

Control of 3' splice site selection by the yeast splicing factor Fyv6

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Katherine A Senn, Karli A Lipinski, Natalie J Zeps, Amory F Griffin, Max E Wilkinson , Aaron A Hoskins 

Department of Biochemistry, University of Wisconsin-Madison, Madison, USA • Department of Chemistry, University of Wisconsin-Madison, Madison, USA • MRC Laboratory of Molecular Biology, Cambridge, UK

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This **important** study addresses how 3' splice site choice is modulated by the conserved spliceosome-associated protein Fyv6. The authors provide **compelling** evidence that Fyv6 functions to enable selection of 3' splice sites distal to a branch point and in doing so antagonizes more proximal, suboptimal 3' splice sites.

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Abstract

Pre-mRNA splicing is catalyzed in two steps: 5' splice site (SS) cleavage and exon ligation. A number of proteins transiently associate with spliceosomes to specifically impact these steps (1st and 2nd step factors). We recently identified Fyv6 (FAM192A in humans) as a 2nd step factor in *S. cerevisiae*; however, we did not determine how widespread Fyv6's impact is on the transcriptome. To answer this question, we have used RNA-seq to analyze changes in splicing. These results show that loss of Fyv6 results in activation of non-consensus, branch point (BP) proximal 3' SS transcriptome-wide. To identify the molecular basis of these observations, we determined a high-resolution cryo-EM structure of a yeast product complex spliceosome containing Fyv6 at 2.3 Å. The structure reveals that Fyv6 is the only 2nd step factor that contacts the Prp22 ATPase and that Fyv6 binding is mutually exclusive with that of the 1st step factor Yju2. We then use this structure to dissect Fyv6 functional domains and interpret results of a genetic screen for *fyv61* suppressor mutations. The combined transcriptomic, structural, and genetic studies allow us to propose a model in which Yju2/Fyv6 exchange facilitates exon ligation and Fyv6 promotes usage of consensus, BP distal 3' SS.

Introduction

Precursor messenger RNA (pre-mRNA) splicing is catalyzed by a large macromolecular complex called the spliceosome. Spliceosomes are composed of 5 small nuclear ribonucleoproteins (snRNPs), each composed of a small nuclear RNA (snRNA; U1, U2, U4, U5, and U6) and several different protein splicing factors. The snRNPs and dozens of other proteins assemble *de novo* at

each intron to form spliceosomes. Spliceosomes are highly dynamic and form different complexes as proteins and snRNAs join and leave. Many of these complexes have been characterized biochemically, genetically, and by cryo-electron microscopy (cryo-EM) (Plaschka et al., 2019). The splicing reaction itself is carried out in two sequential transesterification reactions (Figure 1A). First, the 5' splice site (5'SS) is cleaved during formation of an intron lariat (1st step). Second, the intron lariat is removed simultaneously with exon ligation at the 3'SS (2nd step).

The integrity of the genetic information contained within a pre-mRNA depends on correct identification of the 5' and 3'SS by the splicing machinery since a single nucleotide shift in either site could destroy a protein reading frame. In yeast, several DExD/H-box ATPases function to limit usage of suboptimal SS by enhancing splicing fidelity (Chung et al., 2023; Semlow and Staley, 2012). Two of these ATPases, Prp16 and Prp22, impact the fidelity of the 1st and 2nd catalytic steps, respectively. In addition to these roles, Prp16 is also required for remodeling of the spliceosome to permit the 1st-to-2nd step transition (Schwer and Guthrie, 1992), and Prp22 is essential for releasing the mRNA product (Company et al., 1991). While Prp16 does not need to be present during the 1st step (Chung et al., 2023), Prp22 is required to be present for exon ligation if the branch point (BP) to 3'SS distance is ≥ 21 nucleotides (nt) (Schwer and Gross, 1998). Despite many biochemical, single molecule, and structural studies, how spliceosomes promote usage of BP distal, Prp22-dependent 3'SS has remained elusive.

Identification of the correct 3'SS is an especially challenging problem given that the consensus sequence in *Saccharomyces cerevisiae* (yeast) and humans is just three nucleotides (YAG, Y=U or C). Cryo-EM structures of spliceosome product (P) complexes have revealed how these nucleotides can be recognized within the spliceosome active site (Bai et al., 2017; Fica et al., 2019; S. Liu et al., 2017; Wilkinson et al., 2017; Zhang et al., 2019). The AG 3'SS dinucleotide is recognized by formation of non-Watson Crick base-pairing interactions with the 5'SS +1G and BP adenosine, while the pyrimidine at the -3 position of the 3'SS is recognized by the Prp8 protein. Given this short consensus sequence, multiple different splicing factors also help to ensure that the correct 3'SS is utilized, including Cwc21 and the 2nd step factors Slu7, Prp18, and Prp22 (Crotti et al., 2007; Frank and Guthrie, 1992; Gautam et al., 2015; Kawashima et al., 2014; Roy et al., 2023; Semlow et al., 2016). It is believed that Slu7 and Prp18 help to stabilize 3'SS docking to the active site and that this contributes to 2nd step efficiency. It has been proposed that Prp22 antagonizes 3'SS docking to permit sampling of different 3'SS to ensure that optimal sequences are used as a proofreading mechanism (Mayas et al., 2006; Semlow et al., 2016). Importantly, identification of the correct 3'SS depends not just on identification of the YAG sequence but also involves choosing which YAG sequence to use. Prp18 appears to aid in selection of BP distal 3'SS and avoidance of BP proximal (and often non-consensus) 3'SS. This observation could be due to Prp18 imposing a BP to 3'SS distance constraint, enforcing use of canonical YAG 3'SS sequences, or a combination of the two activities.

Recently, Fyv6 was identified as a novel 2nd step factor in yeast (Lipinski et al., 2023). Fyv6 is a homolog of the human protein FAM192A, which was identified in pre-C* structures of the human spliceosome (Zhan et al., 2022). While the impact of FAM192A on human spliceosome activity has not yet been well-characterized, loss of Fyv6 in yeast decreases 2nd step splicing efficiency *in vitro* and results in use of an alternative 3'SS in the *SUS1* pre-mRNA *in vivo*. It is likely that unassigned cryo-EM densities in yeast C* (just prior to exon ligation) and P complexes correspond to Fyv6 since these densities are in analogous locations to that for FAM192A in the human pre-C* structure (Zhan et al., 2022). However, the resolutions of the yeast C* and P complex structures make unambiguous assignment of Fyv6 amino acid side chains and interactions difficult.

Here, we use a combination of transcriptomic, structural, biochemical, and genetic assays to elucidate how Fyv6 controls 3'SS usage in yeast. RNA-seq analysis reveals widespread activation of alternative, BP proximal nonconsensus 3'SS in the absence of Fyv6 consistent with biochemical assays that show Fyv6 facilitates splicing of 3'SS located ≥ 21 nt from the BP. To further elucidate

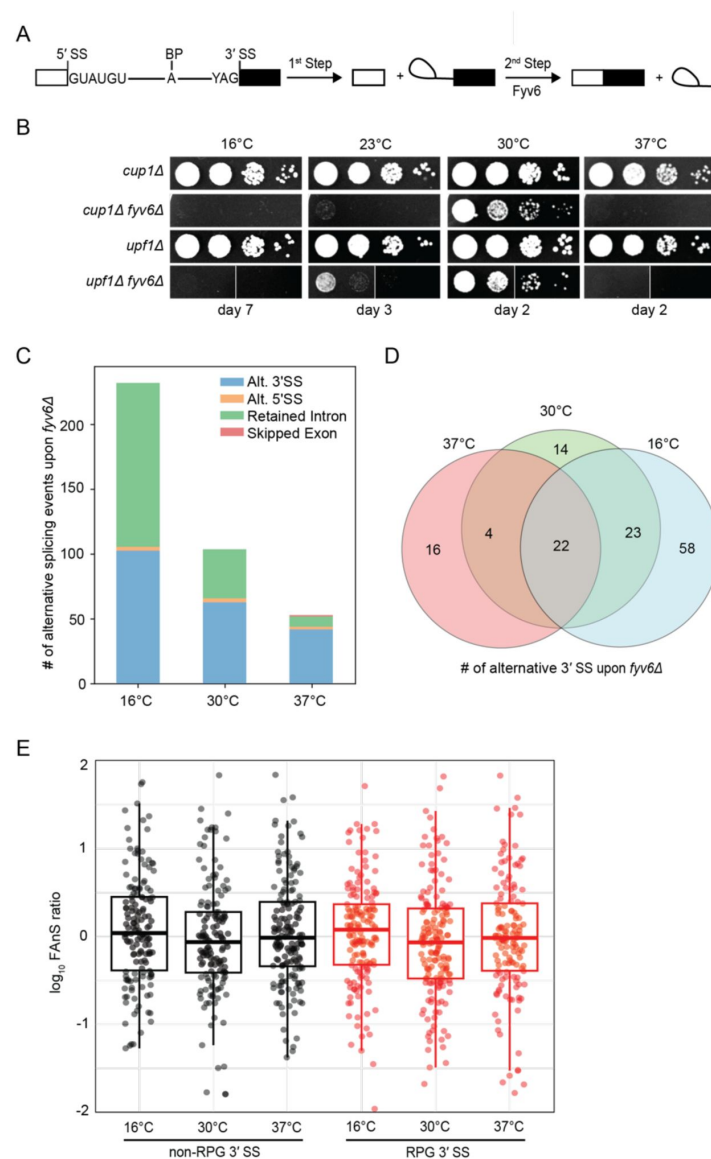


Figure 1.

