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Chapter 6

The Prp4 Kinase: Its Substrates, Function and Regulation in Pre-mRNA Splicing

Martin Lützelberger and Norbert F. Käufer

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http://dx.doi.org/10.5772/48270

1. Introduction

The genome of fission yeast *Schizosaccharomyces pombe* encodes about 107 predicted protein kinases, of which 17 are known to be essential for cell growth. These include the cell cycle regulator Cdc2 as well as several kinases which coordinate cell growth, cell polarity and cell morphogenesis within the cell cycle [1]. Another one of these essential kinases is Prp4. The study of precursor mRNA processing (*prp*) mutants in fission yeast identified Prp4 as the first kinase being involved in the regulation of pre-mRNA splicing in fungi and mammals. Interestingly, all eukaryotic organisms whose genome has been sequenced to date contain a counterpart of Prp4, with the exception of the *hemiascomycetes* to which the other yeast model organism *Saccharomyces cerevisiae* belongs. Here we review the discovery of Prp4 kinase, its genetic interactions and biochemical properties, its substrate specificity *in vitro* and *in vivo*, as well as the molecular consequences of these interactions. We compare and discuss results reported from the counterparts of Prp4 and its substrates in other organisms. We propose a model how Prp4 might be involved in the quality control of pre-mRNA splicing by acting at different "checkpoints" during the recognition of introns and the activation of pre-catalytic spliceosomal complexes.

2. Discovery of Prp4 kinase

More than 25 years ago we and others suggested that the introns found in fission yeast *Schizosaccharomyces pombe* represent a different type than those found in budding yeast *Saccharomyces cerevisiae*. The suggestion was mainly based on the observation that the simian virus 40 (SV40) small T antigen transcript displaying a 66 nt intron was accurately spliced and small T antigen proteins were synthesized in *S. pombe*, but not in *S. cerevisiae* [2]. We used fission yeast and generated temperature-sensitive (ts) mutants in order to screen for those which indicate a defect in pre-mRNA splicing at the restrictive temperature. For these screens

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we constructed an artificial reporter gene using the naturally intron-less *ura4* gene encoding a carboxylase involved in uracil synthesis. The insertion of introns into the *ura4* gene led to the discovery that introns in *S. pombe* are recognized independently of their exon context. This indicated already at that time that introns in *S. pombe* are recognized by a mechanism now called "intron definition" [3–5]. The ts mutants were transformed with a plasmid containing a 108 bp intronic sequence within the *ura4* gene. Then, the mutant strains were compared for the presence of mRNA and pre-mRNA of the *ura4-108I* transcript under growing (permissive, 25 °C) and non-growing (restrictive, 36 °C) conditions, respectively. Out of hundreds of ts mutants, there were three which showed spliced (mRNA) and unspliced (pre-mRNA) transcripts of the *ura4-I108* reporter gene at the permissive temperature, but when shifted to the restrictive temperature a time dependent decrease of mRNA was observed, while pre-mRNA remained stable. This indicated that the *ura4-108I* gene was still transcribed, but the artificial intron was not removed under this condition. In addition, the natural introns of the *cdc2* transcripts were also not removed in these mutants at the restrictive temperature.

Further genetic analysis revealed that two of this mutants belonged to a complementation group of three already existing pre-mRNA processing mutants, called $prp1^{ts}$, $prp2^{ts}$ and $prp3^{ts}$, which were isolated as described in [6]. One mutant allele, however, defined a new complementation group, and therefore was called $prp4\text{-}73^{ts}$ [7]. The $prp4^{+}$ gene was isolated by functional complementation of the $prp4\text{-}73^{ts}$ mutant allele at the restrictive temperature using a genomic library. The construction of a null-allele by replacing the $prp4^{+}$ gene in the genome via homologous recombination with an auxotrophic marker gene revealed that *prp4* is essential for growth [8]. The polypeptide sequence derived from the $prp4^{+}$ gene showed all signature sequences predicting a serine/protein kinase, according to the classification system of Hanks and Hunter [9]. Based on this classification system primers were designed for the T-Loop region of the *prp4* gene, taking into consideration the differences in codon usage of fission yeast and mammalian cells. These primers were used to produce PCR products from a HeLa cDNA library. The PCR products were then used as probes to screen a mouse cDNA library. With this approach cDNAs from human and mouse were obtained. Both show an open reading frame (ORF) encoding a C-terminal kinase domain and an N-terminal domain of unknown function, which consists of about 150 amino acids [10]. Throughout the kinase domain the fission yeast and mammalian sequences share 53 % identical amino acids. The N-termini, however, share less than 20 % identical amino acids. The two mammalian primary amino acid sequences are 98 % identical.

Chimeric mouse/fission yeast gene constructs were used to investigate by functional complementation, whether the mouse kinase is active in *S. pombe*. The mouse kinase domain complemented the $prp4\text{-}73^{ts}$ allele only when the N-terminus of the fission yeast *prp4* gene was fused to it [10]. Meanwhile, identification and characterization of mammalian counterparts of Prp4 by other groups revealed that it has an extented N-terminal region [12–14]. When compared with the sequences of Prp4 homologs in the database, this region shows variable length within different organisms, with fission yeast displaying the shortest N-terminal region [15]. In addition, all eukaryotic organisms whose genome is known contain a counterpart of Prp4 kinase, with the remarkable exception of the *hemiascomycetes* to which the budding yeast *S. cerevisiae* belongs. Therefore, we use for comparison the Prp4 counterpart found in the

