h<sup>+</sup> spprp18<sup>+</sup> leu1::pJK148-nmt81spprp18<sup>+</sup>-V194R ade6<sup>+</sup> his3-D1 ura4-D18 | This study |
| WT pDblet spprp18<sup>+</sup> | h<sup>+</sup> spprp18<sup>+</sup> leu1::pJK148-nmt81spprp18<sup>+</sup>-V194R ade6<sup>+</sup> his3-D1 ura4-D18 pDblet spprp18<sup>+</sup> (ura4<sup>+</sup>) | This study |
| prp18-5 pDblet spprp18<sup>+</sup> | h<sup>+</sup> spprp18<sup>+</sup> leu1::pJK148-nmt81spprp18<sup>+</sup>-V194R ade6<sup>+</sup> his3-D1 ura4-D18 pDblet spprp18<sup>+</sup> | This study |
| WT dbr1Δ | h<sup>+</sup> spprp18Δ::his3<sup>+</sup> leu1::pJK148-nmt81spprp18<sup>+</sup>-V194R ade6<sup>+</sup> his3-D1 ura4-D18 dbr1::KANMX6 | This study |
| prp18-5 dbr1Δ | h<sup>+</sup> spprp18<sup>+</sup> leu1::pJK148-nmt81spprp18<sup>+</sup>-V194R ade6<sup>+</sup> his3-D1 ura4-D18 dbr1::KANMX6 | This study |
| prp18-5 prp2-1 | h<sup>+</sup> spprp18<sup>+</sup> leu1::pJK148-nmt81spprp18<sup>+</sup>-V194R ade6<sup>+</sup> his3-D1 ura4-D18 prp2-1 | This study |
| slu7<sup>+</sup> | h<sup>+</sup> slu7<sup>+</sup> leu1::pJK148-spslu7<sup>+</sup> ade6 his3-D1 ura4-D18 | Banerjee et al. (22) |
| slu7-2 | h<sup>+</sup> slu7<sup>+</sup> leu1::pJK148-spslu7<sup>+</sup>737G ade6 his3-D1 ura4-D18 | Banerjee et al. (22) |

Experimental Procedures

Yeast Strains and Plasmid Constructions—Procedures for genetic analyses in S. pombe were followed as described (60) and on the PombeNet website. spprp18<sup>+</sup> cDNA (1032 bp) was generated by reverse transcription using spprp18 RP on wild-type FY528 RNA. The cDNA was amplified with primers spprp18<sup>+</sup> FP and RP, restricted with BamHI, and cloned into pBS(KS). The spprp18<sup>+</sup> cDNA was excised as a BamHI fragment from pBS(KS)-spprp18<sup>+</sup> cDNA and ligated with BamHI-digested pREP4X to create pREP4X-spprp18<sup>+</sup>. The insert in the pDblet-spprp18<sup>+</sup> clone was obtained by PCR on wild-type FY528 genomic DNA of the ORF and 1-kb upstream sequences using a primer pair (spprp18 5'-UTR FP and spprp18 RP; supplemental Table S1). The ~2-kb PCR product thus obtained was first cloned into the EcoRV site of pBS(KS) and then excised as a KpnI/SacI fragment for cloning into pDblet to generate pDblet-spprp18<sup>+</sup>, which was used for complementation studies. S. pombe strains used here are described in Table 4. spprp18<sup>+</sup> gene disruption was achieved by transforming a 3.1-kb spprp18::his3<sup>+</sup> fragment into S. pombe WT diploid cells to generate the spprp18::his3<sup>+</sup>/spprp18<sup>+</sup> strain. Subsequently, we created the haploid strain spprp18::his3<sup>+</sup>, pREP4X spprp18<sup>+</sup>, where the chromosomal null allele was complemented by the pREP4x spprp18<sup>+</sup> plasmid. Inverse PCR amplifications, for introducing missense mutations G288A/V289A/T290A and K325A/R326E, were done using Vent DNA polymerase (New England Biolabs) on the plasmid pBS(KS)-spprp18<sup>+</sup> cDNA as the template. For each of these mutants, two complementary mutagenic primers of length ~30–35 nucleotides containing the desired mutation(s), flanked by ~15 nucleotides of wild type sequences, were used as primers (supplemental Table S1). The inverse PCR products were DpnI-treated and transformed into Escherichia coli competent cells. Plasmids from E. coli transformants were verified for authenticity of the mutation by sequencing the insert in the plasmids from 2–4 independent colonies. For expression of G288A/V289A/T290A or K325A/R326E mutants or the wild type spprp18<sup>+</sup> cDNA in S. pombe, expression clones were made in the pREP41MH-N or pREP42HA-N vectors. In these recombinants, the desired protein would be overexpressed as an N-terminally tagged fusion driven by the nmt41 promoter. In each case, the full-length wild-type or mutant cDNA was excised as a BamHI fragment from pBS(KS)-spprp18<sup>+</sup>mut cDNA (G288A/V289A/T290A or K325A/R326E) and cloned into BamHI-restricted pREP41MH-N or pREP42HA-N vectors.
Roles for SpPrp18 in Splicing and Cell Division Cycle

This created the plasmid constructs pREP41MH-spprp18<sup>+</sup> or pREP42HA-spprp18<sup>+</sup>, pREP42HA-spprp18mut (G288A/V289A/T290A), and pREP42HA-spprp18mut (K325A/R326E).