Widespread splicing changes due to loss of Fyv6.

A) Pre-mRNA splicing reaction steps and products. **B**) Growth of WT (*cup1Δ*), *fyv6Δ*, *upf1Δ* and *fyv6tiupf1ti* yeast at 16, 23, 30, and 37°C. Images were taken on the indicated days. White lines indicate where images were cropped, as all dilutions for all strains were on the same plate, but not necessarily directly adjacent to one another. **C**) Number of alternative splicing events observed in a *fyv6tiupf1ti* strain relative to a strain containing FYV6 after a temperature shift to 16, 30, and 37°C. **D**) Venn diagram of shared alternative 3' SS splicing events at 16 (blue), 30 (green), and 37°C (red). **E**) Box plots of the $\log_{10}$ of the ratios of *fyv6ti upf1ti* to WT (*upf1ti*) Fraction of Annotated Splicing (FAnS) values for RPGs (red) and non-RPGs (black) for 3' SS splicing events. FAnS values represent the ratio of the alternative splicing event (here, selection of an alternate 3' SS) to the annotated, canonical splice isoform.

Fyv6 function, we determined a 2.3 Å cryo-EM structure of the yeast P complex spliceosome in which Fyv6 can be modeled. The structure reveals interactions between Fyv6 and Prp22 as well as a domain in Syf1 that interacts with either Fyv6 or the 1st step factor Yju2. 3D classification allowed us to identify two additional conformational states of P complex and provides insights into the coupling of 3'SS active site docking and the presence of 2nd step factors. We then used this structural data to dissect the functional domains of Fyv6, interpret the results of a genetic screen for suppressors of *fyv6Δ*, and to probe Fyv6 and Prp22 interactions. Combined, we propose a model in which Fyv6 is recruited to the spliceosome via interactions with the NTC component Syf1 in order to promote usage of BP distal, and Prp22-dependent, 3'SS.

Results

Deletion of *FYV6* Results in Widespread Use of Alternative 3' Splice Sites

Our previous work showed that loss of *FYV6* causes changes in 3'SS usage in the *SUS1* pre-mRNA. To probe for changes transcriptome-wide, we used nonsense mediated decay (NMD)-deficient (*upf11*, WT) yeast cells expressing or lacking Fyv6. By suppressing NMD, we hoped to limit degradation of alternatively spliced mRNA isoforms generated in the absence of Fyv6 (Sayani et al., 2008). Consistent with results from *fyv61* strains, the *fyv61 upf11* double mutant strain also showed reduced growth at 30°C and cold-sensitive (cs) and temperature-sensitive (ts) phenotypes relative to a *upf11* control strain (Figure 1B). We then used RNA-Seq to analyze the isolated RNAs from these strains. As expected, many more changes in splicing were observed in the *fyv61 upf11* strain relative to the *fyv61* strain (Figure 1-figure supplement 1A, 1B; supplementary tables 1, 2). This is consistent with many mRNA isoforms generated due to loss of Fyv6 being substrates for NMD. Loss of Fyv6 resulted in a number of changes in gene expression with many non-intron containing genes also being up-or down-regulated (Figure 1-figure supplement 1C).

We compared and classified changes in splicing between strains containing or lacking *FYV6* under permissive growth conditions (30°C) or after the yeast had been shifted to a non-permissive temperature (16 or 37°C) for 1 h (Figure 1C). In this case, we only considered events resulting in at least a 10% change in the percent spliced in (PSI) value when *FYV6* is deleted. At all temperatures, we see changes in alternative 3'SS usage in the *fyv61* strain relative to the *FYV6* background. For example, we detected use of the alternative 3'SS in the first intron of the *SUS1* transcript as we previously reported (Figure 1-figure supplement 1D; Lipinski et al., 2023). We also saw increased use of a cryptic, nonconsensus GAG 3'SS in the first, but not second, intron of the *YOS1* transcript in the *fyv61* data sets and confirmed this result by RT-PCR (Figure 1-figure supplement 1E, F). We observed several cases of alternative 5'SS. However, the majority of these alternative 5'SS were used with equal efficiencies in the WT and *fyv61* strains (Figure 1-figure supplement 1G).

The strains temperature shifted to 16°C had the largest number of changes due to *FYV6* deletion and the strains shifted to 37°C the least. The temperature shift to 16° caused an increase in the numbers of both alternative 3'SS and retained intron events detected (Figure 1C). The changes in alternative 3'SS used at each temperature were mostly unique with few events detected under all three conditions (Figure 1D). Collectively, we observed that 61 different RNAs (~20% of introns) changed splicing patterns due to loss of Fyv6. For many of these mRNAs, we detected multiple different 3'SS being used.

To investigate these splicing events more closely, we analyzed changes in usage of alternative splice junctions by calculating the Fraction of Annotated Splicing (FAnS) in the presence and absence of Fyv6 (Roy et al., 2023). FAnS ratios report the relative abundance of an alternative splicing event in relation to the main spliced isoform. We calculated FAnS values for all exon junction reads

corresponding to usage of a canonical 5'SS and an alternative 3'SS for WT and *fyv6Δ* strains at each temperature. We then calculated the ratios of the *fyv6Δ*/WT FAnS values for each transcript. These results confirm changes in alternative 3'SS usage for both ribosomal protein gene (RPG) and non-RPG transcripts at all conditions (**Figure 1E** [↗](#)). We could not detect any significant differences between RPG and non-RPG splicing with this analysis, suggesting that differences in gene expression and splicing efficiencies between these two classes are not correlated with alternative 3'SS usage. Combined these results show that Fyv6 plays a critical role in defining mRNA isoform production in yeast, particularly under non-optimal growth conditions such as temperature stress.

Fyv6 Facilitates Usage of Branch Point Distal 3' Splice Sites

We next analyzed the features of the 3'SS impacted by Fyv6 loss. We used RNAs collected from yeast grown at 30°C and sequenced the libraries at higher depth than those used for the temperature-shift experiment described in **Figure 1** [↗](#) (~400 million vs. 200 million reads; supplementary table 2). We first identified alternative 3'SS used in each strain, annotated each as being upstream (5) or downstream (3) of the canonical 3'SS (Grate and Ares, 2002 [↗](#)), and then calculated the FAnS ratio for each site. This analysis shows that the majority of the alternative 3'SS arising from Fyv6 deletion are upstream (**Figure 2A** [↗](#), pink points above the diagonal). When these alternative 3'SS are mapped according to their nucleotide distance from the canonical site, most of these are found within ~40 nt upstream (**Figure 2B** [↗](#), purple violin). Plotting these alternative 3'SS sites relative to their distance from the annotated BP reveals that the highest density of sites is ≤ 20 nt from the BP for transcripts with the highest FAnS ratios (**Figure 2C** [↗](#), purple violin). It is important to note that we did not map BP in Fyv6 deletion strains, and it is possible that in some cases a shift in 3'SS usage could also coincide with a shift in the BP. Indeed, a small number of alternative 3'SS detected in the Fyv6 data sets are located upstream of the annotated BP, suggesting activation of an alternative BP and 3'SS. However, we believe that the changes are predominantly due to usage of alternative 3'SS located between the annotated BP and canonical 3'SS.

The above results suggest that Fyv6 is important for splicing of 3'SS located distant from the BP. To test this directly and systematically, we created a series of ACT1-CUP1 reporters with various BP to 3'SS distances (9-50 nt, **Figure 2D** [↗](#), supplementary table 3) based on sequences used in previous studies of 3'SS selection (Brys and Schwer, 1996 [↗](#); Frank and Guthrie, 1992 [↗](#); Schwer and Gross, 1998 [↗](#)). We transformed strains containing or lacking Fyv6 and without debranchase (Dbr1) to limit degradation of lariat intermediates. We then detected RNA products by primer extension and calculated the 2nd step (exon ligation) efficiency for each reporter (**Figure 2E, F** [↗](#)). While loss of Fyv6 has minimal impact on exon ligation when BP to 3'SS distances are short (9-15 nt), exon ligation efficiency decreases significantly at distances of 21 nt or greater. In addition, we do not see evidence of cryptic BP usage in these assays. This result is consistent with the RNA-seq analysis and supports a function for Fyv6 in facilitating splicing at BP distal 3'SS.