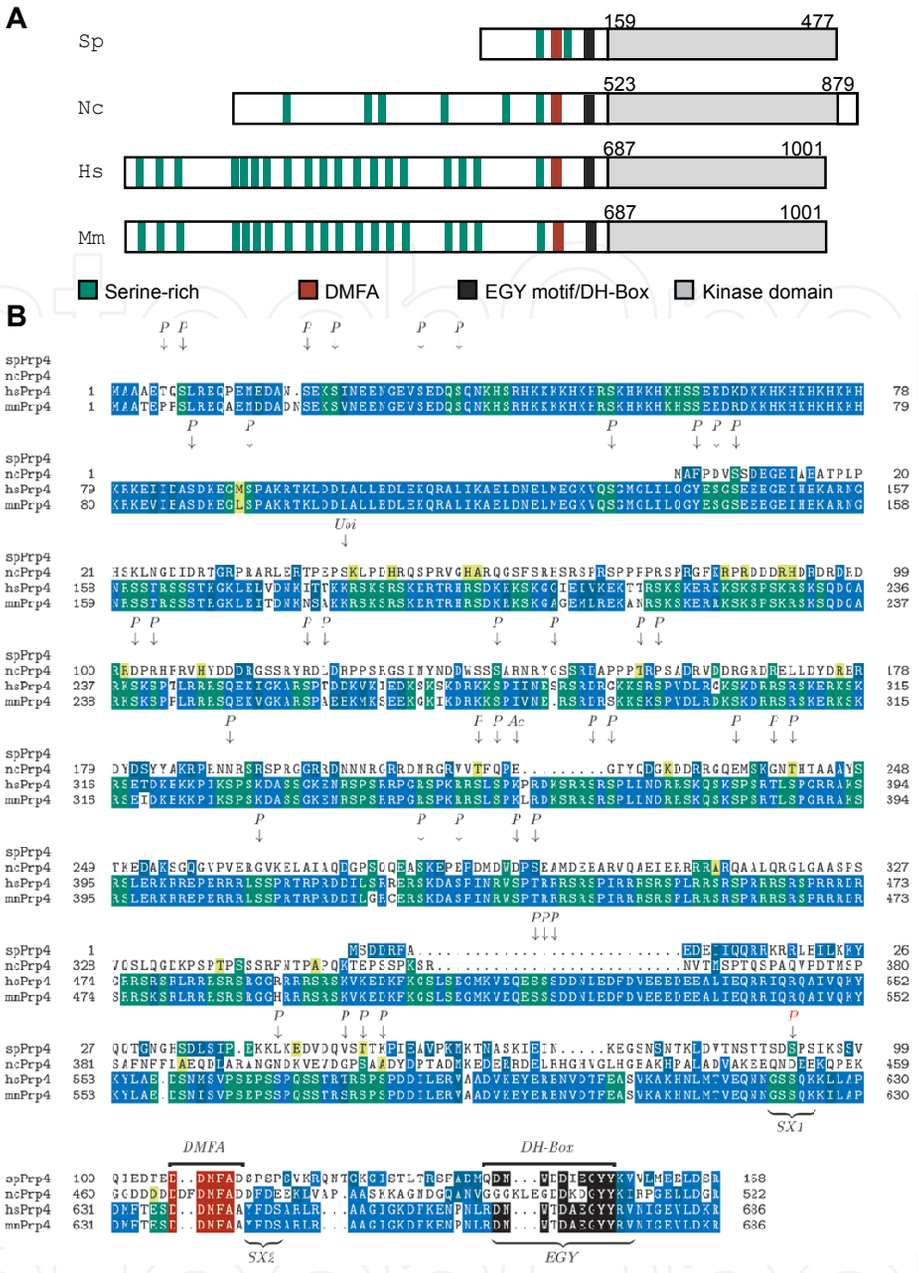


Figure 1. Structure of the Prp4 kinase. **(A)** Schematic representation of the Prp4 homologs from *S. pombe*, *N. crassa*, *M. musculus* and *H. sapiens*. Numbers on top indicate positions of the kinase domain and total length of the proteins, respectively. **(B)** ClustalX alignment of the N-terminus of Prp4 kinase homologs. Serine-rich elements matching the search pattern [SPRKEQLG]X_{0-1}S are highlighted in green. Sequence motifs of spPrp4, which were characterized previously in reference [10], are marked with braces underneath the alignment. The highly conserved DMFA motif and the DYRK homology box (DH-Box) are marked with lines. Amino acid residues of the human Prp4 kinase altered by post-translational modification, such as phosphorylation (P), acetylation (Ac) or ubiquitinylation (Ubi), are marked with arrows (↓). Positions were retrieved from the GeneBank entry of human Prp4 kinase. The phosphorylated S92 of spPrp4 is shown in red. GeneBank accession numbers of the aligned sequences are for spPrp4: CAA20718, ncPrp4: XP001728078, mmPrp4: AAM19102 and hsPrp4: Q13523. Sequences were aligned with ClustalX using default parameters. Shading and labeling of the alignment was done with TEXshade [11].

filamentous fungus *Neurospora crassa*. The N-terminal domain of Prp4 in *S. pombe*, *N. crassa*, *M. musculus* and *H. sapiens* comprises 158, 522 and 686 amino acids, respectively (Figure 1).

A mutational analysis of the N-terminus of the fission yeast kinase, testing the effect of the mutations by functional complementation led to the discovery of three short elements, essential for functioning of the kinase. Two elements, SDSPSI and SPSPSV at position 90–95 and 112–117, respectively, were called the serine elements (SX1 and SX2). The third element EGY at position 144–147 is located proximal to the kinase domain [10]. In all Prp4 counterparts this sequence is highly conserved (Figure 1). Alignment analyses led to the classification of Prp4 as a dual specificity tyrosine-regulated kinase (DYRK) family member. The element (EGY), called DH-box in these analyses, is part of the signature which assigns the Prp4 kinases to the third subfamily of the DYRK family [16]. Based on our sequence comparison there is one more highly conserved element in the N-terminal region close to the kinase domain. The sequence DDMFA at position 106–110 is highly conserved in all Prp4 sequences and may represent together with the DH-Box a signature in the N-terminus, indicating the archetypal Prp4 kinase (Figure 1). One serine element (SX2) is located next to DDMFA and shows no conservation between the *N. crassa* and the mammalian sequence at that position. The same is true for the other serine element (SX1). Neither the fungal nor the mammalian sequence is conserved in this position. However, in the *N. crassa* and the mammalian sequence serine elements similar to those in the N-terminus of the fission yeast sequence were found. Particularly, the mammalian sequence shows serine elements containing prolines, for example, SVPSEPSSP or SRSPSPD proximal to the kinase domain. In the extended N-terminal sequence serine elements of iterated serine/proline and serine/arginine dipeptides such as SRRSRSP are prevalent. Phosphoproteome analysis of fission yeast indicated that element SX1 is phosphorylated at the serine in position 92 which is followed by a proline [17]. Several of the serine elements in the mammalian sequence are phosphorylated. The phosphorylated serines were frequently found next to a proline or an arginine (Figure 1).

3. Prp4 kinase and its substrates

Both protein kinases, that is the fission yeast Prp4 kinase and the chimeric mouse Prp4 kinase, consisting of the mouse kinase domain preceded by the *S. pombe* Prp4 N-terminus, phosphorylated *in vitro* the arginine/serine-rich (RS) domain of the mammalian protein ASF/SF2 [10]. This protein belongs to the SR superfamily of splicing factors. SR proteins consist of one or two N-terminal RNA-binding domains (RBDs) and a C-terminal arginine/serine-rich (RS) domain. Members of the SR protein superfamily in mammals are involved in constitutive splicing and are specific modulators of alternative splicing. They serve as well as chaperones to couple splicing with transcription and RNA export. The versatile functions of SR proteins are modulated by reversible phosphorylation [18]. In fission yeast two SR proteins have been identified [19, 20]. Srp1 contains one RBD at the N-terminus followed by three serine elements, which we called RS1, RS2 and RS3, respectively, containing a various number of SR and SP dipeptides. Phosphoproteome analysis of fission yeast revealed several phosphorylated serines in these three RS elements (Figure 2). Srp2 contains two N-terminal RBDs and two SR and SP dipeptide displaying elements in the C-terminus, which we called SR1 and SR2. Again, phosphoprotein analysis detected phosphorylated

serines in these elements (Figure 2). An extensive mutational analysis of both (SR1 and SR2) elements by replacing the serines with other amino acids and testing the effect of the mutations *in vivo*, revealed that when in both elements the serines were mutated, the GFP-Srp2 fusion protein failed to enter the nucleus and was found instead in distinct dots distributed in the cell [20].

Over the last ten years then, it has been shown that Srp1 and Srp2 are authentic targets of the kinase Dsk1 and that the phosphorylation status influences the distribution of Srp1 and Srp2 between cytoplasm and nucleus. For example, efficient localization of Srp2 in the nucleus requires Dsk1 kinase activity [21]. Dsk1 is the ortholog of the human SR protein-specific kinase 1 (SRPK1). While Dsk1 was detected in the cytoplasm and nucleus, Prp4 is localized predominantly in the nucleus [22]. As mentioned above, initial experiments showed that Prp4 kinase of fission yeast phosphorylated the human SR protein ASF/SF2 *in vitro*. Other experiments indicated, that overexpression of ASF/SF2 leads to the partial suppression of the splicing defect of intron containing genes in a *prp4-73*^{ts} background at the restrictive temperature [10, 19]. Based on these observations we checked whether Prp4 kinase phosphorylates *in vitro* Srp1 and/or Srp2. Notably, Srp2, representing the SR family member displaying two RBDs, was phosphorylated by Prp4, but Srp1 displaying one RBD was not (Figure 2). In contrast, Dsk1 kinase phosphorylated Srp1 as well as Srp2, however, which peptides are phosphorylated has not been determined so far [23, 24]. Therefore, we used and combined mutants in which serines were replaced with other amino acids at different positions in the SR1 and SR2 elements of Srp2, and produced the mutant proteins in bacteria followed by affinity purification. In order to determine which serines become phosphorylated *in vitro* by these kinases, the Srp2 mutant proteins were then employed in kinase assays using either bacterially produced Prp4 or Dsk1 kinase, respectively. Intriguingly, both kinases appear *in vitro* to phosphorylate the same dipeptides. In the SR1 element only one serine at position 188 followed by a proline is phosphorylated by both kinases. In the SR2 element we do not know which serines are phosphorylated. However, phosphoproteome analysis *in vivo* revealed that all of them are phosphorylated [17]. Consistently, our analysis shows that both kinases phosphorylate the SR2 element *in vitro*, as well as two serines to the left and right of SR2 displaying the tripeptides RSR and PSP, respectively (Figure 2).

