Nineteen complex–related factor Prp45 is required for the early stages of cotranscriptional spliceosome assembly

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ABSTRACT

Splicing in *S. cerevisiae* has been shown to proceed cotranscriptionally, but the nature of the coupling remains a subject of debate. Here, we examine the effect of nineteen complex–related splicing factor Prp45 (a homolog of SNW1/SKIP) on cotranscriptional splicing. RNA-sequencing and RT-qPCR showed elevated pre-mRNA levels but only limited reduction of spliced mRNAs in cells expressing C-terminally truncated Prp45, Prp45(1–169). Assays with a series of reporters containing the *AMA1* intron with regulatable splicing confirmed decreased splicing efficiency and showed the leakage of unspliced RNAs in *prp45*(1–169) cells. We also measured pre-mRNA accumulation of the meiotic *MER2* gene, which depends on the expression of Mer1 factor for splicing. *prp45*(1–169) cells accumulated approximately threefold higher levels of *MER2* pre-mRNA than WT cells only when splicing was induced. To monitor cotranscriptional splicing, we determined the presence of early spliceosome assembly factors and snRNP complexes along the *ECM33* and *ACT1* genes. We found that *prp45*(1–169) hampered the cotranscriptional recruitment of U2 and, to a larger extent, U5 and NTC, while the U1 profile was unaffected. The recruitment of Prp45(1–169) was impaired similarly to U5 snRNP and NTC. Our results imply that Prp45 is required for timely formation of complex A, prior to stable physical association of U5/NTC with the emerging pre-mRNA substrate. We suggest that Prp45 facilitates conformational rearrangements and/or contacts that couple U1 snRNP-recognition to downstream assembly events.

Keywords: cotranscriptional splicing; spliceosome assembly; nineteen complex; Prp8; RES complex; chromatin immunoprecipitation

INTRODUCTION

Introns are removed from protein-coding transcripts based on the combination of splicing sequences and inputs from other complexes involved in gene expression, all of which need to be interpreted during the splicing process. The spliceosome in budding yeast consists of five conserved U-rich small nuclear RNAs (snRNAs) and almost 100 proteins; the overall architecture as well as most of the splicing factors is conserved in higher eukaryotes (Bessonov et al. 2008; Fabrizio et al. 2009). These snRNAs form the active site of the spliceosome and base pair with transcripts, directly contributing to intron recognition and splicing catalysis (Valadkhan and Jaladat 2010; Hang et al. 2015). Proteins have structural, enzymatic, and regulatory functions (Jurica and Moore 2003; Will and Lührmann 2011) and also support a dynamic interaction network which links splicing with all other stages of gene expression (Alexander and Beggs 2010; Bentley 2014). However, the functioning of this network

and its role in gene expression regulation are still far from understood.

The spliceosome assembles de novo on each intron in a stepwise fashion. Proper execution of the rearrangements required to reach the catalytically competent spliceosome depends on DExD/H ATPases, which couple ATP hydrolysis with structural alterations. Early in assembly, U1 snRNP binds the 5' splice site (5'ss), forming the commitment complex 1 (CC1) and is then joined by Msl5 (branchpoint binding protein; BBP), which recognizes the branch site (Ruby and Abelson 1988; Berglund et al. 1997). Subsequently, Msl5 and its heterodimer partner Mud2 are displaced in favor of U2 snRNP, after which U4/U6.U5 tri-snRNP is incorporated into the splicing complex. U1 snRNP is then exchanged for U6 and U5 snRNPs at 5'ss (see Wang and Burge 2008 and references therein). A complex of Prp19-associated factors (nineteen complex; NTC) together with “NTC-related” factors (Prp17, Prp45, and Prp46, among others) are

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FIGURE 1. Impairment of Prp45 function leads to pre-mRNA accumulation. (A–C) Transcriptome analysis of *prp45(1–169)* cells in comparison with WT in two biological replicates. (A) pre-mRNA is accumulated in the *prp45* mutant (left panel), while the mRNA levels of intron-containing genes are affected only to a limited extent (right panel). The bottom and top of the box represent the second and third quartiles, respectively; the line represents the median. Whiskers extend up to $1.5\times$ of the interquartile range. (B) The overall relative splicing efficiency is impaired in *prp45(1–169)* cells. However, this drop in relative splicing efficiency does not show any tendency with respect to the branch point (C) or 5' splice site sequence (D). Graphs C and D show results from one biological replicate. Ratios of mutant/WT splicing efficiencies are plotted. (E,F) RT-qPCR quantification of splicing of selected genes. Spliced (E) and unspliced (F) transcripts were quantified by RT-qPCR and normalized to *TOM22*. Data are expressed relative to WT levels. Error bars represent standard deviations (SD) of at least three biological experiments.

the defining intronic *cis*-acting features. There was no correlation between relative splicing efficiency in the mutant and branch point to 3' splice site distance, overall intron length, transcript abundance (transreads), or ORF length (data not shown). Apparently, longer pre-mRNA-mediated interactions of the

(pre)spliceosome with RNA polymerase II (RNA Pol II; on genes with long introns) or more RNA Pol II complexes (on highly expressed genes) were not able to compensate for the deficient assembly process. To verify the RNA-seq results, we measured the levels of unspliced precursors and spliced mRNAs of several endogenous genes by RT-qPCR. We confirmed a three- to sixfold accumulation of all pre-mRNAs tested in *prp45(1–169)* cells (Fig. 1E,F).

Our data set also contained transreads corresponding to unannotated alternatively spliced RNAs as reported previously (Volanakis et al. 2013; Kawashima et al. 2014; Schreiber et al. 2015). In agreement with the earlier reports, these isoforms represented a minor proportion of spliced RNAs of the particular gene (typically $<5\%$). We detected 60 such previously published alternative splice isoforms, 36 of which were less abundant in the *prp45* mutant in comparison to WT (0.5 – 0.1 -fold change; data not shown). It seems that these low-frequency alternative splicing events are more sensitive to *prp45(1–169)*.