Finally, we analyzed the sequence features of the alternative 3'SS with increased use after loss of Fyv6 (**Figure 2G, H** [↗](#)). For sites located upstream of the canonical 3'SS, we observed much greater sequence variability relative to the canonical site. We detected increased use of highly variable 3'SS including those with atypical HAU (H=A,C,U) and BG (B=C,U,G) motifs and confirmed generation of new RNA isoforms for several of these by RT-PCR (**Figure 2H** [↗](#), figure 2-figure supplement 1). Increased use of variable 3'SS was also observed when the 2nd step factor *PRP18* was deleted (Roy et al., 2023 [↗](#)). However, the subset of RNAs most impacted by splicing factor deletion appear to be different. For example, we were not able to detect changes in 3'SS usage for the *UBC12*, *MAF1*, *MUD1*, *PHO85*, *SPT14*, or *YCL002C* transcripts in the *fyv6Δ* data sets as was reported for *prp18Δ* (not shown). Prp18 and Fyv6 likely have transcript-specific effects on splicing outcomes. Combined our results strongly support Fyv6 as a 2nd step splicing factor that facilitates usage of consensus sites located distal to the BP *in vivo*.

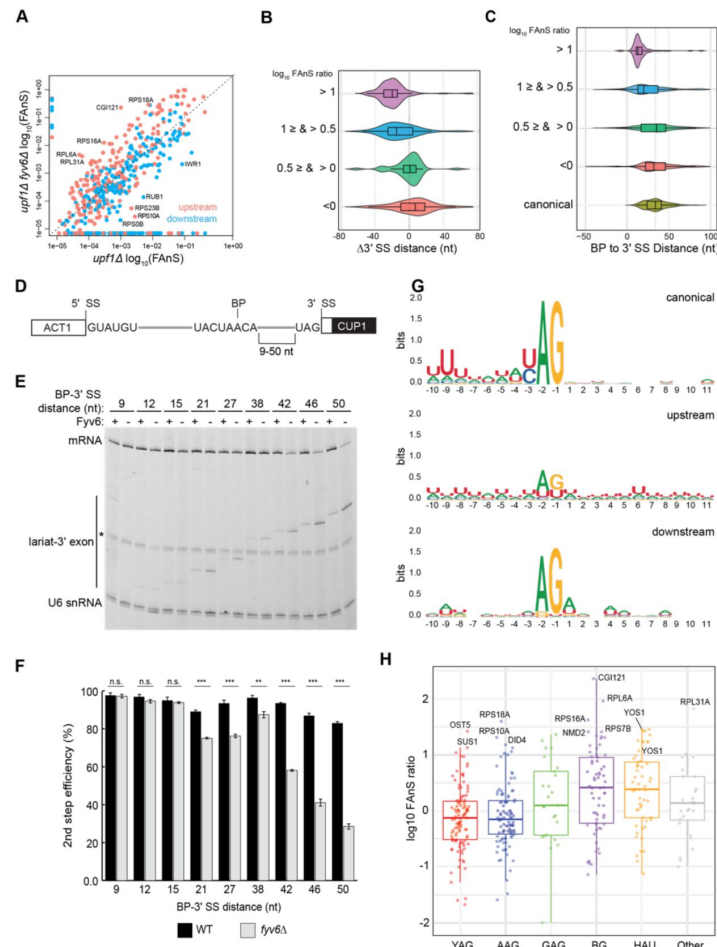


Figure 2

Loss of Fyv6 activates BP proximal, non-consensus 3'SS.

A) Plot of the $\log_{10}$ of Fraction of Annotated Splicing (FANs) values for differences between alternative 3'SS usage in $fyv6\Delta$ $upf1\Delta$ and $upf1\Delta$ strains. High FANs values indicate increased usage of the alternate 3'SS relative to the annotated site. Points are colored based on whether they are upstream (pink) or downstream (blue) to the canonical 3'SS. **B**) Violin plots of the ratios of $fyv6\Delta upf1\Delta$ to the $upf1\Delta$ FANs values based on the distances between the alternative and canonical 3'SS ($\Delta 3'SS = 3'SS_{Alt} - 3'SS_{canonical}$). FANs ratios >0 are indicative of the site being upregulated in $fyv6\Delta$. **C**) Violin plots of the ratios of $fyv6\Delta upf1\Delta$ to the $upf1\Delta$ FANs values based on the distances between the alternative 3'SS and the annotated BP. FANs ratios >0 are indicative of the site being upregulated in $fyv6\Delta$. **D**) Diagram of ACT1-CUP1 reporter showing BP-3'SS distances. **E**) Representative primer extension analysis of RNA products generated from splicing of the ACT1-CUP1 reporter in the presence (WT) or absence of Fyv6 ($fyv6\Delta$). Bands for fully spliced mRNA and lariat intermediate are indicated. U6 snRNA was detected as a loading control. The * indicates an unknown product present in every lane. **F**) Quantification of the primer extension results from $N=3$ replicates represented by the ratio of band intensities for mRNA/(mRNA+lariat intermediate). Bars represent the average ratio of the replicates $\pm$ SD. Means between WT and $fyv6\Delta$ groups for each reporter were compared with an unpaired Welch's two-tailed t-test. Significance is indicated: n.s. no significance; ** $p<0.01$; *** $p<0.001$. **G**) Sequence logos of alternative 3'SS with FANs >0 sorted by either upstream or downstream of the canonical 3'SS compared against the canonical 3'SS for genes with FANs >0 . **H**) The $\log_{10}$ of the FANs ratio for alternative 3'SS sorted by the sequences of the 3'SS.

A High-Resolution Spliceosome Structure Reveals Fyv6 Interactions

Previous structures of the yeast P-complex spliceosome were solved with resolutions at the core ranging from 3.3 Å to 3.7 Å (Bai et al., 2017 [DOI](#); S. Liu et al., 2017 [DOI](#); Wilkinson et al., 2017 [DOI](#)), and with peripheral components such as the Prp22 helicase, U2 snRNP, and Prp19 Complex (NTC) at much lower resolutions (5-10 Å) that precluded detailed investigation. To improve the resolution, we purified P complex as previously described by stalling spliceosome disassembly with a dominant negative mutant of Prp22 (S635A) defective in mRNA release (Schwer and Meszaros, 2000 [DOI](#); Wilkinson et al., 2017 [DOI](#)). However, by collecting a much larger cryo-EM dataset on a more modern electron detector, we were able to resolve the structure to 2.3 Å within the catalytic core, as well as visualize peripheral components at resolutions from 3.0 Å – 3.7 Å (**Figure 3A, B** [DOI](#); figure 3-figure supplements 1-4; supplementary table 4). This is the highest resolution spliceosome structure to date. The high-quality density at the active site shows the positions of bases unambiguously, confirming the manner of the non-Watson-Crick base pairing that mediates 3'SS recognition (S. Liu et al., 2017 [DOI](#); Wilkinson et al., 2017 [DOI](#)). Monovalent ions bound at the active site were previously shown to be important during the 1st step of splicing (Wilkinson et al., 2021 [DOI](#)), and our data indicate that the positions of these ions are preserved after exon ligation. Additionally, the density also shows a metal ion, likely potassium, that bridges the base pair between +1G of the 5'SS and -1G of the 3'SS (**Figure 3C** [DOI](#)).

The 2nd-step splicing factor Prp22 is resolved at 3.0 Å resolution, allowing atomic interpretation for the first time (**Figure 3B** [DOI](#) and later figures). Bases 13 – 21 of the 3'exon are visible between the two RecA domains of Prp22 consistent with biochemical footprinting data (Schwer, 2008 [DOI](#)) (figure 3-figure supplement 4). The S635A mutation used to stall P complex is also visible; however, the origin of defective mRNA release due to this mutation is not apparent (Schwer and Meszaros, 2000 [DOI](#)). The loop containing S635A is in a similar conformation in this structure and in the structure of WT human Prp22 (Felisberto-Rodrigues et al., 2019 [DOI](#)) (figure 3-figure supplement 4). Part of the extensive N-terminal domain of Prp22 is resolved and constitutes a long helix that bridges between the Prp22 RecA2 domain and the C-terminal domain of Cwc22; this helix was unassigned in previous P-complex structures (**Figure 4B** [DOI](#)).

To improve the resolution of the peripheral, flexible regions, we used a new data-driven regularization strategy (Kimanus et al., 2024 [DOI](#)) that allows focused refinements of much smaller domains than previously possible (figure 3-figure supplement 2, 4). Combined with AlphaFold2-assisted modelling, we were able to obtain more accurate models for the U2 snRNP, NTC, U5 Sm ring, and Cwc22 N-terminal domains. Our model for the NTC within the P complex is similar to our previous model for the NTC within C and Cⁱ complexes (Wilkinson et al., 2021 [DOI](#)) (**Figure 4C** [DOI](#)). Notably however, we observed a star-shaped density coordinated by several conserved lysines from Clf1 and Ntc20, which we were able to model as inositol hexakisphosphate (IP₆) (**Figure 4D** [DOI](#)). The same density, at lower resolution, is visible in the C complex (Wilkinson et al., 2021 [DOI](#)). A distinct, separate IP₆ molecule had previously been observed within all catalytic spliceosomes between the B^{act} and P-complex stages coordinated by Prp8 and (for C* and P-complexes) Slu7 (figure 3-figure supplement 4). We do not believe that the NTC-bound IP₆ is essential for splicing since deletion of the Clf1 tetratricopeptide repeat that coordinates IP₆ does not result in growth or splicing phenotypes in yeast and Ntc20 is a non-essential splicing factor (Chen et al., 2001 [DOI](#); Chung et al., 1999 [DOI](#)). Nonetheless, it is possible that IP₆ may regulate splicing in some manner via interactions with both Prp8 and the NTC.