4. Function of Prp4 in pre-mRNA splicing

We began to address the role of Prp4 kinase in spliceosome assembly *in vivo* by monitoring the co-transcriptional recruitment of spliceosomal and non-spliceosomal components including Srp2 using ChIP (Chromatin Immuno Precipitation, [25]). Varying the kinase activity with an ATP analog in a fission yeast strain expressing an (analog-sensitive) asPrp4 kinase indicates that recruitment of Srp2 to nascent intron containing transcripts depends on Prp4 kinase activity (D. Eckert and N. F. Käufer, unpublished results). It has been shown that Srp2 promotes the splicing of reporter genes by binding to purine-rich exonic splicing enhancer (ESE) sequences [26]. Based on these observations, we hypothesize that phosphorylation within the SR elements of Srp2 by Prp4 kinase in the nucleus may serve to target Srp2 to ESE containing pre-mRNAs, thereby modulating their splicing efficiency. Human Prp4 kinase phosphorylates *in vitro* the serine/arginine rich domain of the SR protein ASF/SF2 and has

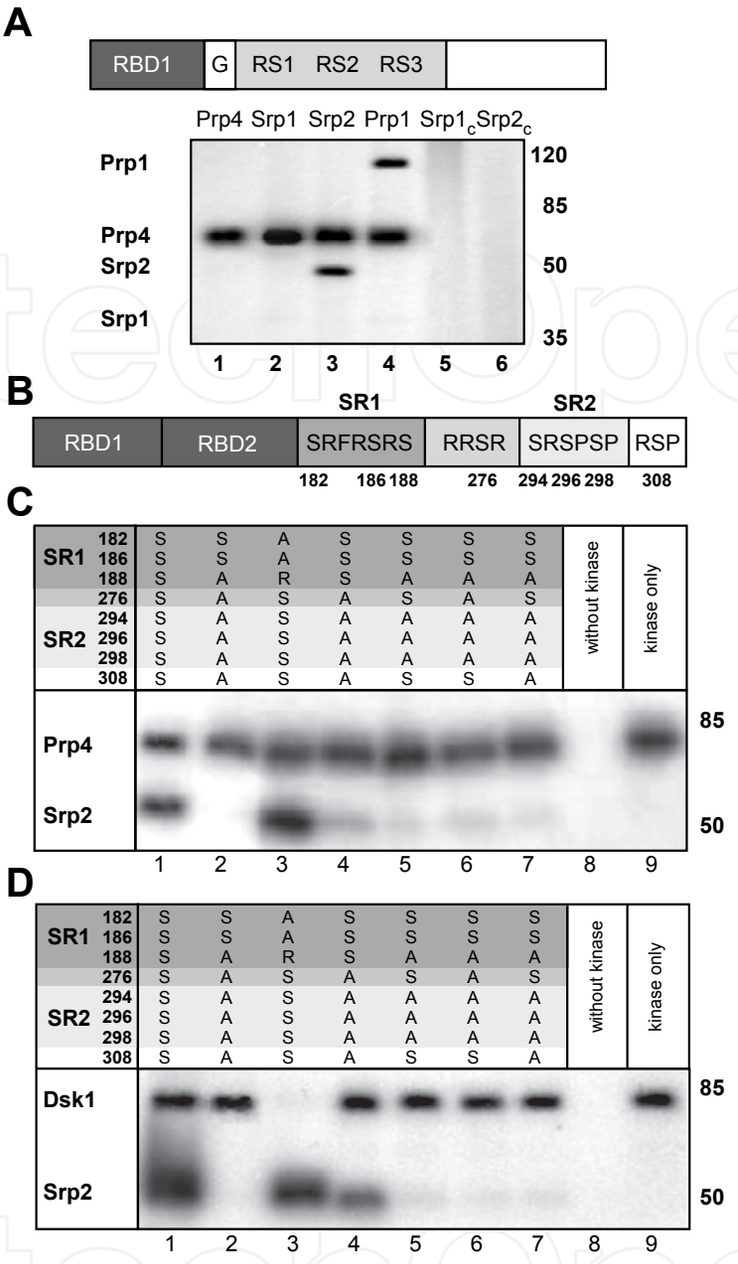


Figure 2. Phosphorylation of SR proteins by Prp4 kinase *in vitro*. **(A)** Schematic representation of Srp1 from fission yeast. Srp1 consists of an N-terminal RNA-binding domain (RBD1), followed by a glycine-rich domain (G) and a domain containing three arginine/serine-rich elements (RS1–3). In order to test whether Srp1 or Srp2 are phosphorylated by Prp4 kinase, recombinant proteins purified from *E. coli* were incubated in the presence of $\gamma[^{32}P]$ATP and separated by SDS-PAGE. Phosphorylated proteins were detected by auto-radiography. Prp1, which has been previously identified as a substrate of Prp4 kinase served as a positive control. Lanes 5 & 6: negative control without addition of the kinase. **(B)** Schematic representation of Srp2 from fission yeast. Srp2 consists of two N-terminal RNA-binding domains (RBD1, RBD2) and a C-terminal domain containing two serine/arginine-rich elements (SR1, SR2). Numbers below indicate positions of the serine residues within the SR elements. **(C)** *In vitro* kinase assay of recombinant Srp2 proteins, mutated within the SR elements as indicated in the table, with recombinant Prp4 kinase. **(D)** *In vitro* kinase assay of mutated Srp2 proteins with recombinant Dsk1 kinase.

been shown to co-localize in HeLa cells with SR family members, including ASF/SF2, in molecular structures called speckles [13]. In addition, Clk1 (Cdc2-like kinase 1) as well as SRPK1, a protein kinase phosphorylating SR-rich domains in SR proteins, have been shown to phosphorylate *in vitro* several of the serines within the SRSP elements that are located in the extended N-terminus of hsPrp4 (Figure 1, ↓, P). Phosphorylation of the serine elements in this region may be in mammalian cells part of the process to move and target hsPrp4 kinase to the location of its action [13]. In mammals the SR protein ASF/SF2 is involved in the regulation of alternatively spliced genes. Intriguingly, based on results with human immunodeficiency virus type 1 (HIV-1), it has been proposed that the interaction of hsPrp4 kinase with the HIV-1 Gag polyprotein may lead to the down-regulation of alternative splicing through reduced phosphorylation of ASF/SF2 by hsPrp4 at late stages of infection to support production of unspliced viral genomic RNA [27].

We took advantage of the powerful genetic system available with fission yeast and produced an epistasis map, particularly, by screening for genetic interactions with the *prp4-73^{ts}* allele. With this approach we identified Prp8/U5-220K and Brr2/U5-200K [29, 30]. Both proteins are highly conserved between yeast and human and play key regulatory roles in the activation of spliceosomes. Prp8/U5-220K interacts directly with the splice sites and branch region of pre-mRNAs [31]. Brr2/U5-200K belongs to the DEAD/DEXH-box family of ATP-dependent RNA helicases containing two helicase domains. Brr2/U5-200K has been implicated in the unwinding of U4/U6 molecules as a prerequisite for the snRNA rearrangements necessary for the activation of a spliceosome [32]. As mentioned above, we started to investigate with ChIP analysis the association of spliceosomal and non-spliceosomal proteins with nascent transcripts. Brr2, represents a specific U5 snRNP protein. The recruitment of Brr2 to nascent intron containing transcripts appears to be strongly influenced by active Prp4 kinase. (D. Eckert and N. F. Käufer, unpublished results). Genetic interactions with the *prp4-73^{ts}* allele indicate by no means that the interaction partner is a substrate of Prp4 kinase. However, the multiple genetic interactions we determined between Prp8, Brr2, Prp1 and Prp4 kinase, and among themselves, unambiguously pointed to Prp8, Brr2 and Prp1 as a structural center of the spliceosome which is targeted by Prp4 kinase [15, 29, 30]. While there is no indication that Prp4 kinase is a stable spliceosomal component, Prp8, Brr2 and Prp1 have been determined as *bona fide* spliceosomal components on all accounts present together in pre-catalytic spliceosomes. We identified Prp1 as a physiological substrate of Prp4 kinase in fission yeast by demonstrating that *in vivo* Prp1 was not phosphorylated in the genetic background of *prp4-73^{ts}* at the restrictive temperature. In addition, immunoprecipitated (IP) and recombinant Prp4 kinase phosphorylated recombinant Prp1 *in vitro* [33].