Prp45(1–169) causes increased leakage of unspliced reporters

To monitor pre-mRNA accumulation under conditions of regulated splicing, we used a set of *AMA1-CUP1*-based reporter constructs expressed in *cup1 Δ* cells (Fig. 2A). *AMA1*, together with the *MER2/REC107*, *MER3/HFM1*, and *SPO22* genes, is spliced efficiently only in meiotic Mer1-expressing cells. Mer1 binds to the intron's splicing enhancer (Spingola and Ares 2000) and facilitates the recruitment of U1 snRNP, Mud2, and U2 snRNP to pre-mRNA (Spingola et al. 2004; Balzer and Henry 2008). In vegetative cells, the splicing of *AMA1* intron-containing reporters can be activated by ectopic expression of Mer1. The reporters provide complementary information: (i) The splicing reporter (SpR) yields functional Cup1 protein only when spliced; (ii) the export reporter (ExR) is translated to Cup1 only when unspliced; and (iii) the control ExR-AU reporter, which has a modified 5' splice site and cannot be spliced, yields Cup1 irrespective of changes in splicing. We also used modified

FIGURE 2. Splicing/export reporters indicate a defect before the first transesterification in *prp45(1-169)* cells. (A) Principle of assay using the AMA1 intron-splicing/export reporter substrates (Scherrer and Spingola 2006). AMA1 is a meiotic gene; its splicing in vegetative cells depends on the presence of Mer1, a first-step accessory factor. The reporters SpR and ExR contain STOP codons in different positions; their transcripts can be translated to functional proteins (CUP1 or β -galactosidase) only when spliced (SpR) or not spliced (ExR). The control ExR-AU reporter with mutated 5'ss (GU to AU) is not recognized by the spliceosome and serves as a control to exclude export-specific defects. (B) CUP1-based reporters suggest that *prp45(1-169)* cells are impaired before the first splicing step. Mid-log cells (*cup1Δ* background) expressing the indicated reporter plasmid in the absence or presence of Mer1 were harvested and spotted in eightfold dilution series on media containing increasing concentrations of copper ions. "EV" cells bear an empty vector. Compare the growth of SpR/ExR-expressing cells between *prp45(1-169)* and WT strains in the presence of Mer1 (reporter intron is recognized by the spliceosome) and in its absence. (C) ONPG assay. Mid-log cells expressing the indicated reporter plasmid in the absence or presence of Mer1 were harvested and β -galactosidase activity was measured by ONPG assay. Values were normalized to the activity measured for the ExR-AU reporter in the respective strains in the absence of Mer1. Error bars represent SD of at least three independent experiments.

reporters, which enable the CUP1 sequence to be replaced by the *lacZ* gene encoding β -galactosidase, to obtain an independent and quantifiable readout of the system (Fig. 2C).

The spot-tests shown in Figure 2B display the differences in reporter-conferred Cu²⁺-resistance between *prp45(1-169)* and WT cells in the presence or absence of Mer1. *prp45(1-169)* cells show impaired splicing in comparison to WT, which is documented in the complementary readouts of SpR and ExR reporters in Mer1-expressing cells. No differences between mutant and WT were observed in the ExR-AU expressing cells, ruling out unrelated splicing defects. The *lacZ* reporters confirmed the results of CUP1 reporters in *prp45(1-169)* cells (Fig. 2C). The comparison of the

prp45(1-169) mutant with cells that lack Mud2, a protein involved in the early stages of spliceosome assembly (Abovich et al. 1994), revealed a similar pattern of effects. In contrast, the deletion of the nuclear exosome component RRP6 had an opposite impact on the reporter readouts. The splicing-dependent accumulation of unspliced ExR in *prp45(1-169)* cells is in agreement with a defect preceding the first splicing step. A defect affecting only the second step, i.e., the accumulation of intron-exon 2 lariat, would not influence the ExR signal.

To support the data obtained on reporter genes by the measurement of an endogenous gene, we monitored the splicing of *MER2* by RT-qPCR. We compared *MER2* pre-

mRNA and mRNA levels in WT and *prp45(1–169)* in the absence or presence of Mer1. Splicing proceeded efficiently only in the presence of the splicing activator Mer1, as revealed by high mRNA levels in both strains (Fig. 3). However, the *MER2* pre-mRNA level dropped to about one half in the presence of Mer1 in WT cells, but remained unchanged in *prp45(1–169)* cells. We conclude that the unspliced *MER2* accumulated in *prp45(1–169)* cells, in comparison to WT, only under conditions when the splicing was active. These observations demonstrate that pre-mRNA accumulation in *prp45(1–169)* cells is associated with active splicing.

Truncation of Prp45 decreases its recruitment to intron-containing genes and disrupts cotranscriptional splicing

ChIP assays of tagged spliceosomal components have been successfully used to prove the cotranscriptional character of spliceosome recruitment and splicing in both yeast and mammalian systems (Görnemann et al. 2005; Tardiff and Rosbash 2006; Price et al. 2014). The ChIP signals along the gene can be interpreted as the “time” of the stable association of a particular splicing component (Kotovic et al. 2003; Görnemann et al. 2005). Such experiments as well as single-molecule studies also provide evidence in favor of the stepwise spliceosome assembly model (Hoskins et al. 2011; Carillo Oesterreich et al. 2016). To address the association of Prp45 with intron-containing genes, we prepared two strains expressing either C-terminally HA-tagged Prp45 or Prp45(1–169) and performed ChIP-qPCR analysis using an anti-HA antibody. We designed primer pairs to span *ECM33* and *ACT1* genes with PCR amplicons positioned along the genes (Fig. 4A,J). Prp45 was detected with a maximum signal in the middle of exon 2 (Fig. 4B,K). In contrast, the Prp45(1–169) signal was dramatically lower in all Prp45-positive amplicons, with the tendency to grow gradually toward the 3′ end of genes.