We were able to unambiguously assign three, connected long α -helices to Fyv6 (**Figure 4A** [DOI](#)). These helices were visible in previous cryo-EM reconstructions of yeast C* and P complexes but were either unassigned or misassigned. In the cases of misassigned densities, Fyv6 was previously attributed to the C-terminal domain of the 1st step factor Yju2 (Wilkinson et al., 2017 [DOI](#)). Yju2 also has an elongated helical architecture and adopts a similar position on C complex (Wilkinson et al., 2021 [DOI](#)) and the post-P complex Intron Lariat Spliceosome (ILS) (Wan et al., 2017 [DOI](#)). We closely

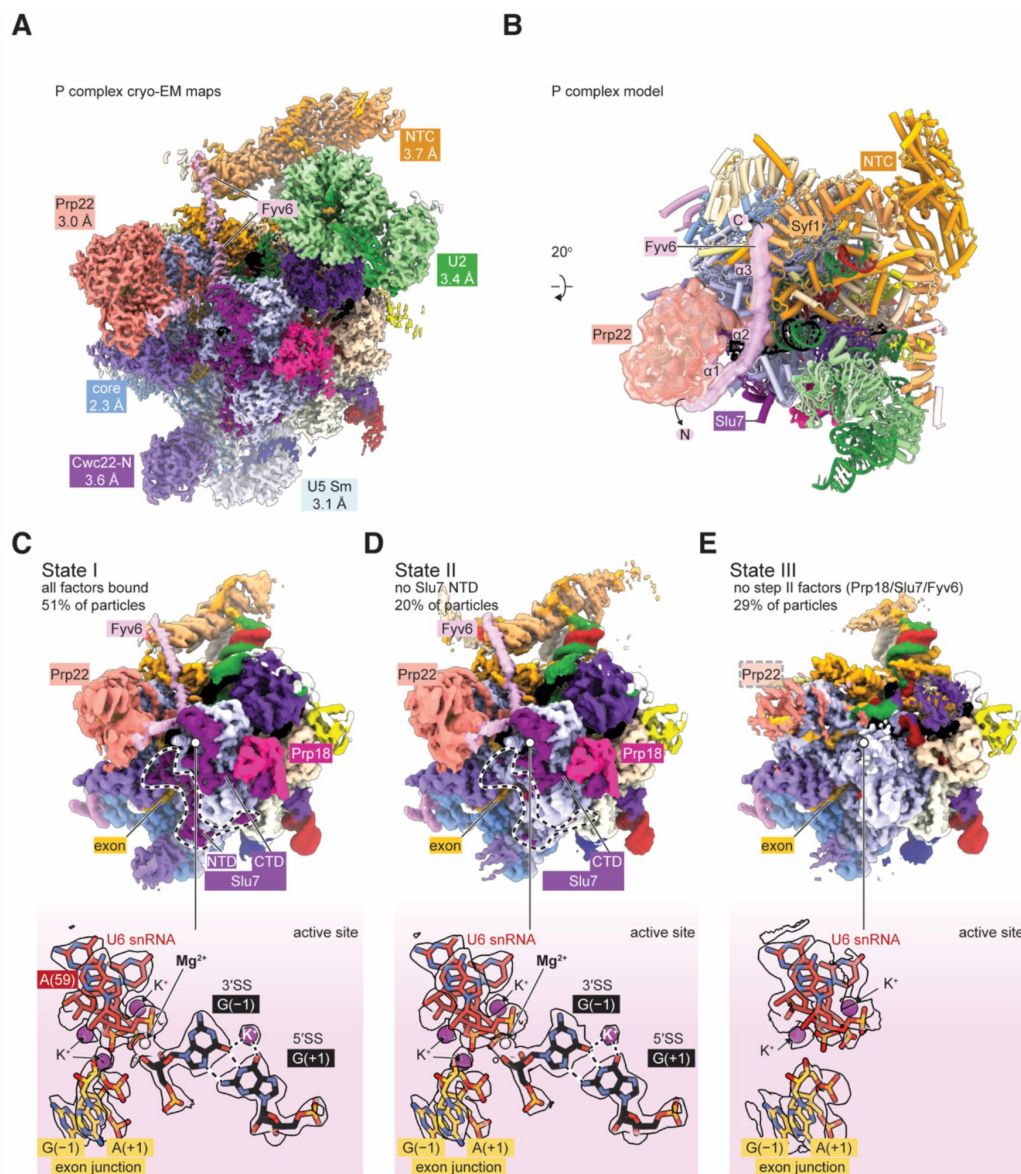


Figure 3.

Cryo-EM structure of the yeast P complex spliceosome at 2.3 Å resolution.

A) A composite density map for P complex showing focused refinements of the Prp22, NTC, U2 snRNP, U5 Sm ring, and Cwc22 N-terminal domain regions. **B)** Overall model for the P complex spliceosome. **C)** Cryo-EM density for state I of P complex (above, low-pass filtered) and for the active site and 3'SS (below, sharpened). **D,E)** As for **C)** but for states II (**D**) and III (**E**).

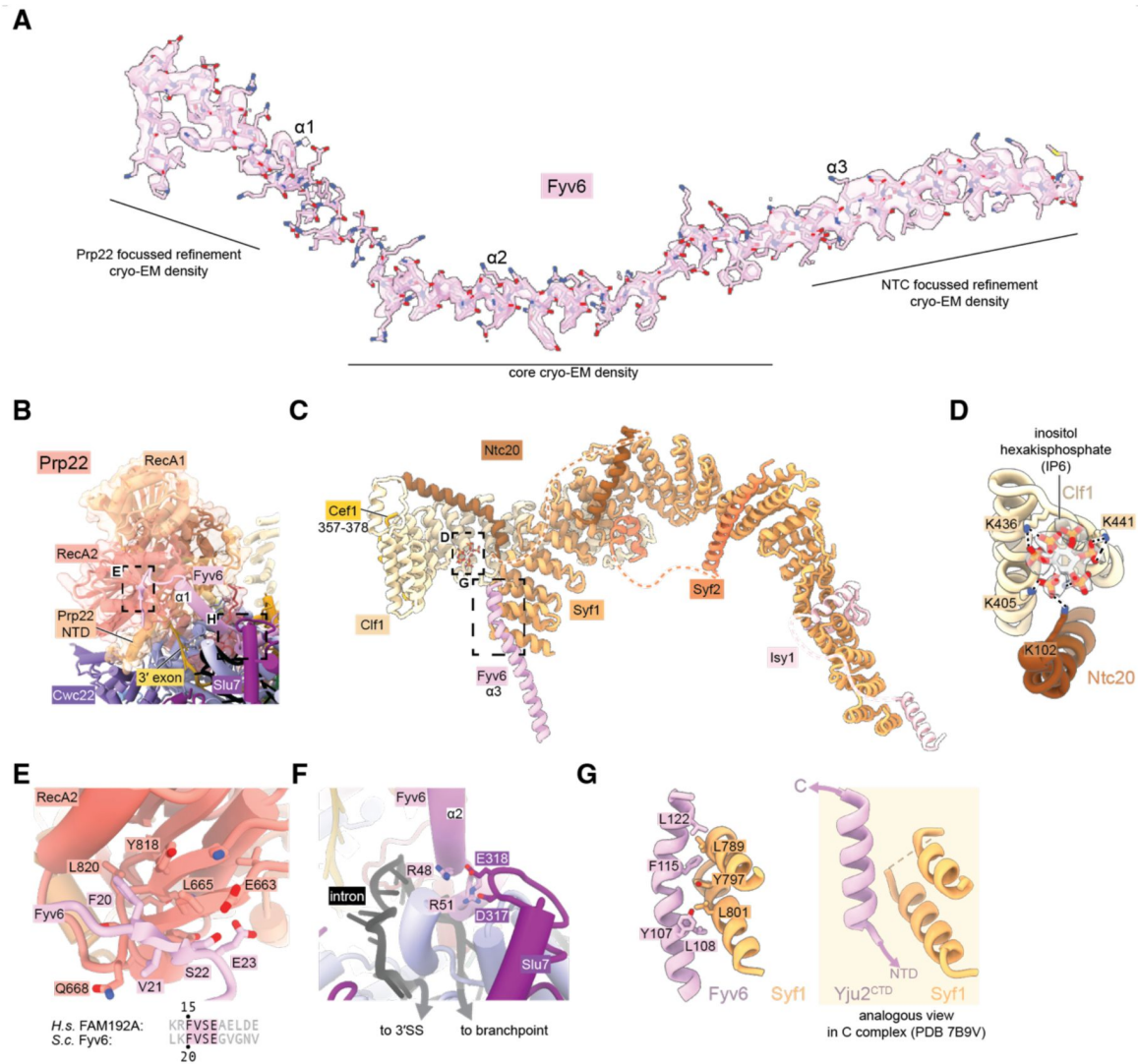


Figure 4.