To avoid confusion, we discuss here shortly the nomenclature of the orthologs of Prp1 in the literature and suggest a standardization. The ortholog of Prp1 in *S. cerevisiae* is called Prp6, whereas the ortholog in human is called hsPrp6 or U5-102K, referring to a protein with a molecular weight of 102 kDa, which is associated with the spliceosomal snRNP U5 in mammalian cells. Prp1 (Prp6/U5-102K) is a protein consisting of multiple direct repeats called tetra-tricopeptide repeats (TPRs), which are listed as HATs (half a TPR) in UniProtKB (Q12381, PRP1_SCHPO; P19735, PRP6_YEAST; Q872D2_NEUCS ; O94906, PRP6_HUMAN). In this report, we analysed Prp1 of fission yeast and its homologs in other eukaryotes

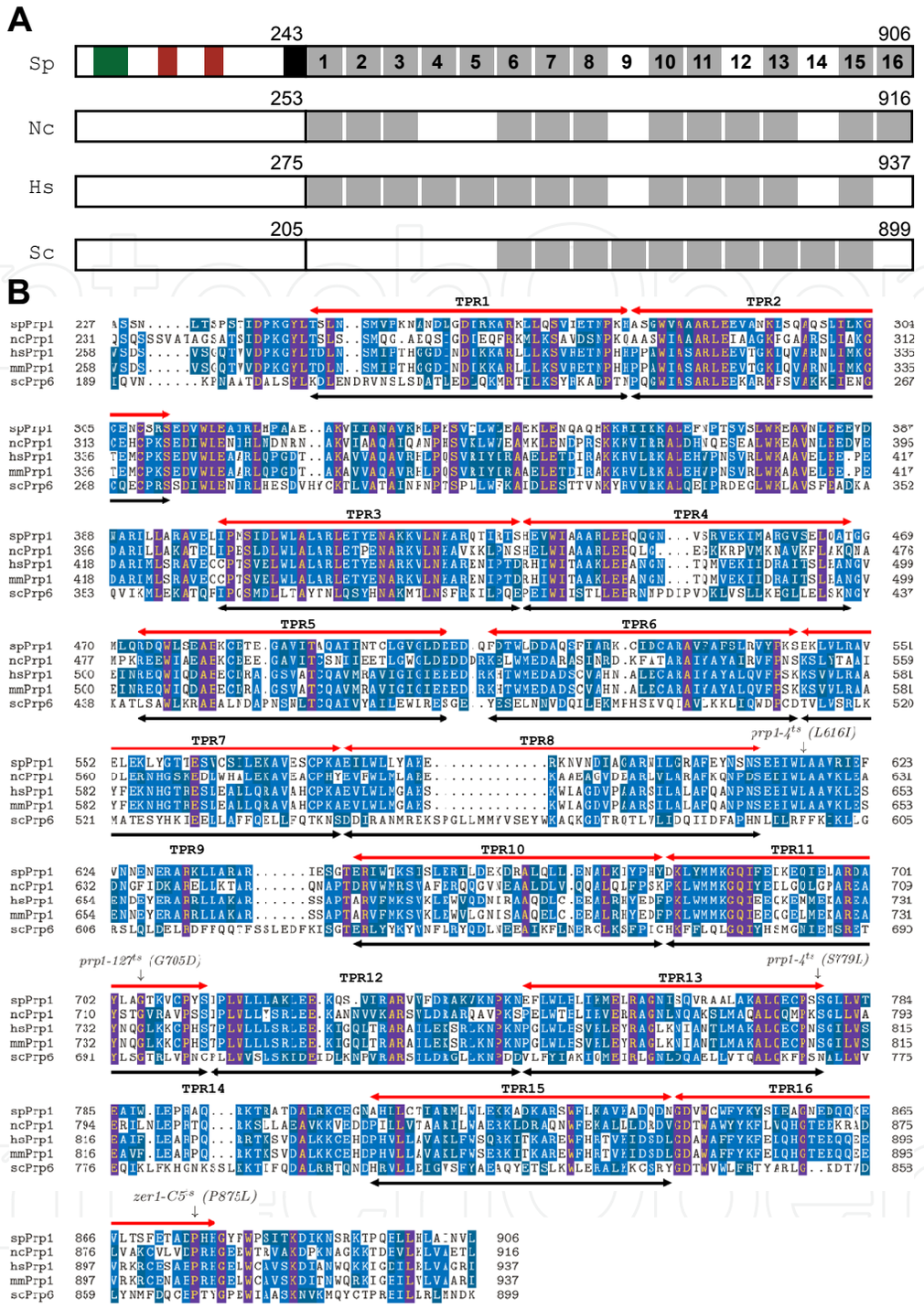


Figure 3. (A) Schematic representation of Prp1 from *S. pombe*, *N. crassa*, *H. sapiens* and *S. cerevisiae*. Conserved sequence elements (see Fig. 3) within the N-terminus are coloured in green, red and black. Tetratrico peptide repeats (TPRs) within the C-terminus are drawn as grey boxes. Positions of the TPR sequences were determined using the program TPRpred [28]. Only TPRs with a P-value lower than P = 0.01 are shown. **(B)** ClustalX alignment of the Prp1 C-terminal domain. Red arrows (↔) above the alignment mark TPR sequences within spPrp1, black arrows (↔) below indicate TPR positions of hsPrp1. Mutated amino acid residues of the spPrp1 temperature-sensitive alleles *prp1-127*^{ts}, *prp1-4*^{ts}, and *zer1-C5*^{ts} are indicated with arrows (↓).

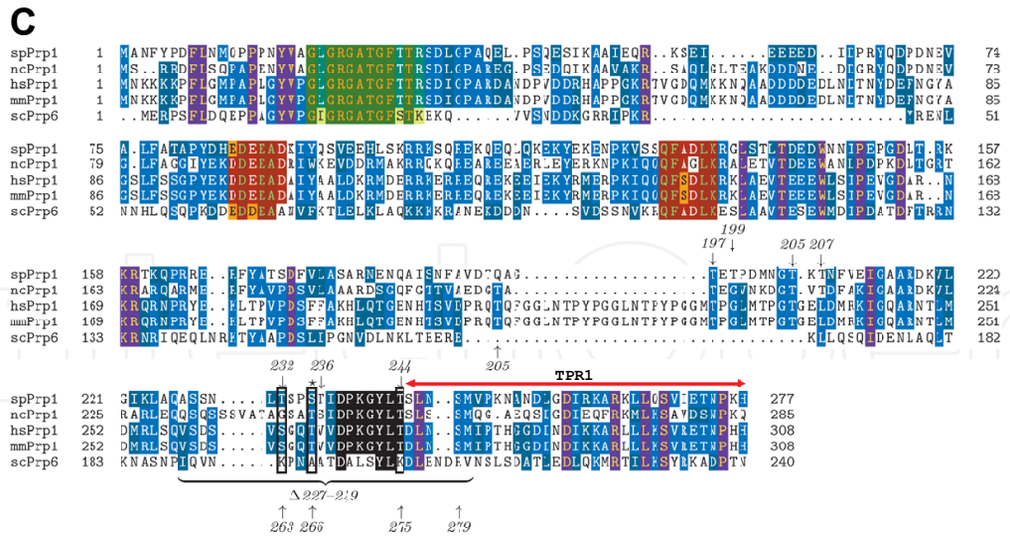


Figure 3. (C) ClustalX alignment of the Prp1 N-terminus. Conserved sequence elements are coloured in green, red and black. Positions of spPrp4 kinase *in vitro* phosphorylation sites are marked with arrows (↓) above the alignment. The *in vivo* phosphorylation site at pos. 235 of spPrp1, determined by phospho-proteome analysis [17] is marked with a star (⋆). Phosphorylation sites within hsPrp1 determined by [34] are indicated below the alignment (arrows, ↑). Amino acid residues which are phosphorylated in both spPrp1 and hsPrp1 are marked with a frame. The region containing most Prp1 phosphorylation sites, whose function was previously analysed by deletion mapping in [35], is marked with a brace (Δ227–249). GeneBank accession numbers of the sequences shown in the alignment are: spPrp1, CAA17050; ncPrp1, XP_958849; hsPrp1, NP_036601; mmPrp1, AAH23691; scPrp6, CAA84998.