FIGURE 3. *prp45(1–169)* cells accumulate an excess of *MER2* pre-mRNA only when splicing is active. Mer1, a meiotic factor indispensable for *MER2* splicing, was ectopically expressed in vegetative cells. The levels of *MER2* mRNA (A) and pre-mRNA (B) were quantified by RT-qPCR. *prp45(1–169)* cells accumulated more *MER2* precursors than WT cells only when Mer1 was present (see pre-mRNA levels, B) and splicing was active (see mRNA levels, A). Error bars represent SD of three biological experiments.

We used the HA-tagged splicing factors Prp42, Msl5, Mud2, Msl1, Brr2, and Prp19 to monitor the presence of U1 snRNP, BBP, Mud2, U2 snRNP, U5 snRNP, and NTC, respectively, along the *ECM33* and *ACT1* genes. The profiles of U1 snRNP and Msl5/Mud2 showed maxima at the beginning of exon 2, which is in agreement with their roles in the early phases of spliceosome assembly (CC2). The profiles of U2 and U5 snRNPs peaked afterward. The levels of NTC were highest in the second half of exon 2. These data are in agreement with previously reported ChIP analyses (Görnemann et al. 2005; Lacadie and Rosbash 2005). *prp45(1–169)* mutant cells did not show any alterations of either U1 snRNP (Fig. 4D,M) or Mud2 recruitment (Fig. 4F,O). The profile of Msl5 was also similar to WT; we observed only a slightly higher signal from the middle of exon 2 in *prp45* mutants (Fig. 4E,N). In contrast, *prp45(1–169)* hampered the cotranscriptional recruitment of U2 snRNP (Fig. 4G,P), the U5 snRNP component Brr2 (Fig. 4H,Q), and the NTC member Prp19 (Fig. 4I,R). The profile of Prp19 resembled the behavior of Prp45 and showed a similar decrease of recruitment in *prp45(1–169)* cells (Fig. 4B,K). ChIP on *DBP2* gave principally the same data but of lower resolution, because of the short second exon of this gene (data not shown). We also measured RNA Pol II ChIP to complement the spliceosome recruitment data. RNA Pol II was evenly distributed along the genes with slightly lower signals in mutant cells; we did not observe any accumulation of the RNA Pol II signal on the tested intron-containing genes in the *prp45* mutant. Our ChIP results show that Prp45 affects U2 snRNP recruitment and subsequent spliceosome formation events, which supports the conclusion that this factor plays a role in the early stages of spliceosome assembly.

DISCUSSION

Extensive truncation of Prp45 has a mild splicing phenotype in yeast

The RNA-seq data analyzed in this paper document that the *prp45(1–169)* allele resulted in a seven- to eightfold increase of pre-mRNA levels, whereas most of the corresponding mRNAs were only subtly affected. When comparing the relative splicing efficiencies of genes with noncanonical splice sites against the changes induced by the *prp45* mutation, we did not observe any splice site sequence-specific effects (Fig. 1C,D). We examined pre-mRNA and mRNA levels of *AMA1*-based splicing and export reporters, which depend on Mer1 for 5′ss recognition and splicing. In these assays, which measure the proportion of spliced and leaked (unspliced) pre-mRNAs relative to WT, *prp45(1–169)* and *mud2Δ* mutants showed a similar defect, namely a decreased proportion of mRNA-rendered versus leaked pre-mRNA molecules (Fig. 2C). In a separate experiment, we analyzed the pre-mRNA and mRNA levels of the endogenous *MER2*

FIGURE 4. *prp45(1–169)* delays cotranscriptional spliceosome assembly. ChIP-qPCR analysis of cotranscriptional recruitment of spliceosomal components was performed on the *ECM33* and *ACT1* genes (A,J; positions of PCR amplicons are indicated). Cells expressing HA-tagged Prp45 or Prp45 (1–169) were used for ChIP-qPCR with the anti-HA antibody. Signals were normalized to input values and expressed as the fold enrichment relative to the signal from exon 1 in the Prp45-HA strain. The strain without the tagged protein served as a negative control (B,K). RNA Pol II ChIP signals were obtained using the anti-Rpb3 antibody. Cotranscriptional spliceosome assembly was monitored via the recruitment of HA-tagged components of commitment complex 1 using HA-tagged Prp42 (D,M), Msl5 (E,N), and Mud2 (F,O). Subsequent stages of spliceosome formation were followed using HA-tagged members of U2 snRNP (Msl1; G,P), U5 snRNP (Brr2; H,Q), and NTC (Prp19; I,R). Signals were normalized to input values and expressed as the fold enrichment relative to the signal obtained from the telomeric region (TELVIR; graphs C–I and L–R). Error bars represent SD from at least four independent experiments.

gene. *prp45(1–169)* cells accumulated a relative excess of pre-mRNA only when splicing was active (Fig. 3B).

The phenotypes of mutant *PRP45*, i.e., elevated pre-mRNA levels, decreased splicing efficiency of first-step limiting substrates (Gahura et al. 2009), and impaired nuclear retention of pre-mRNA, are similar to factors involved in the early stages of prespliceosome formation, such as cap-binding complex (CBC) or Mud2 (Colot et al. 1996; Rain and

Legrain 1997). *MSL5* (BBP) mutants also affect the splicing of nonconsensus introns and cause leakage of pre-mRNA into the cytoplasm, albeit to a much larger extent than *prp45(1–169)* (Rutz and Séraphin 2000). The early role of the NTC-related component *PRP45* is also supported by the findings of yeast two-hybrid interactions between Snw1, the Prp45/SKIP ortholog in *S. pombe*, and U2AF23, as well as human SKIP and both U2AF65/35 subunits (the

major interaction being with U2AF65) (Ambrozková et al. 2001; Chen et al. 2011). The U2AF65-related protein of yeast, Mud2, has been found to interact with the NTC component Clf1 (Chung et al. 1999). Intriguingly, none of the MS analyses of purified staged spliceosomal complexes have presented an assembly where the U2AF subunits and the NTC-related component Prp45/SKIP were found together (Jurica et al. 2002; Makarov et al. 2002; Ohi et al. 2002; Fabrizio et al. 2009).