Fyv6 and its interactors in the P complex cryo-EM structure.

A) Cryo-EM density segmented around Fyv6. **B)** Structure of Prp22. **C)** Structure of the NTC within P complex. **D)** IP6 site and cryo-EM density in NTC. **E)** Interaction of the hook domain of Fyv6 with the Prp22 RecA2 domain. **F)** Interaction of Fyv6 with Slu7 and the region of the intron between the BP and 3'SS. **G)** Interaction of Fyv6 with Syf1 and an analogous view of the interaction of Syf1 with Yju2 in C complex (Wilkinson et al., 2021 [DOI](#)).

inspected the density from previously determined spliceosome structures and concluded that the Yju2 C-terminus is still a better fit than Fyv6 for densities in B*, C, and ILS complexes, whereas Fyv6 is a better fit than Yju2 for densities in C* and P complexes (figure 4-figure supplement 1). Therefore, this position of Fyv6 seems to be characteristic of a 2nd step conformation of the spliceosome and consistent with the position of its distant human homolog FAM192A in the human pre-C* complex (Zhan et al., 2022 [DOI](#)).

Fyv6 makes a multitude of interactions with essential splicing factors (**Figures 3B** [DOI](#); **4E, F, G**). One conserved region of Fyv6 is the N-terminus with a FVSE motif (figure 4-figure supplement 2). This motif forms a ‘hook’ that interacts with a hydrophobic patch on the surface of the Prp22 RecA2 domain, where it also forms hydrogen bonds by β -sheet augmentation (**Figure 4E** [DOI](#)). The hook is followed by three α -helices. The start of helix 2 sits on top of the intron between the BP and the active site docked 3'SS, where it may act as a steric block to prevent 3'SS undocking and promote exon ligation (**Figure 4F** [DOI](#)) (S. Liu et al., 2017 [DOI](#)). Helix 2 also contains a patch of conserved arginines that form salt bridges with Asp317 and Glu318 of Slu7 (**Figure 4F** [DOI](#)). The linker between helices 2 and 3 interacts with the NTC component Cef1, and helix 3 interacts with NTC component Syf1 (**Figure 4G** [DOI](#)). The binding interface with Syf1 overlaps with the Syf1/Yju2 interaction in the C complex (Wilkinson et al., 2021 [DOI](#)), suggesting these interactions are mutually exclusive (**Figure 4G** [DOI](#)). Fyv6 thus provides a direct link between the Prp22 ATPase, NTC components, and other essential splicing factors over a distance of ~ 100 Å.

By 3D classification, we were able to resolve a total of three states of the P-complex spliceosome (**Figure 3C-E** [DOI](#)). State I, represented by 51% of particles, is the most complete and has all the above-described factors visible, including a stably-docked 3'SS. State II, represented by 20% of particles, is similar to State I but lacks density for the N-terminal half of the 2nd step factor Slu7. The endonuclease-like domain of Prp8, to which this domain of Slu7 binds, is correspondingly shifted. Interestingly, this state retains strong density for the 3'SS, suggesting the N-terminal regions of Slu7 are not involved in maintaining 3'SS docking after exon ligation. Finally, State III, containing 29% of particles, entirely lacks density for the 2nd step factors Slu7, Prp18, and Fyv6. Prp17 and the RNase H domain of Prp8 are still present but are weaker than in States I and II, suggesting they are more flexible in State III. Most of Prp22 can still be observed with weaker density except for the C-terminal domain that interacts with Prp8, which lacks density entirely. Importantly, State III also lacks density for the 3'SS but has density for the 3'exon, suggesting it is indeed post-catalytic and represents a state after loss of 2nd step factors and undocking of the 3'SS from the catalytic core. The coincidence of loss of Fyv6 with loss of Prp18 and Slu7 reinforces the notion that these three factors act together and are important for 3'SS docking, maintaining the 3'SS in the active site after exon ligation, and for stabilization of Prp17, Prp22, and the RNase H domain of Prp8.

The Fyv6/Syf1 Interaction is Critical for Suppressing *fyv61* Phenotypes

To determine the functionally critical regions and interactions of Fyv6, we used the cryo-EM structure to design five truncation mutants (**Figure 5A** [DOI](#)). The first three truncations remove amino acids from the N-terminus of Fyv6: deletion of the first 16 amino acids, which are not resolved in the cryo-EM structure ($\Delta 1-16$); deletion of the first 23 amino acids, which removes the conserved hook region of Fyv6 that interacts with the Prp22 RecA2 domain ($\Delta 1-23$); and deletion of amino acids 1 to 51, which additionally removes the first α -helix and interactions with Prp22, Prp8, and Slu7 ($\Delta 1-51$). The remaining two mutants are C-terminal truncations. In the first, amino acids 134 to 173, which do not have clear density in the cryo-EM structure and are not modeled but which do contain a predicted nuclear localization signal (NLS), are deleted ($\Delta 134-173$) (Nguyen Ba et al., 2009 [DOI](#)). In the second, amino acids 103 to 173 are deleted ($\Delta 103-173$), which additionally removes the region of the protein that interacts with Syf1.

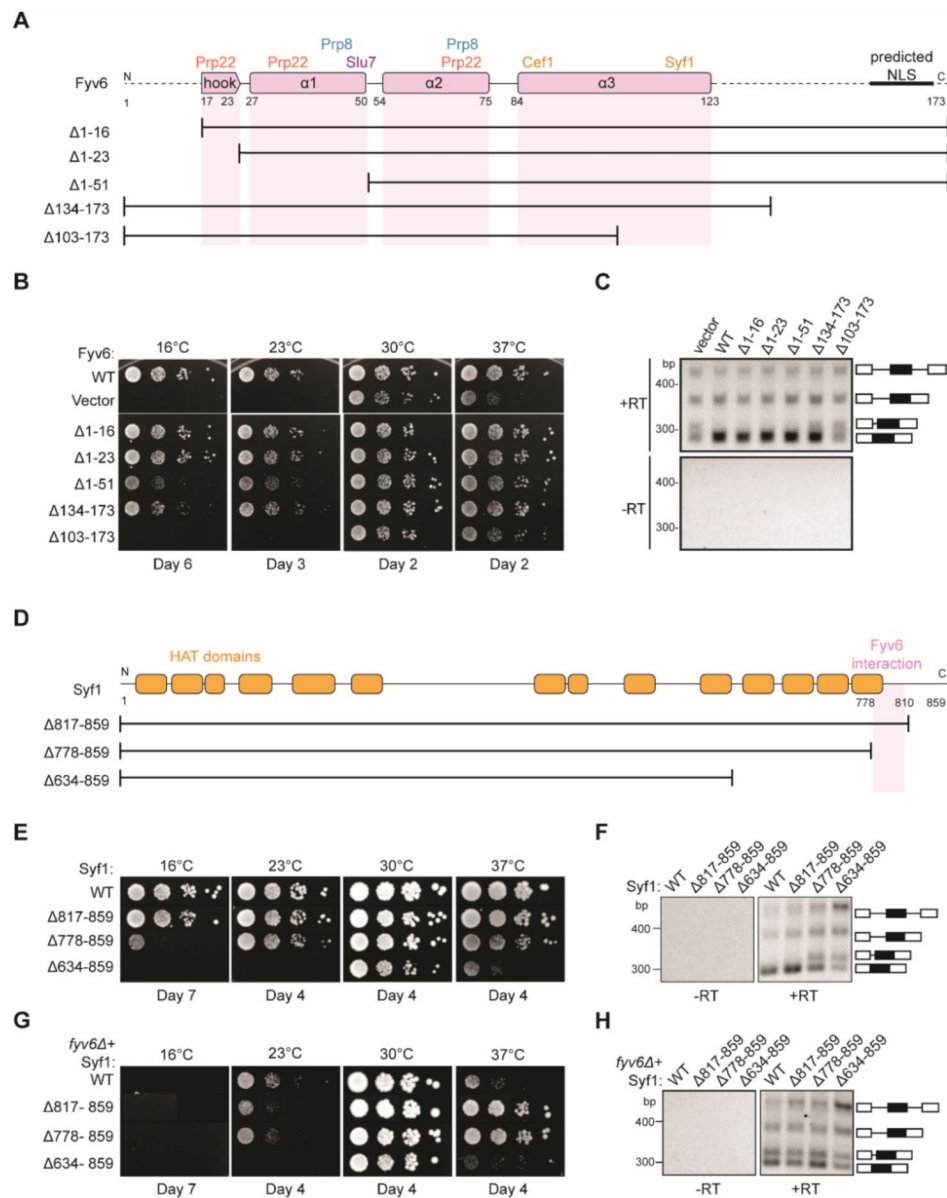


Figure 5.

Structure-based analysis of Fyv6 domain function.