including Prp6 of *S. cerevisiae* using TPRpred, a computer algorithm recently developed to find tetra-tricopeptide repeats [28]. Based on this analysis, Prp1 family members display 16 TPR positions in the C-terminus preceded by an N-terminal region of about 250 amino acids. Prp1 of fission yeast and the mammalian orthologs show 13 TPR repeats, of which 12 share the same positions. The sequences of *N. crassa* and *S. cerevisiae* display 12 and 10 TPR repeats, respectively (Figure 3A and 3B). A comparison of the N- termini revealed that there is only one highly conserved region comprising 32 amino acids with an identical core, GRGATGF, of seven amino acids proximately to the start codon. Two further conserved regions with the signatures, DEEAD and QFADLK at positions 85 and 129, respectively, were detected (Figure 3C). Most intriguingly, however, the fourth highly conserved signature sequence in the N-terminus, DPKGYLT, is directly next to the first TPR domain of fission yeast, *N. crassa* and the mammalian sequence, whereas the sequence in *S. cerevisiae* in this region is diverged and does not reveal a TPR repeat according to the algorithm used (Figure 3C). In addition, phosphoproteome analysis of fission yeast and human proteins revealed that the threonine in DPKGYLT is phosphorylated as documented in UniProtKB (PRP1_SCHPO, PRP6_HUMAN). Mapping the *in vitro* phosphorylation sites in Prp1 of fission yeast by recombinant Prp4 kinase using peptide fingerprinting revealed this site and additional sites close to this threonine [35]. Mass spectrometry analyses of *in vitro* assembled and purified pre-catalytic B complexes from HeLa cells treated with recombinant human Prp4K also show additional phosphorylation sites in this region [34]. The results of both analyses are shown in Figure 3C.

5. Participation of mammalian Prp4K in other cellular processes

Mammalian Prp4K has been shown to interact and colocalize with many diverse cellular structures and defined proteins, such as speckles, spliceosomal particles, chromatin organizing complexes and with proteins at the kinetocore organizing the spindle assembly checkpoint [12–14, 27, 36–39].

6. Role of Prp4 kinase in the activation process of pre-catalytic spliceosomes

The results discussed above, presenting the genetic, molecular and biochemical interactions of Prp4 kinase in fission yeast demonstrate genetic interactions with several major regulatory spliceosomal components, however, up to date a stable association with spliceosomal particles and any other cellular complexes was not detected. Prp4 kinase molecules in whole cell extract (WCE) sediment around 9S [30, 35, 40–42].

Particularly the lack of an *in vitro* splicing system in fission yeast, led us to use extensively the inducible and repressible expression systems available to switch-off and -on spliceosomal components and to express mutant versions for studying their molecular consequences *in vivo*. With this approach, we discovered that the expression of mutations in the N-terminus of Prp1 leads to the appearance of spliceosomal complexes containing the five snRNAs U1, U2, U5 and U4/U6 and pre-mRNAs. The mutations in the N-terminus, which prevent splicing to occur, include the identified phosphorylation sites of Prp4 kinase, as well as the other three highly conserved regions in the N-terminus (Figure 3C). This substantial mutational analysis revealed that the structural integrity of the N-terminus is required to mediate a splicing event, but that it is not necessary for the assembly of a pre-catalytic spliceosome. The purification of spliceosomal complexes by the tandem affinity purification (TAP) method, using the TAP-tagged spliceosomal component Prp31 of a strain expressing solely the N-terminal Prp1 mutant version in which the phosphorylation sites were deleted, yielded spliceosomal complexes containing pre-mRNA, the five snRNAs, Prp31-TAP, MycΔNPrp1 and HA-Cdc5, indicating that this complex is frozen or stalled in a pre-catalytic stage [35]. In fact, mass spectroscopy revealed a spliceosomal protein content clearly suggestive for a pre-catalytic complex containing all five snRNPs and the Prp19 complex (NTC) as shown for *in vitro* assembled mammalian B complexes (Lützelberger and Käufer, unpublished, [43]). Based on these observations, we hypothesized that phosphorylation of Prp1 by Prp4 kinase is involved in the activation of spliceosomes. However, it is not clear as of yet, whether this phosphorylation is involved directly, that is, inducing the rearrangements of the snRNPs for catalysis, or indirectly by recruiting other components necessary for the switch.

This in mind we employed again classical genetics to screen for suppressors and synthetic lethals of the three temperature sensitive alleles *prp1-127^{ts}*, *prp1-4^{ts}* and *zer1-C5^{ts}*, respectively (Figure 3B). For *prp1-127^{ts}*, displaying one point mutation, we found two extragenic suppressors, but none for the two other alleles [49]. In addition, we found a strong epistatic interaction between *prp1-4^{ts}*, displaying two point mutations, and a gene in fission yeast called *cdc28-P8^{ts}* [50]. This genetic interaction implicates that for proper processive action of Cdc28 a functional Prp1 is a prerequisite. The *cdc28* gene encodes the ortholog of Prp2 in *S.*