The early effects of Prp45 on the association of U2 snRNP with pre-mRNA

We used HA-tagged versions of yeast splicing factors and Prp45 to gain information on the impact of its truncation on cotranscriptional splicing. Prp45(1–169) severely delayed cotranscriptional recruitment of a subset of splicing subcomplexes to *ACT1* and *ECM33* pre-mRNAs. Because catalysis can occur after the stable association of tri-snRNP and NTC, our results suggest that splicing in *prp45*(1–169) cells proceeds only after RNA Pol II reaches the end of the gene. The transcriptome analysis [performed with poly(A)-enriched RNA] shows that splicing is eventually accomplished, albeit post-transcriptionally and with lower efficiency (higher steady-state levels of pre-mRNA). Notably, the *ACT1* gene, where we observed the delay, shows no second-step splicing defect in *prp45*(1–169) cells using *ACT1* reporter-based primer extension assays (Gahura et al. 2009).

Composition analyses of purified splicing complexes using MS indicated that Prp45 was present as part of the NTC-containing spliceosome (i.e., B complex and later stages) (Jurica et al. 2002; Makarov et al. 2002), but not at earlier points of the splicing cycle (CC2 or A complex) (Hartmuth et al. 2002). However, the truncation of Prp45 affected the spliceosome before tri-snRNP, and NTC are observed to be stably associated with pre-mRNA. The purification schemes used in these MS studies may have precluded the isolation of transiently associating components, including highly flexible Prp45. For example, recent in vitro analysis of human spliceosomes stalled because a mutation in Prp28/DHX8 identified a complex that contained U1, U2, and loosely associated tri-snRNP (called pre-B complex) (Boesler et al. 2016). This particle, which apparently precedes the formation of B complex, evaded identification previously.

Early effects on assembly, i.e., effects preceding the stage when a protein becomes detectable as part of the (pre) spliceosome, have been reported for Prp8, Prp28 (Li et al. 2013; Price et al. 2014), as well as the NTC component Clf1 (Chung et al. 2002; Wang et al. 2003), among others. The mutant form of U5 snRNP-associated DEAD-box ATPase Prp28, Prp28-1, hampers the formation of CC2 complex and subsequent U2 recruitment. Prp28-1 also leads to the delayed dissociation of U1. Importantly, mutations in the bromodomain-like domain of Prp8 are able to suppress both these effects of Prp28-1 (Price et al. 2014). These results

argue that Prp8 must gain access to Prp28 during its ATP-independent role in CC2 formation and/or to U1 snRNP (Li et al. 2013). U5 and Prp8 have been repeatedly found cross-linked to 5' ss during the early phase of splicing, both in yeast (Newman et al. 1995) and in Metazoa (Wyatt et al. 1992; Ast and Weiner 1997). The interaction of Prp8 with 5' ss requires U4 and U6, which suggests that the interacting entity is in the form of tri-snRNP (Maroney et al. 2000). The defect in the cotranscriptional recruitment of U2 snRNP caused by Prp45 truncation bears resemblance to the early effects of Prp8 and Prp28. Intriguingly, Prp45 is found in many contacts with Prp8 (see below).

The dissociation of U1 is uncoupled from downstream binding events in *prp45*(1–169) cells

The profile of U1 was identical for WT and mutant, while the stable association of U2, U5, and NTC was delayed in *prp45*(1–169) cells (U2<U5<NTC). This implies that U1 displacement is not coordinated with tri-snRNP binding (U6–5' ss pairing) (Lacadie and Rosbash 2005; Tardiff and Rosbash 2006). Information in the literature suggests that U1 recruitment and dissociation are dependent on many factors, including later-acting ones, such as Prp28 or Prp8 (Price et al. 2014). In cotranscriptional assays, the interaction of U1 with pre-mRNA was extended toward the 3' end of exon 2 in deletion mutants of the cap-binding complex (CBC) (Görnemann et al. 2005) or after U2 snRNA depletion (Tardiff and Rosbash 2006). In contrast, the dissociation of Msl5/Mud2 has not been reported to be directly coupled to U2 binding. Temperature-sensitive mutations of Msl5, which block the formation of CC2 in vitro at nonpermissive temperatures, do not affect prespliceosome assembly (U2 association) (Rutz and Séraphin 1999, 2000). The observed lack of U1 stabilization on exon 2 in the context of reduced U2 binding in *prp45*(1–169) cells suggests that Prp45 contributes to the coupling of U1 dissociation to subsequent binding events.

U1 snRNP occupancy may decrease too early for the delayed U2/tri-snRNP to bind efficiently. Splicing can be accomplished without U1 only in genes with specific *cis* elements (Crispino et al. 1996). Overexpression of SR proteins can compensate for the depletion of U1 in HeLa cells (Crispino et al. 1994). Intriguingly, the organism that lacks the Prp45 homolog, *Cyanidioschyzon merolae*, has a reduced splicing system without U1 snRNP, NTC, and RES proteins (see also below; Stark et al. 2015). Splicing in this reduced setting probably proceeds through the formation of the U2/tri-snRNP complex without the U1 recognition event.