The buried amino acids Val-194, Leu-239, Ile-259, and Leu-324 in spprp18<sup>+</sup> were predicted using the program PREDBUR (35). Random mutagenesis of V194X was done by inverse PCR on genomic DNA. Confirmed by PCR on genomic DNA.

Double mutant was generated by free spore analysis after mat-19 c after mat-19 c. G418-resistant haploid spores were chosen, and the wild-type and mutant spore. Haploid double mutant, and the his3 diploid. Sporulation was done to obtain viable spprp18Δ haploids with plasmids carrying various spprp18V194X random mutants. These were screened for growth phenotypes. Plasmid from one of the several ts colonies was sequenced to identify the V194R mutation. Subsequently, the wild-type and mutant (V194R) spprp18 open reading frames were cloned into the plasmid pRS418 vector for integration at the leu1-32 locus and expression from the nmt81 promoter. The plasmid pJK148 nmt81 spprp18<sup>+</sup>/spprp18V194R integrants at the leu1-32 locus were confirmed by PCR on genomic DNA.

The spprp18:sis3<sup>+</sup> leu1::nmt81 prp18V194R prp2-1 haploid double mutant was generated by free spore analysis after mating prp2-1 9c (derived from prp2-1) and prp18-5. We screened for spores that were his<sup>+</sup> leu<sup>+</sup> ts at 37 °C and obtained the haploid double mutant, and the prp2-1 mutation was sequence-verified. The spprp18:sis3<sup>+</sup> leu1::nmt81 prp18V194R dbr1Δ haploid double mutant was generated by tetrad dissection analysis. After mating dbr1::KANMX4 with prp18-5, his<sup>+</sup> leu<sup>+</sup> G418-resistant haploid spores were chosen, and spprp18<sup>+</sup> and dbr1 gene disruptions were verified by PCR in the double mutant spore.

Comparative Modeling of SpPrp18—We performed multiple sequence alignment of homologous Prp18 proteins using ClustalW. The alignment obtained was used with ScPrp18A79 structure (PDB code 1DVK) as a template for homology modeling using MODELLER 9v7 or Swiss MODEL (61). The default spatial constraints for distances, angles, dihedral angles, pairs of dihedral angles, and other spatial features defined by atoms and pseudo-atoms (62) were used. The models were validated by means of PROCHECK (63) and VERIFY3D (64). The obtained models were saved in PDB format and visualized using PyMOL or chimera version 1.8.1.

Far UV CD Spectrometry and Intrinsic Tryptophan Fluorescence Studies—The E. coli C-41 cells harboring the clones (WT and V194R) in pET15b vector were grown at 37 °C to late log phase. This was followed by induction with 0.1 mm isopropyl 1-thio-β-d-galactopyranoside and further growth for 6 h at 25 °C. The proteins obtained in the soluble fraction were purified using nickel-nitrilotriacetic acid affinity columns and used for the biophysical studies. Proteins were exposed to temperatures ranging from 20 to 90 °C and were heated at a constant rate of 1°C/min. Circular dichroism measurements were made with a JASCO J-715 spectropolarimeter fitted with a Jasco Polarimetry type temperature controller (PTC-348W1). Spectra were recorded with a scan speed of 20 nm/min and with a response time of 1 s. Far UV CD spectra were taken in the wavelength range of 200–250 nm, at a protein concentration of 12 nm with a 2-mm path length cell.

Fluorescence measurements were carried out on a Hitachi spectrofluorometer (F-7000) equipped with a data recorder DR-3. The fluorescence spectra were measured at a protein concentration of 9.8 μM with a 1-cm path length cuvette at a temperature range of 20–90 °C. To determine intrinsic tryptophan fluorescence, the excitation wavelength was set at 295 nm, and the emission spectrum was recorded in the range of 300–450 nm with 5- and 10-nm slit width for excitation and emission, respectively.

Probe Design, Sample Preparation, Microarray Hybridization, and Data Acquisition—The design of various probes for splicing sensitive microarray analysis was as described (22). RNA was isolated from two biological replicates of WT and prp18-5 cells grown in the presence and absence of 15 μM thiamine for 36 h and then harvested. The total RNA was prepared using TRI-reagent (Sigma). The RNA samples were labeled using an Agilent Quick-Amp labeling kit. 500 ng each of the untreated and treated samples were reverse transcribed at 40 °C using oligo(dT) primer with a T7 polymerase promoter and random hexamer primer with a T7 polymerase promoter in two individual reactions and converted to double-stranded cDNA. Synthesized double-stranded cDNA was used as a template for cDNA synthesis. cDNA was generated by in vitro transcription, and the dye Cy3 CTP was incorporated during this step. The cDNA synthesis and in vitro transcription steps were carried out at 40 °C. 700 ng of the Cy3-labeled cDNA samples (600 ng of oligo(dT)-labeled + 100 ng of random hexamer-labeled samples) were fragmented at 60 °C and hybridized onto customized S. pombe, 8X60K arrays (Agilent Technologies). Fragmentation of labeled cRNA and hybridization were done using a gene expression hybridization kit, and hybridization was carried out at 65 °C for 16 h. The hybridized slides were washed using gene expression wash buffers and scanned using the Agilent microarray scanner at 3 μm resolution.