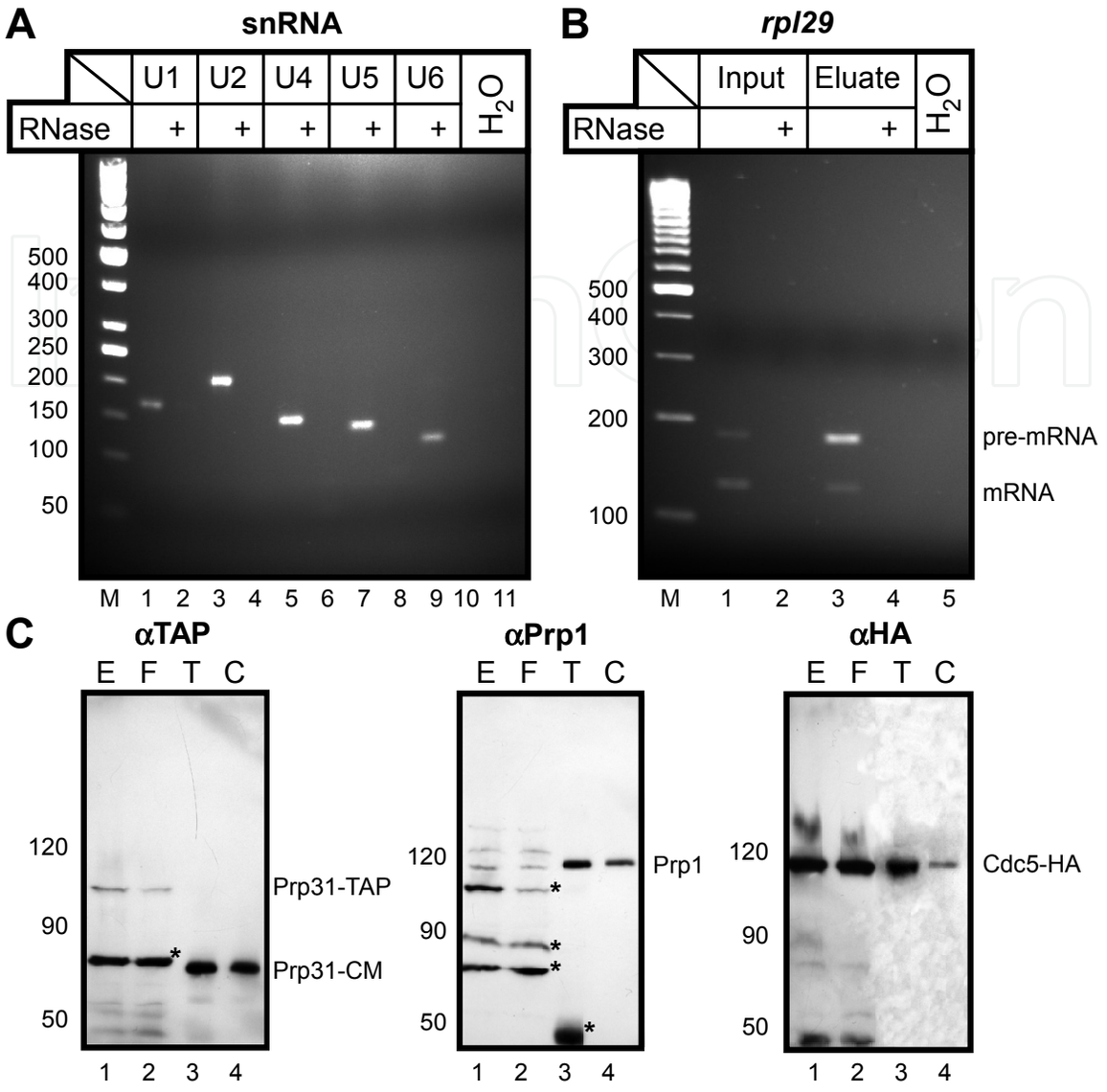


Figure 4. Tandem affinity purification (TAP) of pre-catalytic spliceosomal complexes. For this experiment a strain with the genotype h^{-s} *prp31 int::prp31-CTAP leu1-32 int::pMLtet*^{ON} *prp2*^{H539D} *cdc5 int::HA-cdc5* was used. In this strain, expression of a mutated Prp2 helicase is driven by a tet-inducible CaMV35S (tet^{ON}) promotor [44]. It has been shown that expression of Prp2^{H539D} leads to growth arrest by inhibition of pre-mRNA splicing [45–47] in yeast and human cells. TAP was performed as described in reference [48], after 3 hours of induction with 6 μM anhydro-tetracycline. RNA purified from the TAP eluate by phenol-extraction was analysed by RT-PCR in **(A)** using primer pairs amplifying the five snRNAs (U1–U6) and in **(B)** with a primer pair amplifying RNA expressed from the ribosomal protein gene *rpl29*, which contains a single intron of 53 nt. The PCR products of snRNA U1, U2, U4, U5 and U6 have a size of 166, 207, 149, 139 and 119 bp, respectively. The PCR products of *rpl29* pre-mRNA and mRNA migrate at 182 and 129 bp. Prior to reverse transcription, all samples were treated with DNase I. Samples were treated with RNase A as indicated (+), to verify that they were not contaminated with genomic DNA. **(C)** Whole cell extract, E, flow-through, F, eluate of the first-, T, and second TAP affinity column, C, were analysed for the presence of Prp31-CTAP (90.3 kDa; Prp31-CM after treatment with TEV protease, 62.6 kDa), Prp1 (103 kDa) and HA-Cdc5 (89.8 kDa) with a Western blot, using anti-TAP, anti-Prp1 and anti-HA antibodies as indicated on the top of each blot. Unspecifically recognized bands and degradation products of Prp31 and Prp1 are marked with asterisks ($\star$).

cerevisiae, DHX16 (hPrp2) in mammals and belongs to the DEAH subgroup of DExD/H-box ATPases/RNA helicases. Prp2 is required for the first catalytic step of the splicing pathway, and thus, is involved in the early activation process of spliceosomes [45, 46]. The stalled pre-catalytic complex found in fission yeast when ΔNPrp1 mutations were expressed did not contain Prp2, suggesting that, as observed for spliceosomes in mammals, the interaction of Prp2 with the spliceosome is transient.

Therefore, to study how this helicase affects pre-mRNA splicing in fission yeast, we made mutations in the conserved DEAH-box of Prp2 (spCdc28). In yeast and human, this mutation ($Prp2^{H539D}$) leads to a diminished helicase activity and inhibition of splicing, when overexpressed [45–47]. When we expressed the mutation with one of the inducible expression systems mentioned above, cells stopped mitotic growth within three hours. At this time point spliceosomes were purified using the TAP method [51, 52]. Spliceosomes purified under these conditions contained Prp31-TAP, Prp1, HA-Cdc5, all five snRNAs U1–U6 and pre-mRNA indicating the arrest of spliceosomes in a pre-catalytic stage (Figure 4). We have reason to believe that the protein composition of this spliceosomal complex is equivalent to the stalled spliceosomes purified after expressing the ΔNPrp1 mutations described above [35]. First, the results presented are consistent with the notion, that Prp1 operates in the activation center of a pre-catalytic spliceosome and emphasize that phosphorylation of Prp1 might be involved to recruit chaperones to induce the dynamic rearrangements of a spliceosome for activation. Second, and most intriguingly, the overexpression of mammalian DHX16/hPrp2 mutant versions in human cell lines led to the retention of unspliced pre-mRNA in the nucleus. It has been suggested that this nuclear retention is directly related to the fact that DHX16/hPrp2 functions after spliceosome formation [46, 53].

7. Inhibition of Prp4 kinase activity affects splicing of intron-containing genes differentially

Based on the observation that a relatively small number of intron-containing transcripts accumulated in cells expressing mutant versions, it was speculated that DHX16/hPrp2 might affect the splicing of different introns or different genes [46, 53]. These considerations prompted us to ask the question: Does the inhibition of Prp4 kinase in fission yeast affect splicing of intron-containing genes differentially?

Therefore we constructed a conditional analog sensitive (as) allele *prp4-as2* which allows to reversibly inhibit Prp4 kinase with ATP analogs [54]. Adding the inhibitor 1-NM-PP1 to a growing cell culture expressing Prp4-as2 leads to growth arrest [1]. We performed semi-quantitative RT-PCR analyses to detect mRNA and pre-mRNA of different intron containing genes, using RNA of a growing culture prepared 10, 20 and 30 minutes after adding the kinase inhibitor. Here we present selected data of the most interesting observations we made in these series of experiments.

We used, for example, *tbp1* encoding the TATA-binding protein. This gene contains three introns in the open reading frame (ORF). Within 30 min after adding the inhibitor, the ratio of *tbp1* mRNA and pre-mRNA is reversed, indicating that pre-mRNA is accumulating and mRNA degraded. In contrast, the ribosomal protein gene *rpl29* containing one intron of 53 bp

is hardly affected, showing no significant change in mRNA concentration and a slight increase in pre-mRNA (Figure 5). We also used the genes *res1* and *res2* containing one intron in a similar 5'-position of the ORF with a size of 127 bp and 164 bp, respectively. Both genes, *res1* and *res2*, encode components of the Mlu1-binding factor (MBF). In fission yeast MBF is required together with the cyclin-dependent kinase Cdc2 for passage through Start in G1 phase by activating the transcription of genes for S phase. At this point the cell is in each cycle faced with a critical decision between vegetative proliferation by cell division or sexual conjugation followed by meiosis [55].

In case of *res1* only pre-mRNA is detected after 10 min exposure to the kinase inhibitor. Thus, splicing of this pre-mRNA is inhibited immediately and pre-mRNA accumulates, whereas the mRNA is rapidly degraded. On the contrary, *res2* is still efficiently spliced (Figure 5). These examples demonstrate that fission yeast contains genes with introns whose removal appears strictly dependent on Prp4 kinase activity, and also contains genes whose intron removal seems independent of kinase activity. The observed differences in mRNA and pre-mRNA concentrations detected indicate that there are major differences in pre-mRNA and mRNA stability of each gene (Figure 5).

It has been shown for several precursor pre-mRNA (*prp*) temperature sensitive mutants, that introns of different genes show a range of splicing defects in these strains. There are introns which are spliced independently of the large subunit of the U2 auxiliary factor ($U2AF^{59}$). Noteworthy here, $U2AF^{59}$ is essential for growth [56–58]. Moreover, there is some evidence that the recruitment of $U2AF^{59}$ to auxiliary factor dependent introns might be mediated by the SR protein Srp2, which has been shown above to be an *in vitro* substrate of Prp4 kinase [26]. However, we have no data, whether $U2AF^{59}$ dependent introns are also Prp4 kinase dependent introns.