Truncated Prp45 lacks parts that contact U2 components

Prp45 is an essential protein and its extensive truncation, which lacks the conserved SNWKN motif as well as the C-terminal 210 amino acids of the protein, is compatible with

growth (Gahura et al. 2009). According to available spliceosome structures of *S. cerevisiae*, *S. pombe*, and *H. sapiens* (Yan et al. 2015, 2016; Bertram et al. 2017), Prp45/Snw1/SKIP interacts with a number of proteins across the spliceosome, with contacts to Prp8 occurring along the entire length of Prp45/Snw1/SKIP (Fig. 5A). The NTD domain of Prp8 is bound through the conserved DPL and SNWKN motifs of Prp45 (amino acids 120–122 and 170–174, respectively), and the RT domain is contacted by the C-terminal helix of Prp45. The structure of the *S. cerevisiae* B^{act} complex (unlike other available spliceosome structures) also contains structural data for part of the C terminus of Prp45 (amino acids 303–350). In the B^{act} spliceosome, amino acids 303–350 of Prp45 contact with the linker domain and the RH domain of Prp8 (the amino acids 303–320 and 329–350 of Prp45, respectively). Interestingly, the RH domain changes its position with respect to other domains of Prp8 during B^{act}–C–C* transitions (Bertram et al. 2017). The contacts and position

of the C terminus of Prp45 (amino acids 329–350) stabilized by the RH domain are thus likely to be stage-specific.

With respect to other proteins, Prp45(1–169) extensively contacts Prp46 (wrapping around its globular fold) and Slr11/Ecm2. In addition, contacts also occur with Cef1, Clf1, Cwc15, and Syf2, all of which are NTC components. The portion of Prp45 that is ablated (amino acids 170–379) has closer interactions with Prp8, Syf2, and Cef1, and, in addition, contacts the SF3b scaffold Hsh155 and the RES proteins Ist3, Cwc26/Bud13, and Pml1 (see Yan et al. 2017). Truncated Prp45 ends only N-terminally at the highly conserved SNWKN motif (amino acids 170–174), thus lacking a contact which is present in both *S. cerevisiae* and *S. pombe* structures between this motif and the U-rich loop of U2 snRNA of the B^{act} complex (Yan et al. 2015, 2016). Remarkably, despite the loss of this interaction, the ablation of the SNWKN motif is compatible with splicing. The more extensive C-terminal truncation of *prp45*(1–131) is viable,

but the cells are already severely growth-impaired (K Abrahámová, unpubl.). The contacts of the Prp45 chain between amino acids 131 and 169 include Prp46, Cef1, and Clf1. N-terminal truncations (implicating the lack of Prp46-binding interface parts) are also viable, provided, however, that the C-terminal part is retained (Martínková et al. 2002; Albers et al. 2003).

Because of its intrinsically disordered character and nonglobular nature, it is likely that Prp45 acts in a complex with its partners, not as a free protein. The truncated form may exist in a complex with Prp8 and NTC, similar to the WT protein. However, based on the structural information from the B^{act} complex, the mutant should be deficient in mediating contacts or regulatory inputs to U2 snRNP and the RES complex (Fig. 5A).

After assembly, Prp45 is present in the spliceosome through the whole splicing cycle (Albers et al. 2003). Using chemical crosslinking, Prp45 has been found to be in the vicinity of intronic RNA in the C complex, where it overlaps the contacts of the RES complex subunit: Snu17, Hsh155, and Prp8 (Schneider et al. 2015). In the human C* complex, the C-terminal helix of SKIP contacts with Prp22 helicase (DHX8) (Bertram et al. 2017). Accordingly, biochemical evidence implicates Prp45 in the recruitment of Prp22 (Gahura et al. 2009). SNW1/SKIP thus not only spans the whole spliceosomal core, but also

FIGURE 5. Truncation of Prp45 opens a gap between U1 dissociation and U2/U5 snRNP recruitment. (A) Scheme of Prp45 conformation and contacts within the spliceosome. The ribbon diagram combines information from the cryo-EM structure of the *S. cerevisiae* B^{act} (5GM6) and human C* (5MQF) complexes (Yan et al. 2016; Bertram et al. 2017). Amino acids missing in the Prp45(1–169) mutant are labeled red (i.e., 170–379). Regions of close contact for Prp45 and proteins/RNA (i.e., encompassing <4 Å intermolecular distances) are indicated by gray ovals. (B) Scheme summarizing the effect of the *prp45*(1–169) allele on cotranscriptional splicing. The amplicons used for ChIP analysis are indicated by lines under the gene diagram.

connects it with effector enzymes positioned at the periphery—with Prp22 helicase via its C-terminal helix (Bertram et al. 2017) and PPIL1 isomerase through its N terminus (Skrůžný et al. 2001; Xu et al. 2006).

Post-transcriptional splicing is compatible with growth in *S. cerevisiae*

Data from single-molecule intron-tracking experiments document that the speeds of splicing and transcription are tuned with respect to each other, so that splicing occurs in close vicinity of the RNA Pol II holocomplex (Carillo Oesterreich et al. 2016). This physical proximity may be important for the availability of some splicing factors/regulators. Increasing the distance between the two complexes also changes the circumstances, which are important for splice site choice. For example, more alternative splice sites or regulatory *cis* sequences can compete (as they emerge from the RNA Pol II exit channel) (Carillo Oesterreich et al. 2016 and references therein). Truncation of Prp45 hinders spliceosome assembly so that splicing can no longer keep pace with transcribing RNA Pol II. Nevertheless, the defect is still compatible with viability at 30°C. While *S. cerevisiae* cells tolerate extensive truncations of Prp45, *S. pombe* cells do not [Snw1(1–280) does not complement SNW1 deletion; data not shown]. It is possible that *S. pombe* is more dependent on the coordination of transcription and splicing, as it has less conserved splicing sequences, more alternative splice sites, and more Metazoan-like organization of its intronome (Wood et al. 2002; Kuhn and Käufer 2003; Carillo Oesterreich et al. 2016).

We conclude that the C-terminal part of Prp45 is important for both U2 and tri-snRNP association kinetics, perhaps enabling them to attain an association-competent state (Fig. 5B). These results thus add Prp45 to the list of spliceosomal components that have been shown to affect spliceosome assembly “from afar,” even though not yet incorporated into the prespliceosome–pre-mRNA complex (Price et al. 2014). This suggests that the interactions that must be considered in order to better explain splice site recognition and spliceosome assembly encompass not only the firmly bound snRNP particles but also the surrounding compartment with transiently interacting components.