A) Diagram of Fyv6 protein structure, protein interactors, and protein truncations. NLS= nuclear localization signal **B)** Spot dilution assay of strains with Fyv6 truncations on -Trp DO plates at different temperatures. Plates were imaged after the number of days indicated. **C)** Representative gel image of *SUS1* RT-PCR in strains with an empty vector or expressing the indicated Fyv6 variant. (+RT reactions contain reverse transcriptase; -RT control reactions do not contain reverse transcriptase.) **D)** Diagram of Syf1 protein structure and truncations. **E)** Spot dilution assay of strains with Syf1 truncations on -Trp DO plates at different temperatures. Plates were imaged after the number of days indicated. **F)** Representative gel image of *SUS1* RT-PCR in strains with an empty vector or expressing the indicated Syf1 variant. **G)** Temperature growth assay of strains with Syf1 truncations in a *fyv6Δ* background on YPD plates. Plates were imaged after the number of days indicated. **H)** Representative gel image of RT-PCR of *SUS1* in strains with plasmids containing WT Syf1 or a Syf1 truncation in a *fyv6Δ* background.

We tested each truncation mutant for its ability to suppress the *cs* and *ts* phenotypes of the *fvy6Δ* strain when expressed from a plasmid under the native promoter (**Figure 5B**). All of the N-terminal truncation mutations could suppress the *cs* or *ts* phenotypes to some degree. Surprisingly, a strain with the complete deletion of the conserved hook domain that interacts with Prp22 (Δ1-23) grew similarly to the strain expressing WT Fyv6. We wondered if splicing changes could still be present even if the growth phenotype was suppressed; so, we assayed *SUS1* isoforms by RT-PCR. We previously showed that loss of Fyv6 causes use of an alternative 3'SS in the first intron of this pre-mRNA (Lipinski et al., 2023). In addition to suppression of the *fvy6Δ* growth phenotype, the N-terminal truncation mutants also showed no evidence for changes in *SUS1* splicing (**Figure 5C**). The N-terminal region of Fyv6 composed of the hook domain and the first α-helix is not required for suppression of either the temperature sensitivity or splicing phenotypes observed when Fyv6 is lost despite its conservation and interactions with critical splicing factors (Prp8, Prp22, and Slu7).

For the C-terminal truncations, the Δ134-173 truncation was also able to suppress the *cs* and *ts* phenotypes, indicating that the predicted NLS is also not essential (**Figure 5B**). However, this mutant grew more poorly at 16°C than did the Δ1-16 and Δ1-23 truncations and showed some evidence of alternative 3'SS usage in *SUS1* (**Figure 5C**). The largest effects were seen when the C-terminus was truncated further. The Δ103-173 truncation strain phenocopied the *fvy6Δ* strain and grew poorly at 37°C and was dead at 23° or 16°C (**Figure 5B**). Additionally, RT-PCR showed increased use of the alternative 3'SS in *SUS1* with this truncation (**Figure 5C**). To determine if this phenotype could be due to loss of protein expression, we assayed expression of the epitope-tagged proteins by western blot (figure 5-figure supplement 1A). In all cases, we could detect protein; however, the abundance of the N-terminally epitope-tagged Δ103-173 variant was much lower than the others. Therefore, we also constructed a C-terminally epitope-tagged version of Δ103-173 (figure 5-figure supplement 1B). The C-terminally-tagged protein expressed at much higher levels but also failed to suppress the *cs* and *ts* phenotypes. These data suggest that the C-terminal region of Fyv6 that includes a portion of α-helix 3 and the Syf1 interaction domain is critical for function.

To obtain additional evidence for the importance of the Fyv6/Syf1 interaction, we created a *syf1Δ* shuffle strain containing WT Fyv6 and expressed C-terminal Syf1 truncations (**Figure 5D**). We hypothesized that loss of the Fyv6-interacting domain on Syf1 would phenocopy *fvy6Δ*. We observed just this effect. Loss of the Fyv6-interacting region in Syf1 Δ778-859 and Δ634-859 mutants resulted in either modest (Δ778-859) or severe (Δ634-859) *cs* and *ts* phenotypes. In addition, these Syf1 truncation mutants also triggered use of the alternative 3'SS in *SUS1* (**Figure 5F**). Interestingly, if the Δ778-859 and Δ817-859 Syf1 mutants are expressed in a *fvy6Δ* background, it results in suppression of the *fvy6Δ ts* phenotype (**Figure 5G**; *c.f.* **5B**). However, the *SUS1* alternative 3'SS is still used (**Figure 5H**). This indicates that *ts* phenotype suppression and triggering usage of the alternative 3'SS in *SUS1* are separable processes.

When considered together, these results likely originate from mutually exclusive Fyv6/Syf1 and Yju2/Syf1 interactions. It is possible that in the absence of Fyv6, Syf1 inappropriately binds Yju2 during exon ligation. The presence of Yju2 during the 2nd step could then lead to growth defects that are suppressed when the Yju2-binding site on Syf1 is deleted. Alternatively or in addition, the absence of Fyv6 could perturb the equilibrium between the 1st- and 2nd-step conformations of the spliceosome and result in favoring of the 1st step (L. Liu et al., 2007). Loss of this equilibrium could lead to growth defects that are suppressed by destabilization of the 1st-step conformation by removing a binding site for Yju2 on Syf1 and restoring the equilibrium. In both cases, truncation of Syf1 suppresses growth defects but not *SUS1* splicing defects since Fyv6 function is still needed during the 2nd step.

Mutations in Many Different Splicing Factors Can Suppress *fyv6Δ* Phenotypes

To gain additional insights into Fyv6 function, we carried out a genetic screen to detect spontaneously arising suppressors that can correct temperature-dependent growth defects (**Figure 6A**). This method has previously been used to illuminate many aspects of spliceosome biochemistry and non-silent mutations within splicing factors are likely causative for suppression (Montemayor et al. 2014; Kuhn and Brow, 2000; Brow, 2019).

We plated saturated *fyv6Δ* yeast cultures onto 20 different YPD plates and incubated them at 37°C until colonies arose. Within several days, colonies of various sizes appeared and were isolated. A subset of picked colonies were then tested for suppression of the *ts* phenotype. All of these colonies were able to suppress growth defects at 37°C, and many also suppressed the *cs* phenotype at 23°C (figure 6-figure supplement 1). We then performed whole genome sequencing on the largest of the picked colonies as these were expected to be the strongest suppressors. In 19 of 20 sequenced strains, we were able to identify at least one non-silent mutation in a splicing factor (**Figure 6B**, supplementary table 5). When we carried out a similar experiment aimed at identifying *cs* suppressors, we were unable to identify any likely causative mutations in splicing factors. Therefore, we focused our analysis on the *ts* suppressor mutations.

Collectively, the identified mutations tended to fall into two categories: gain of positive charge or substitution with a bulkier side chain. Gain of positive charge mutations were found in Prp8 (G1868R) as well as the second step factors Slu7 (A24R, and N28K), and Prp22 (I1133R). Structurally, amino acids within Prp8 and Prp22 are proximal to RNA within the spliceosome and addition of charged amino acid side chains may stabilize interactions with the phosphodiester RNA backbone. The N-terminus of Slu7 containing A24 and N28 is not resolved in any spliceosome structure to our knowledge and normally contains many negatively charged residues. The majority of identified suppressors involve a mutation to a bulkier side chain including several in Prp8 (S1594F/Y, V1862L), Prp17 (A251V, G386W), Cef1 (A37V), and Clf1 (T33I). The increase in hydrophobic surface area provided by these mutations may help to stabilize interactions with nearby proteins, compensating for structural destabilization due to the absence of Fyv6. Alternatively, since Prp8, Prp17, Cef1, and Clf1 are all present during 5'SS cleavage, it is possible that these bulky mutations could destabilize a 1st step conformation of the spliceosome and suppress phenotypes due to the loss of Fyv6 by facilitating formation of the 2nd step conformation.

We isolated one suppressor mutation within the U2 snRNA (A25G) near where the C-terminal domain of Prp22 inserts towards the active site and located beneath the Fyv6 binding site. It has previously been observed that mutation of this specific U2 snRNA nucleotide is not deleterious to growth (McPheeters and Abelson, 1992) and is therefore well-tolerated by yeast. The mechanism of suppression by this mutation is not clear; however, it is interesting to note that we also observed suppressor mutations in the U2 snRNP component Rse1 (supplementary table 5) which is released from the spliceosome prior to catalysis. The Rse1 mutations always occurred coincident with mutations in other splicing factors. It is possible that suppressors in factors present prior to or after exon ligation are able to suppress (or enhance suppression of) *fyv6Δ* by changing how pre-mRNAs compete for a limited set of splicing factors or by reducing blocks to pre-spliceosome assembly caused by accumulation of stalled complexes (Mendoza-Ochoa et al., 2019; Munding et al., 2013). Together these data indicate that mutations in many different splicing factors including second-step factors and the U2 snRNA can be isolated from *fyv6Δ* suppressor strains.