This is the first report demonstrating Prp4 kinase dependent removal of introns from essential genes, such as *tbp1* and *res1*, respectively. Furthermore, inhibition of Prp4 kinase by the ATP-analog leads to growth arrest, and cells in this cell population are specifically arrested in G1 and G2 phase, respectively (Figure 5). These results are consistent with the notion that those cells in which the *res1* intron is not spliced out specifically arrest in G1 and, therefore, splicing control of *res1* might be involved in the regulatory switch between vegetative proliferation by cell division and sexual conjugation [59].

Apparently only few cases of pre-mRNA splicing regulation exist in fission yeast. A meiotic cyclin, Rem1, is only expressed when a 87-nt intron is spliced out of the *rem1* pre-mRNA. Splicing of *rem1* pre-mRNA is dependent on the meiosis specific transcription factor Mei4 regulating transcription of the *rem1* gene and recruiting the splicing machinery to the transcribed locus [60, 61]. About 45 % of the genes contain introns, whereas the genes contain from one to more than 10 introns. The introns are small with a mean size of 78 nt [62]. There is no known case of regulated alternative splicing in fission yeast producing different isoforms of a protein from the same open reading frame.

We have suggested that fission yeast represents the archetype of the pre-mRNA splicing machinery in eukaryotes [63]. And, we proposed that phosphorylation of Prp1 by Prp4 kinase is part of the process in which spliceosomes are activated: either the phosphorylation by Prp4

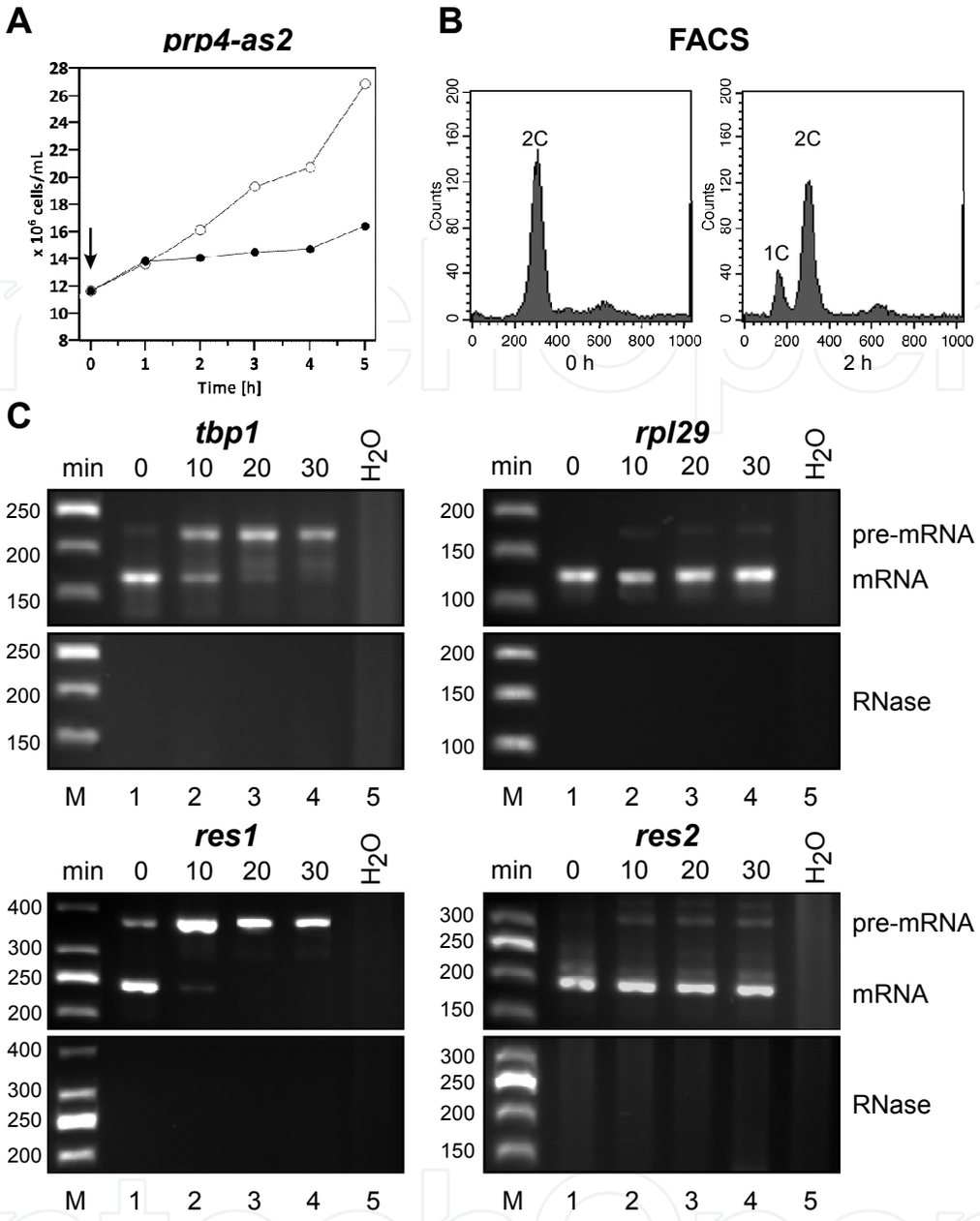


Figure 5. Inhibition of Prp4 kinase activity with 1-NM-PP1. **(A)** A strain with the genotype h^{-s} *prp4-as2* was grown to early log-phase. Then, 1-NM-PP1 was added to the culture medium (0 hours, arrow, ↓) at a final concentration of 10 μM. Growth of the culture was monitored by counting the number of cells/mL with a haemocytometer every hour (closed circles, ●) and compared to a culture growing in absence of the inhibitor (open circles, ○). **(B)** Immediately before (0 hours) and 2 hours after addition of 1-NM-PP1, cells were analysed by FACS for their DNA content (1C, 2C). **(C)** RNA prepared after 0, 10, 20 and 30 min of inhibition with 1-NM-PP1 was analysed by RT-PCR, using primer pairs detecting *tbp1* (second intron), *rpl29*, *res1* and *res2* RNA, resulting in PCR products with a size of (pre-mRNA/mRNA) 225/173 bp for *tbp1*, 182/129 bp for *rpl29*, 374/247 bp for *res1* and 366/203 bp for *res2*. Prior to RT-PCR, all RNA samples were treated with DNase I. To verify that contaminating genomic DNA was completely removed, samples were treated with RNase as indicated. The analog-sensitive *prp4-as2* kinase allele was generated by introducing a point mutation within the kinase domain at position 238, changing the so-called "gate-keeper"amino acid [54] from phenylalanine to alanine.

kinase is part of a mechanism which signals that an intron is occupied by a splicing competent spliceosome in a sense of quality control and/or the phosphorylation is directly involved in inducing the rearrangements for catalysis [30, 35]. The results received since then, including those reported here, allow now a discussion which will help further to pinpoint the function of Prp4 kinase in pre-mRNA splicing.

8. Is Prp4 a pre-mRNA splicing checkpoint kinase?

The Prp4 kinase dependent genes we detected do not reveal any obvious sequence motifs indicating their Prp4 dependency. For example, the comparison of *res1* and *res2* exon and intron sequences did not offer anything clearly pointing into this direction. However, there is one tendency to observe: The removal of introns from transcripts which are encoded by genes that are essential for growth appears to be Prp4 kinase dependent.

In consideration of all the facts and thoughts presented above, we reason that in general, removal of introns in fission yeast during mitotic growth is constitutive by default. That means, the splicing machinery is recruited to the pre-mRNA and assembled on introns to form a splicing competent spliceosome. Spliceosomal ATPases/RNA helicases, including Prp2, have been identified as the candidates of a pre-mRNA splicing proofreading system in eukaryotes. They control stepwise induced conformational changes to turn a competent spliceosome into a catalytically active one [64–66]. Therefore, it is conceivable that phosphorylation of Prp1 by Prp4 kinase enhances the interaction of Prp1 with the ATPase/RNA helicase Prp2 to induce conformational changes in a pre-catalytic spliceosome. This suggestion does neither imply, nor exclude that the phosphorylation of Prp1 is the cause for the recruitment of Prp2 helicase. As a matter of fact, the Lührmann laboratory showed by using the mammalian HeLa cell splicing system that *in vitro* assembled pre-catalytic B-complexes are stabilized when Prp1 is phosphorylated by the Prp4K kinase *in vitro* [34]. This is consistent with our suggestion that stabilizing the pre-catalytic spliceosome *in vivo* by phosphorylation of Prp1 might increase time and chance for the ATPase(s) to operate as chaperone(s) at the pre-catalytic spliceosome.