MATERIALS AND METHODS

Yeast strains, plasmids, and media

Yeast strains are listed in Table 1. The strains were grown in synthetic (SD) or YPAD media. The *PRP45* truncation was generated using an integration cassette obtained by PCR with pFA6-3HA-NatMX6 (Van Driessche et al. 2005) or pFA6-3HA-kanMX6 (Longtine et al. 1998) as a template, or by replacing the *PRP45* coding sequence for amino acids 170–379 using the *delitto perfetto* method (Storici and Resnick 2006).

Plasmids R1070 (Mer1-expressing plasmid), R1130 (empty vector), SpR-AMA1-CUP1, ExR-AMA1-CUP1, ExR-AU-AMA1-CUP1, SpR-AMA1-lacZ, and ExR-AMA1-lacZ were generously gifted to us by Marc Spingola (Engbrecht et al. 1991; Scherrer and Spingola 2006). Plasmid ExR-AU-AMA1-lacZ was prepared from ExR-AU-AMA1-CUP1 by replacing the *CUP1* gene using the KpnI fragment encoding *lacZ*.

TABLE 1. Strains used in this study

Strain	Genotype	Reference
EGY48	<i>MATα ura3 his3 trp1 LexA(6xop)-LEU2</i>	Golemis et al. 1998
KAY02	<i>MATα ura3 his3 trp1 LexA(6xop)-LEU2, prp45(1–169)-3HA::KanMX6</i>	Gahura et al. 2009
BY4741	<i>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Euroscarf
AVY17	<i>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 prp45(1–169)-3HA::NatMX6</i>	This study
46ΔCUP	<i>MATα ade2 cup1Δ::URA3 his3 leu2 lys2 trp1 ura3 GAL+</i>	Lesser and Guthrie 1993
MHY04	<i>MATα prp45(1–169)-HA::KanMX6 ade2 cup1Δ::URA3 his3 leu2 lys2 trp1 ura3 GAL+</i>	This study
<i>mud2Δ</i>	<i>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YKL074c::KanMX4</i>	Euroscarf
<i>rrp6Δ</i>	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YOR001w::KanMX4</i>	Euroscarf
Prp42-HA	<i>MATα ura3 his3-11 leu2-3,112 trp1-1 can1-100 prp42::prp42HA/KanMX6 rpo21::rpo21MYC/HIS</i>	Kotovic et al. 2003
KAY21	<i>MATα ura3 his3-11 leu2-3,112 trp1-1 can1-100 prp42::prp42HA/KanMX6 rpo21::rpo21MYC/HIS prp45(1–169)::NatMX6</i>	This study
Msl5-HA	<i>MATα ura3 his3-11 leu2-3,112 trp1-1 can1-100 MSL5-HA/TRP</i>	Görnemann et al. 2005
KAY22	<i>MATα ura3 his3-11 leu2-3,112 trp1-1 can1-100 MSL5-HA/TRP prp45(1–169)::NatMX6</i>	This study
Mud2-HA	<i>MATα ura3 his3-11 leu2-3,112 trp1-1 can1-100 MUD2-HA/TRP</i>	Görnemann et al. 2005
KAY23	<i>MATα ura3 his3-11 leu2-3,112 trp1-1 can1-100 MUD2-HA/TRP prp45(1–169)::NatMX6</i>	This study
Msl1-HA	<i>MATα ura3 his3-11 leu2-3,112 trp1-1 can1-100 MSL1-HA/TRP</i>	Görnemann et al. 2005
KAY24	<i>MATα ura3 his3-11 leu2-3,112 trp1-1 can1-100 MSL1-HA/TRP prp45(1–169)::NatMX6</i>	This study
Brr2-HA	<i>MATα ura3 his3-11 leu2-3,112 trp1-1 can1-100 BRR2-HA/TRP DBP2-GFP/Kanr (JG12)</i>	Görnemann et al. 2005
MHY15	<i>MATα ura3 his3-11 leu2-3,112 trp1-1 can1-100 BRR2-HA/TRP DBP2-GFP/Kanr (JG12) prp45(1–169)</i>	This study
Prp19-HA	<i>MATα ura3 his3-11 leu2-3,112 trp1-1 can1-100 PRP19-HA DBP2-GFP (JG12)</i>	Görnemann et al. 2005
MHY16	<i>MATα ura3 his3-11 leu2-3,112 trp1-1 can1-100 PRP19-HA DBP2-GFP (JG12) prp45(1–169)</i>	This study

Nucleic acid extraction for transcriptome analysis

Yeast cultures were grown at 30°C in a YPAD medium. Cells (8 mL) were harvested by centrifugation (1000g, 3 min, room temperature) during the exponential growth phase. Cell pellets were stored at –80°C. The pellets were thawed on ice and then 400 µL of extraction buffer (0.1 M Tris–HCl pH 7.5, 1 mM EDTA, 0.1 M LiCl, 1% SDS), 400 µL glass beads, and 400 µL of acid phenol (pH 4.3):chloroform:isoamyl alcohol (25:24:1) solution were added. Cells were disrupted using FastPrep-24 (MP Biomedicals), lysates were spun, and the total RNA was precipitated from the aqueous layer using 96% EtOH. One hundred and fifty micrograms of RNA was taken and adjusted with DEPC-treated water to 175 µL of the total volume. To remove DNA, 20 µL of the DNase buffer and 5 µL of DNase from a MasterPure Yeast RNA Purification Kit (Epicentre Biotechnologies) were added; further steps were performed according to the manufacturer's instructions. After rinsing in 70% EtOH, 500 µL of 70% EtOH was added to the RNA pellets and stored at –20°C.