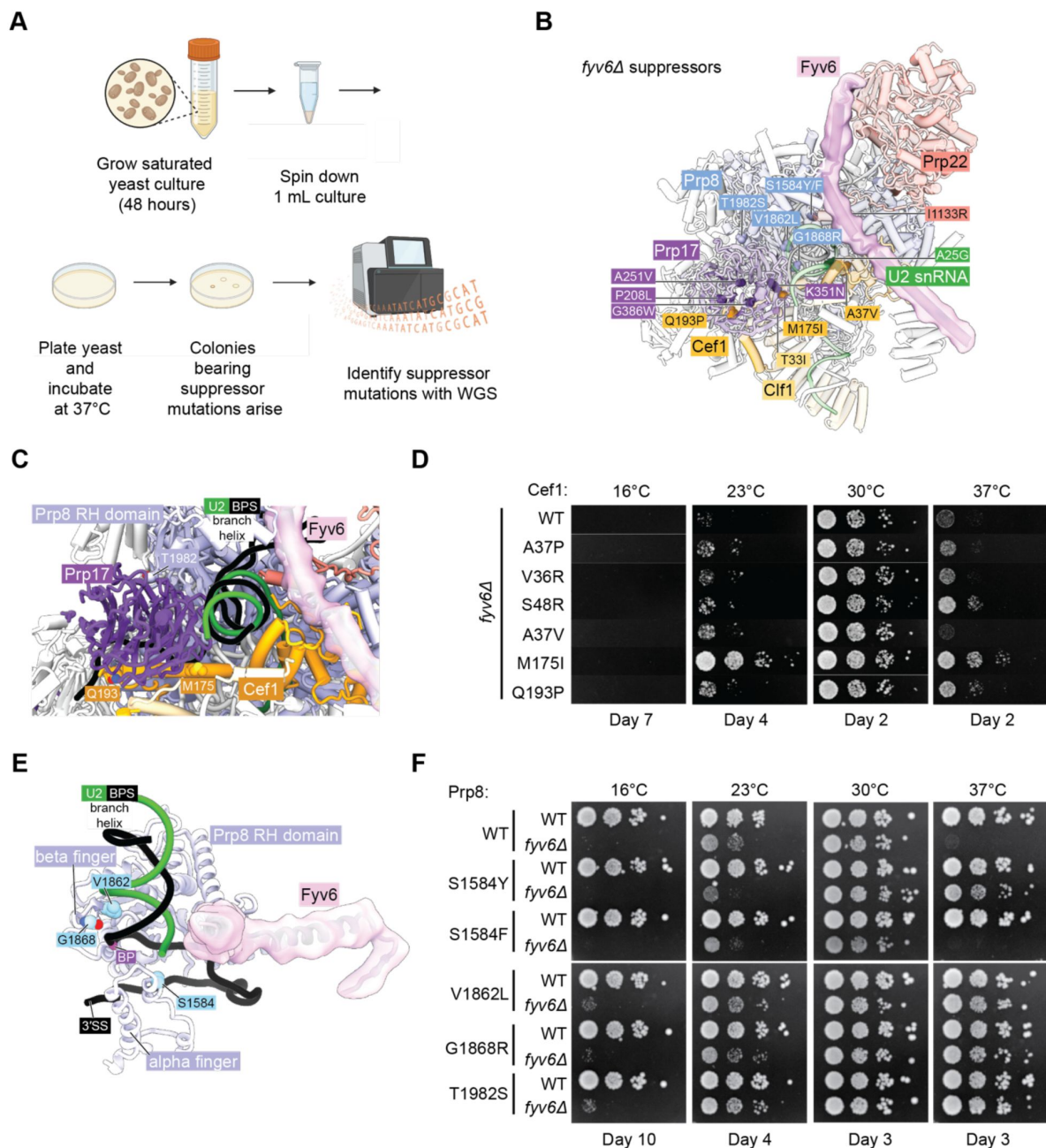


Figure 6.

Identification of novel suppressors of *fyv6ti* temperature sensitivity.

A) Workflow for the suppressor screen. **B**) P complex spliceosome structure with *fyv6ti* suppressor mutations labelled. (See Supplemental Table S5) **C**) Close-up view of Cef1 and Prp8 suppressor mutations in P complex. **D**) Spot dilution temperature growth assay with Cef1 mutants on YPD plates. Plates were imaged after the number of days indicated. **E**) Close-up view of Prp8 mutations in P complex. **F**) Spot dilution temperature growth assay with Prp8 mutants on YPD plates. Plates were imaged after the number of days indicated.

Multiple Second-step Splicing Defects can be Rescued by the Same Suppressor

We decided to study several of these mutations in greater detail and to confirm that they were sufficient for *fvy6Δ* *ts* phenotype suppression. In the case of Cef1, we obtained several mutants located near the interface between Cef1 and the 2nd step factor Prp17: A37V, M175I, and Q193P (**Figure 6C**). This interface has only been observed in structures of C* and P-complex spliceosomes, suggesting its importance for exon ligation. In addition, it has previously been reported that another mutant of Cef1 A37—A37P—is able to suppress splicing defects due to a BS adenine to guanosine substitution (BS-G) which causes stalling after 5' SS cleavage (Query and Konarska, 2012). Therefore, we wondered if other Cef1 mutants capable of suppressing defects due to BS-G could also suppress *fvy6Δ* (Cef1 A37P, V36R, and S48R). We created *fvy6Δ* shuffle strains for Cef1 and tested each mutant's ability to suppress *cs* and *ts* phenotypes (**Fig. 6D**). All of these Cef1 mutants, both those isolated from our suppressor screen and those identified as BS-G suppressors, could at least weakly suppress the *cs* phenotype at 23°C with our isolated M175I mutant being a particularly strong suppressor. In addition, all of the mutants except Cef1 A37V could suppress the *ts* phenotype at 37°C to varying degrees. These results confirm that single point mutants in Cef1 can suppress *fvy6Δ* growth phenotypes and that at least some BS-G suppressors in Cef1 also suppress *fvy6Δ*. A common mechanism could be that these mutations stabilize the exon ligation conformation of the spliceosome to favor the 2nd step either when a BS-G is present or Fyv6 is absent.

In the case of Prp8, we tested five different suppressor mutations in *prp8* shuffle strains (**Figure 6E, F**). Two of these are located within the Prp8 a-finger domain (S1584F,Y; **Figure 6E**) while the remainder are located within the RNase H-like domain β-finger (V1862L, G1868R, T1982S; **Figure 6C**, **6E**). We confirmed that four of the identified Prp8 suppressor mutations could partially suppress either or both of the *cs* and *ts fvy6Δ* phenotypes (**Figure 6F**; S1574Y, V1862L, G1868R, and T1982S). Even though we isolated the Prp8 S1584F mutant in our screen and this substitution is very similar to S1584Y, we were not able to observe suppression due to this mutant in these strains. Identification of mutants in Prp8 that suppress *fvy6Δ* is not surprising since many different alleles of Prp8 are known to modulate the conformational equilibrium between the 1st and 2nd steps (Fica and Nagai, 2017; L. Liu et al., 2007; Query and Konarska, 2004) or other steps in splicing. Indeed, suppressors in Prp8 have previously been identified at some of these same positions: V1862A/Y/D and T1982A were identified as suppressors of, respectively, the *U4-cs1* allele (Kuhn and Brow, 2000; Kuhn et al., 1999) or the 5' SS U2A mutant (Siatecka et al., 1999). Finally, given our observations with the Cef1 and Prp8 mutants, we wondered about the generality of 2nd step splicing factor suppression. Several dominant mutants in the 2nd step factor Prp18 were previously shown to suppress *ts* phenotypes due to various mutations in *Slu7* (Aronova et al., 2007). We wondered if these Prp18 mutants would also suppress the *ts* (or *cs*) phenotype due to Fyv6 loss. Indeed, the Prp18 V191A suppressor of *slu7* alleles was able to improve growth at 37°C of *fvy6Δ* yeast (figure 6-figure supplement 2). Together our results from the suppressor analysis support a model in which a given 2nd step suppressor mutation may be able to restore splicing activity lost by a variety of mechanisms.