In addition, while probing different transcripts containing multiple introns for their splicing defects, we discovered that pre-mRNAs containing introns larger than 250 nt never accumulate when splicing is inhibited *in vivo*. This occurred neither in cells where Prp4 kinase was inhibited by 1-NM-PP1 nor in cells containing the ts alleles *prp1-127*^{ts} or *prp1-4*^{ts} grown at the restrictive temperature, respectively (Eckert and Käufer, unpublished). It appears that transcripts containing introns of this size are rapidly degraded as a consequence of the splicing defect. This observation is consistent with the notion that this degradation of pre-mRNA most likely takes place in the nucleus. Noteworthy here, fission yeast contains only 9 % introns in the size range of 250–800 bp [62]. Preliminary results indicate that Rrp6, a subunit of the nuclear exosome, might be a candidate involved in this degradation process (Zock-Emmenthal and Käufer, unpublished).

Not much is known yet about the mechanisms of pre-mRNA degradation in the nucleus. However, distinct pathways of poly(A)-dependent mRNA degradation involving the nuclear poly(A)-binding protein (Pab2) and Rrp6 have been described in fission yeast [67–71].

Eukaryotic cells have developed several RNA surveillance pathways to prevent the expression of aberrant transcripts [72–74]. An early surveillance checkpoint acts at the transcription site and prevents the release of mRNAs that carry processing defects [75]. In mammals, the exosome including the Rrp6 subunit homolog PM/SCl-100, has been shown to be involved in many different RNA processing pathways. Based on these interactions of the exosome with multiple RNA processing machineries, it has been speculated that the production of a fully functional mRNA may be ensured through several different checkpoints [76].

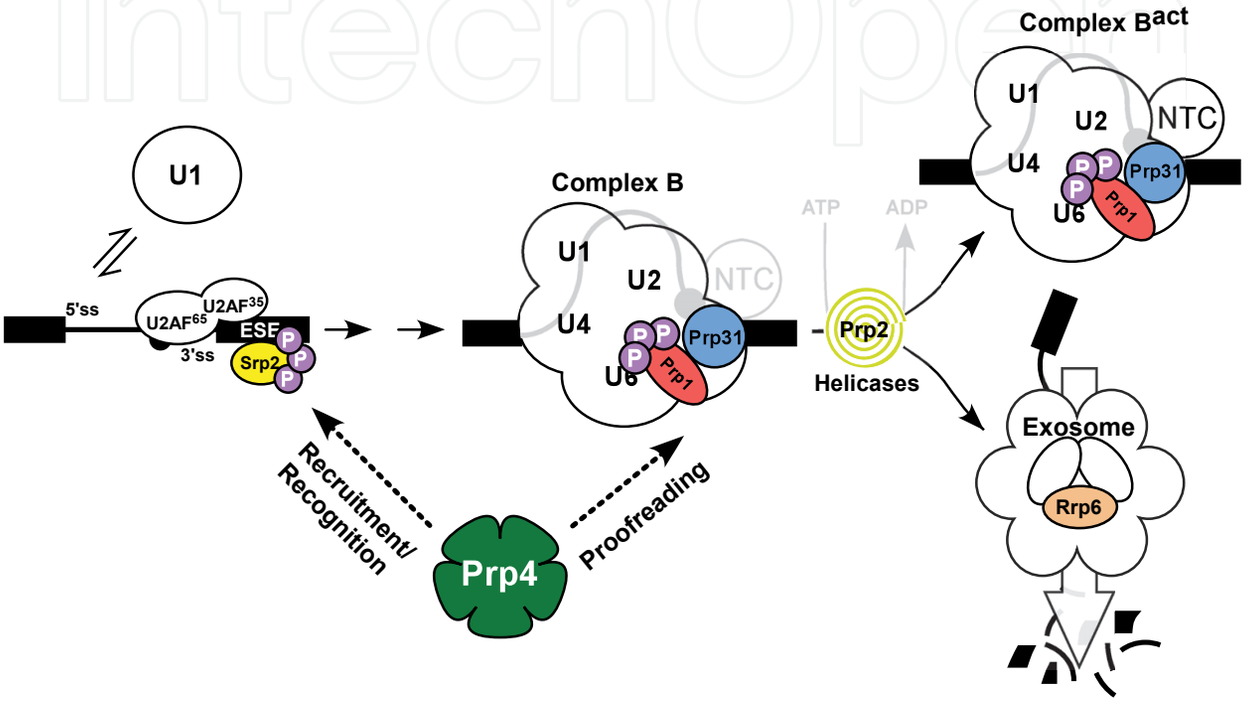


Figure 6. Checkpoints controlled by Prp4 kinase during splicing of a pre-mRNA. Phosphorylation of splicing factors, such as Srp2 may modulate their recruitment/binding to specific pre-mRNA substrates, thereby affecting the efficiency of intron recognition and removal. The phosphorylation of Prp1 acts as a "switch" to convert a fully assembled spliceosome into a catalytically active spliceosome. This is achieved, either directly or indirectly, by the recruitment of RNA helicases such as Prp2, which act as chaperones and aid in the proofreading of this process. Prp4 might also contribute in proofreading, i.e. by linking the splicing machinery to the nuclear exosome, which prevents that aberrant or unspliced pre-mRNA accumulates within the nucleus, if the transition from an inactive to an active spliceosome fails or the splicing reaction is unable to proceed. Exons of the pre-mRNA substrate are drawn as filled boxes, connected with a line (intron). Abbreviations: NTC, Nineteen Complex (containing Prp19, Cdc5 and other components, see reference [77]; ESE, Exonic Splicing Enhancer; U2AF, U2 auxiliary factor; ss, splice site.

We propose that Prp4 kinase is the pre-mRNA splicing checkpoint kinase, operating at the pre-catalytic spliceosome to provide splicing competent spliceosomes and to ensure that only properly spliced mRNA is transported out of the nucleus. Recruitment and/or enhancement of interactions between spliceosomal and exosomal components might be dependent on the phosphorylation of Prp1 by Prp4 kinase as suggested above for the ATPases (Figure 6). In the mammalian system Prp4K kinase appears also to phosphorylate hPrp31 *in vitro* which is a direct interaction partner of hPrp1 [34]. Interestingly, mutations in the hPrp31 phosphorylation sites seem to decrease the biochemical interaction capacity of pre-catalytic

spliceosomes with the exosome [76]. Prp4 kinase of fission yeast does neither phosphorylate Prp31 *in vivo* nor *in vitro*. Two of the three mammalian phosphorylation sites are not conserved in the fission yeast homolog.

Collectively, we propose that phosphorylation of Prp1 determines the architecture of a platform for the proofreading and activation action of the ATPases at an assembled pre-catalytic spliceosome. At this level, the surveillance system is in close communication with the nuclear exosome and results in degradation of "aberrant" pre-mRNA at the transcriptional site (Fig. 6). All the questions which come up with our hypothesis can be experimentally approached and are currently under investigation. Further analysis will help to unravel in more detail the mechanism of this early surveillance checkpoint involved in controlling the expression of intron-containing genes.

Acknowledgements

We are grateful to Susanne Zock-Emmenthal, Daniela Eckert and Carolin Vogt for providing invaluable material and data for this chapter. Particularly, we want here to appreciate the input of Carolin Vogt who worked on the Srp2 project for her Masters Thesis (Technische Universität Braunschweig, 2011).

Author details

Martin Lützelberger and Norbert F. Käufer
Technische Universität Braunschweig, Institute of Genetics, Spielmannstr. 7, 38106 Braunschweig, Germany

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