Transcriptome sequencing

Poly(A)-enrichment and strand-specific RNA-seq library preparation and sequencing (Illumina, 100 nt, paired-end) were carried out by BGI, Hong Kong. The sequencing data are available from the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-5149.

RNA-seq data analysis

The quality of sequencing reads was verified with fastQC 0.11.4 (www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads were aligned to the *S. cerevisiae* genome r64 using HISAT 2.0.3-beta (Kim et al. 2015). Nonuniquely mapping reads (MAPQ < 10) were filtered out using SAMtools 1.3.1 (Li et al. 2009). Analysis of differential gene expression was performed using R (www.r-project.org/) and the Bioconductor package DESeq2 (Love et al. 2014; Huber et al. 2015).

Splicing efficiency for each intron was calculated as described previously (Převorovský et al. 2016). Briefly, transreads (reads spanning exon–exon junctions, reflecting the abundance of spliced mRNA) were extracted using regtools 0.2.0 (<https://regtools.readthedocs.io>) and quantified for each junction. The number of reads spanning the most 5'-terminal base of each intron (reflecting unspliced pre-mRNA abundance) was determined using BEDTools 2.25.0 (Quinlan 2014). Splicing efficiency (Fig. 1B) was then calculated as the ratio of transreads (mRNA) to intron-end reads (pre-mRNA).

In the comparisons of pre-mRNA and mRNA abundance of intron-containing genes (Fig. 1A), sequencing read counts were first normalized to account for different sequencing library sizes (i.e., sample sequencing depth). To this end, a group of control intronless genes was selected, which showed unchanged expression between WT and the *prp45*(1–169) mutant according to DESeq2 analyses ($n = 178$ control genes). The median of the mutant/WT mRNA abundance ratios of these control genes was then used to normalize the respective read counts from the mutant to the WT in each biological replicate. Relative pre-mRNA and mRNA abundance for the WT and mutant were then analyzed by comparing the normalized 5' intron-end read counts and transread counts, respectively.

RT-qPCR

Total RNA was extracted using the MasterPure Yeast RNA Purification Kit (Epicentre Biotechnologies) according to the manufacturer's protocol. Complementary DNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific), random hexamer primers, and 2 µg of RNA as a template. qPCR was performed on a LightCycler 480 II (Roche). Each reaction (total volume of 12.5 µL) consisted of 6.25 µL of MESA GREEN qPCR MasterMix Plus for SYBR Assay, no ROX (Eurogentec), 0.3 mM primers, and 0.05 µL of cDNA. Primer sequences are summarized in Table 2. Each sample was run in triplicate. Results were calculated either using the $\Delta\Delta C_t$ method (Livak and Schmittgen 2001) or using a calibration curve calculated from standards included in each run.

ONPG assay

Cells were cultivated to the middle exponential phase (corresponding to OD 0.5–0.8) in the appropriate media and the equivalent of 4 OD was harvested by centrifugation. The cell pellet was washed with Z-buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄ [pH 7.0], 10 mM KCl, 1 mM MgSO₄) and resuspended in the Z-buffer to a final volume of 1 mL. Samples were transferred on ice and 3.4 µL of β -mercaptoethanol was added. The cell suspension (300 µL) was mixed with glass beads and lysed using FastPrep-24 (MP Biomedicals; speed 6; four cycles; cycle duration 15 sec; 5 min on ice between cycles). After lysis, 300 µL of Z-buffer was added and samples were mixed by vortexing. Samples (250 µL each) were incubated with 350 µL of Z-buffer and 80 µL of freshly made ortho-nitrophenyl- β -galactoside (ONPG) solution (4 mg/mL) in Z-buffer at 30°C for 60 min in a shaker. The reaction was then stopped by adding 250 µL of 1 M Na₂CO₃; samples were centrifuged, and supernatants collected. Absorbance was measured at 420 and 550 nm using the spectrophotometer UV Mini 1240 (Shimadzu). Miller units of activity were calculated using the formula $MU = 1000 \times [(A_{420} - 1.75 \times A_{550}) / (T \times V \times OD_{600})]$, where OD₆₀₀ is the optical density of the cell suspension after centrifugation, washing and volume adjustment to 1 mL, T is the incubation time in minutes, and V is the volume of the cell suspension used for the enzymatic reaction in milliliters.

Chromatin immunoprecipitation

Cells were grown in 50 mL YPAD or SD media to OD 0.6 and fixed by shaking for 30 min in 1% formaldehyde (Sigma-Aldrich; 1% solution in medium) at room temperature. Formaldehyde was quenched with 0.125 M glycine for 10 min. Cells were harvested by centrifugation and washed with deionized water; the cell pellet was stored at –80°C. The pellet was thawed in 270 µL of LB buffer (50 mM HEPES pH 7.6, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1 mM Na-DCC, protease inhibitor cocktail [Serva]), mixed with glass beads and lysed using FastPrep-24 (MP Biomedicals; speed 5.5; three cycles; cycle duration 13 sec; 5 min on ice between cycles). The disrupted cells were collected by centrifugation; the pellet was washed with 600 µL of LB buffer and resuspended in 270 µL of the same buffer. Chromatin was fragmented to ~500 bp by sonication (Bioruptor sonicator, Diagenode; “high” settings; 15 cycles; 30 sec on followed by 30 sec off; 2°C water bath). Samples were centrifuged and the supernatant collected. Protein concentration was