Microarray Data Analysis—Feature-extracted data were analyzed using Agilent GeneSpring GX software, and normalization of the data was done using the 75th percentile shift. The log<sub>2</sub> Cy3 fluorescence values for the wild type and mutant were mathematically zero-transformed and analyzed relative to the respective untreated sample (without thiamine, −T). Student’s t test along with a false discovery rate-adjusted (Benjamini and Hochberg) p value calculated using the R statistical program was used to obtain statistically significant values for various probes (p < 0.05) in two biological replicates that were taken for hierarchical clustering. Affected introns were considered to be those with a >0.8-fold (log<sub>2</sub> scale) increase in signal for intronic probes. Also considered were those with a decrease in mRNA levels, minimum 0.8-fold (log<sub>2</sub> scale) splice junction, and intronic probes for both biological replicates of WT−T and WT+T samples are provided (supplemental Data Set 1, Sheet 1). The status of these 253 introns in the prp18-5−T sample as compared with WT−T sample is also provided (supplemental Data Set 1, Sheet 2), represented as a heat map in Fig. 4. Also shown is a list of introns with a >0.8 average -fold change (log<sub>2</sub>) (equivalent to 1.7-fold) (p < 0.05) between decrease in splice junction probe signal and that of the untreated sample. List of the stringent set of 253 introns showing -fold change and raw
intensity values for gene expression, WT−T, and WT+T samples for the intronic probe (supplemental Data Set 1, Sheet 3). Also shown is a list of introns with statistically significant average fold change less than −0.8 (log2 scale) for splice junction probe when WT−T and WT+T samples are compared (supplemental Data Set 1, Sheet 4).

Reverse Transcription and Primer Extension Assays—Total RNA from fission yeast was extracted using TRI-reagent (Sigma). 2–5 μg of DNase I (New England Biolabs)-treated total RNA was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (New England Biolabs) using a reverse primer from a downstream exon. [α-32P]dATP tracer-labeled PCRs on the cDNAs and gel analyses on 8% native PAGE were performed as described (22). The primers used are listed in supplemental Table S1. Primer extension reactions, at 37 °C, were done on 50 μg of RNA and a γ32P-5′-end-labeled 3′ exon reverse primer using Moloney murine leukemia virus RT (New England Biolabs). The single-stranded cDNAs were resolved on 4% 8M urea-polyacrylamide gels.

FACS Analysis—To analyze DNA content, the cells were collected by brief centrifugation, fixed in ice-cold 70% ethanol, and then treated overnight with RNase A (10 mg/ml) in 1× PBS. After staining with propidium iodide (20 μg/ml), the fluorescence intensities were measured by flow cytometry using a BD FACScalibur (BD Biosciences). About 10,000 events were scanned for each analysis.

Immunoblotting of SpPrp18 and SpCdc2 in WT or Mutant Cells—Crude whole cell extracts were prepared from cells of the specified strains grown at 30 °C in the absence or presence of 15 μM thiamine in EMM-selective media as described (65). For Western blotting, 20–25 μl of crude protein lysate corresponding to 20 μg of protein was run on a 10% SDS-PAGE. The separated proteins were electroblotted to a Hybond P (GE Healthcare) membrane. The blot was incubated with rabbit polyclonal anti-SpPrp18 antibodies or monoclonal anti-HA12CA5 antibodies from Roche Applied Science and subsequently with secondary goat anti-rabbit HRP conjugate/anti-mouse HRP conjugate from Bio-Rad. Blot was developed with SuperSignal West Pico chemiluminescent substrate (Millipore), and the image was analyzed using ImageQuant LAS 4000 (GE Healthcare). Immunoblotting was done with Cdc2 p34 Y100.4 mouse monoclonal antibody (Santa Cruz Biotechnology, Inc.) on crude whole cell lysates from WT and prp18Δ cells grown in the absence and presence of thiamine (−T and + T) at 30 °C.

Confocal Imaging—Cells were fixed in 70% ethanol, followed by washing with 1× PBS and finally suspended in 1× PBS. DAPI-stained cells were imaged at 23–25 °C, with a Carl Zeiss LSM 710 confocal microscope. Data were acquired using a ×60 oil immersion objective with 5% laser power (wavelength 405 nm) and ×1.2 zoom. The images were processed by ImageJ software.

Author Contributions—U. V., R. V., P. K., N. V., and G. M. designed the research; N. V., G. M., P. K., and R. K. performed the experiments; R. K. performed and analyzed the biophysical experiments; P. B. performed the microarray data analysis. Data analysis and manuscript preparation was performed by N. V., G. M., P. K., R. V., and U. V. All authors read and approved the final version of the manuscript.

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