TABLE 2. Primers used in this study

Primer ID	Primer sequence
ECM33-mRNA-F	CTCCGCTCTAGCTGCTAAC
ECM33-mRNA-R	ACCGGAGATTTTGTCCAAATCAG
ECM33-pre-mRNA-F	CTGTCATAGGATTAGGGCGAG
ECM33-pre-mRNA-R	GAAGTAGTTGAGTTAGCTGCTTAG
ACT1-mRNA-F	AATTTACTGAATTAACAATGGATTCTGAGG
ACT1-mRNA-R	GATAGATGGGAAGACAGCACG
ACT1-pre-mRNA-F	AATTTACTGAATTAACAATGGATTCTGGTA
ACT1-pre-mRNA-R	AGGTTATGGGAGAGTGAAAAATAGTA
TUB1-mRNA-F	AGAGAAGTTATTAGTATTAATGTC
TUB1-mRNA-R	TCTCTAGATGTCCATCC
TUB1-pre-mRNA-F	AAACAATGAGAGAAGTTATTAGTATTAATGGTA
TUB1-pre-mRNA-R	TTGCGTTGAAGTTAGTAATGAAAGATG
TUB3-mRNA-F	GACAATGAGAGAGGTCATTAGTATTAATGTTG
TUB3-mRNA-R	CCTCCAAATGGCCGTCTTC
TUB3-pre-mRNA-F	GACAATGAGAGAGGTCATTAGTATTAATGGTA
TUB3-pre-mRNA-R	GAATGAATCAAACAGATCAGATCAAAC
APS3-mRNA-F	GTTACACAATGATTCATGCAGTTCTAATATTC
APS3-mRNA-R	AATTCATATACTTGCTCTAATAGCAGTTTTTG
APS3-pre-mRNA-F	GTTACACAATGATTCATGCAGTTCTAATATGT
APS3-pre-mRNA-R	GGTTGGCATTCTTATTGACTATACCAAA
RPO26-mRNA-F	CAGACTACGAGGAGGCGTTTA
RPO26-mRNA-R	GTTTTCTCATAAGTCTCCTCATCAG
RPO26-pre-mRNA-F	AGACTACGAGGAGGCGTATG
RPO26-pre-mRNA-R	GTCGTAAACCTGTATACAATGATAGAAG
IMD4-mRNA-F	TCTGGCTTCCCAGTTACTGAA
IMD4-mRNA-R	AAGAATCATCTTCTAAGAATTGTATATCACGA
IMD4-pre-mRNA-F	CTTTCGTTCTACTGACTG
IMD4-pre-mRNA-R	CACACATAATGGGCTTTC
COF1-mRNA-F	CTAACAAAAGAAGATGTCTAGATCTGGTG
COF1-mRNA-R	GGTTTTAGCATCGTTCAATCCG
COF1-pre-mRNA-F	ACAAAAGAAGATGTCTAGATCTGGGTA
COF1-pre-mRNA-R	GTTCCATGTTAAAAAGGGAGGAAAGA
TOM22-F	CGACGTTGTTCGATGATGAAG
TOM22-R	GCAACGATTCTGTCCAAC
MER2-mRNA-F	ATGCTGCTACGAACGGAAAC
MER2-mRNA-R	TTCTCGTCGCCTTTCGATAAC
MER2-pre-mRNA-F	GATGCTGCTACGAACGGTT
MER2-pre-mRNA-R	TCACGTTTCTATACTACAGTTGTTAG
ECM33-A-F	TTTCGGTAGCGTCTTGAG
ECM33-A-R	GCAAGAGAGGTCCGTTGAT
ECM33-B-F	ATTCAAGAACGCTTTGACTGCT
ECM33-B-R	TCGAGATTTGTGAGGAAAGAGG
ECM33-C-F	GTACTTCTGCCACTGCTACTG
ECM33-C-R	AAGATAGTCAAGGAACCATCAATCTC
ECM33-D-F	TTGGAAAGTGTCTGAAGGTTTCTC
ECM33-D-R	GTAGTGTTGTACCGTTGGAAG
ECM33-E-F	CGACTCTTCTCCAGTAACCTCTC
ECM33-E-R	AGATGTGGCACCGTTCTTG
ACT1-A-F	TACATCAGCTTTTAGATTTTTCACG
ACT1-A-R	GCTAGAACATACCAGAATCCATTG
ACT1-B-F	AACGGTTCTGGTATGTGTAAAGC
ACT1-B-R	ACCGACGATAGATGGGAAGAC
ACT1-C-F	GATGGTGTTACTACGTCGTTT
ACT1-C-R	GTAGTCAGTCAAATCTCTACCGGC
ACT1-D-F	GAAGGTCAAGATCATTGCTCCTC
ACT1-D-R	GAAACACTTGTGGTGAACGATAG
TELVR-F ^a	AAATGCGCAAGGGTAAAAACAG
TELVR-R ^a	TCCGATCACTACACACGGAAAT

^aWyce et al. 2007.

measured by UV absorption at 280 nm using a Nanodrop 2000 (Thermo Scientific) and adjusted to the same level with the LB buffer. Samples were cleared for 1 h with 20 μ L of protein A sepharose (GE Healthcare; blocked with 0.5 mg/mL BSA). An aliquot of the sonicated sample was saved as an input; 240 μ L was mixed with 10 μ L of an anti-HA (HA.11, Covance) or anti-Rbp3 (1Y26 [1Y27], Santa Cruz Biotechnology) antibody. A 1-h preincubation was followed by a 3-h incubation with 40 μ L of protein A sepharose. All sepharose incubations were carried out on a rotator in a cold room. Sepharose beads were washed six times with IP buffer (50 mM Tris-HCl pH 7.5, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40). For decrosslinking and DNA isolation, 100 μ L of 10% Chelex (Bio-Rad) was added to the beads, mixed by vortexing, and incubated for 10 min at 95°C. Samples were then cooled down and incubated with 1 μ L of proteinase K (20 mg/mL) for 30 min at 55°C on a shaker. Proteinase K was inactivated by incubating for 10 min at 95°C. Samples were centrifuged and the DNA containing the supernatant was collected. The slurry was washed once again with 60 μ L of deionized water, centrifuged, and the supernatants pooled. Input DNA was isolated using the same protocol. qPCR analysis was performed as described above with 20 ng of DNA as a template. Primer sequences are summarized in Table 2.

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