A Prp22-Dependent Splicing Stall is Relieved by Fyv6 Deletion

The final *fvy6Δ* suppressor mutation that we studied was the Prp22 I1133R mutant. This mutation is located within the C-terminal domain of Prp22 that enters the spliceosome active site and is located proximal to U2 snRNA nucleotide A31 (**Figure 7A**). Fyv6 bridges over this Prp22-U2 snRNA interaction. The I1133R mutation might stabilize Prp22/U2 snRNA contacts that can compensate for Fyv6 loss and promote exon ligation. The Prp22 I1133R mutant is able to weakly suppress both the *cs* and *ts* phenotypes in *fvy6Δ* yeast (**Figure 7B, C**). Given its location in the C-terminal domain, we wondered if it or *fvy6Δ* would show synthetic genetic interactions or epistasis with mutations in the helicase domain. We tested two helicase domain mutations, R805A and

G810A, both of which are known to impact Prp22-dependent proofreading (Mayas et al., 2006 [↗](#)). The Prp22 R805A mutant is by itself *cs* (Schwer and Meszaros, 2000 [↗](#)) and showed a synthetic lethal interaction with *fyv6Δ* (Figure 7B [↗](#), 7C [↗](#)). This was only slightly rescued by simultaneously introducing I1133R into Prp22. In contrast, Prp22 G810A mutant is viable in the presence and absence of Fyv6 as well as in combination with the I1133R suppressor mutation (Figure 7B [↗](#), 7C [↗](#)). Prp22 G810A by itself does not suppress the *ts fyv6Δ* phenotype; however, the Prp22 G810A/I1133R double mutant is a stronger suppressor of the *ts* phenotype than either mutant alone. These additive genetic interactions suggest that the Prp22 *fyv6Δ* suppressor mutation (I1133R) and the helicase domain mutations (R805A and G810A) impact distinct Prp22 functions. These functions could correspond to Prp22's role in promoting exon ligation during the 2nd step (the C-terminal domain mutation) and its role in promoting ATP-dependent mRNA release and proofreading (the helicase domain mutations).

Recently, it was reported that the Prp22 G810A mutant could activate usage of some alternative 3' SS that are also activated by *PRP18* deletion (Roy et al., 2023 [↗](#)). We therefore wondered if any of the Prp22 mutants could activate alternative 3' SS also activated by *FYV6* deletion. We isolated RNA from these strains and analyzed *SUS1* splicing by RT-PCR (Figure 7D, E [↗](#)). None of the Prp22 mutants activated usage of the *SUS1* alternative 3' SS or enhanced usage of this site beyond what is observed upon Fyv6 deletion. However, we noticed that the Prp22 helicase mutants, R805A and G810A, both caused an accumulation of unspliced pre-mRNA. This is consistent with these mutations causing a block during pre-mRNA splicing and having a different effect on splicing than Prp22 I1133R. Surprisingly, the block due to the G810A mutation was relieved in the *fyv6Δ* strains and little pre-mRNA accumulation was observed in the absence of Fyv6. This result suggests that Fyv6 could act as a negative regulator of splicing of the *SUS1* transcript when a Prp22 helicase domain mutant is present. Removing Fyv6 restores splicing by activation of the *SUS1* BP proximal 3' SS.

To further explore the connections between Fyv6 and Prp22 fidelity, we used the ACT1-CUP1 assay in which yeast growth in the presence of increasing concentrations of Cu²⁺ is proportional to the extent of splicing of a reporter RNA (Figure 7F, G [↗](#); figure 7-figure supplement 1, 2). Since the Prp22 R805A mutant grew poorly, we only assayed the Prp22 G810A, I1133R, and G810A/I1133R (double) mutants in the presence and absence of Fyv6. Consistent with previous results, we found that *fyv6Δ* is deleterious for splicing of the WT ACT1-CUP1 reporter containing consensus splice sites. Copper tolerance was improved when *fyv6Δ* was combined with Prp22 I1133R. Unexpectedly, Prp22 I1133R was deleterious when Fyv6 was present for this reporter. If this Prp22 mutant and Fyv6 both promote the 2nd step, then their combination may have an additive, negative effect on splicing due to over-stabilization of this spliceosome conformation.

Finally, we assayed strains containing a 3' SS UAG to gAG reporter since it was previously shown that Prp22 helicase domain mutants could increase usage of nonconsensus 3' SS due to faulty proofreading activity (Figure 7F, G [↗](#)). In agreement with this, we observed a dramatic increase in copper tolerance when Prp22 contained both the C-terminal domain and helicase mutants (double). This suggests that splicing of nonconsensus 3' SS splice sites is enhanced both by stabilization of the 2nd step conformation (I1133R) and perturbing mRNA release (G810A). This effect on Prp22 is Fyv6-dependent since loss of Fyv6 results in very poor copper tolerance of strains containing the gAG reporter, likely since this 3' SS is located far from the BP (38 nt). These results show that *fyv6Δ* can be suppressed with a Prp22 C-terminal domain mutant and that mutations in the Prp22 C-terminal domain can have distinct functional consequences from those located in the helicase domain. In the case of *SUS1*, stalling of splicing due to the Prp22 G810A mutant can be relieved by removing Fyv6 and activation of a BP proximal 3' SS.

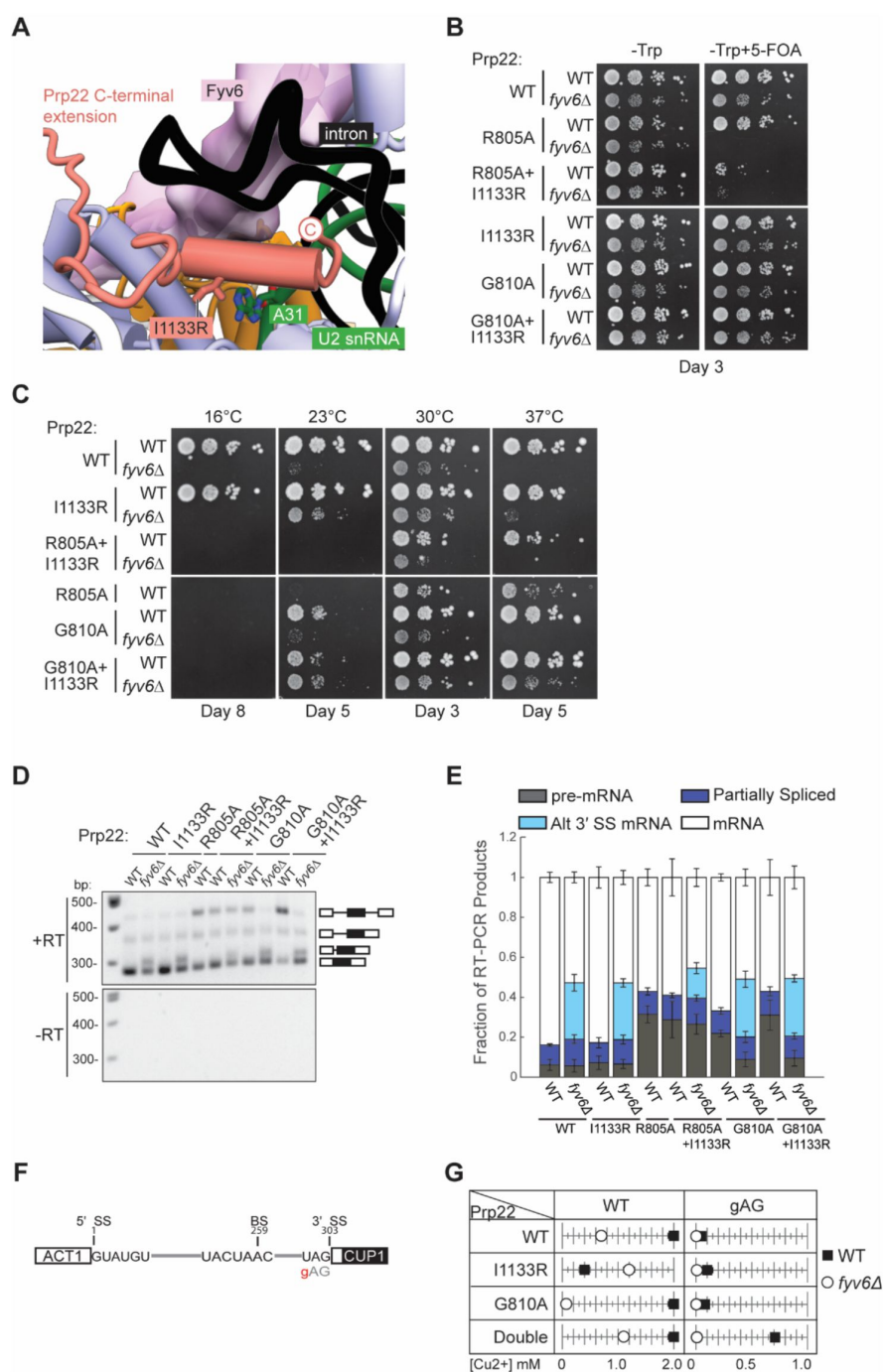


Figure 7.

Genetic interactions between Prp22 and Fyv6.

A) Location of the Prp22 I1133R *fyv6Δ* suppressor in the P complex structure. **B)** Spot dilution growth assay with Prp22 mutants in WT or *fyv6Δ* backgrounds grown on -Trp DO or -Trp+5-FOA plates. Plates were imaged after 3 days at 30°C. **C)** Spot dilution growth assay with strains containing Prp22 mutants on YPD plates at different temperatures. Plates were imaged after the number of days indicated. **D)** Representative gel of *SUS1* RT-PCR products in strains with Prp22 mutants in the presence or absence of Fyv6. **E)** Quantitation of band intensities for each *SUS1* splice isoform as a fraction of total product in the lane. Bars indicate average $\pm$ SD of $N=3$ replicates. **F)** ACT1-CUP1 reporter construct with the location of the 3' SS substitution noted. **G)** Copper tolerance for each ACT1-CUP1 reporter with each Prp22 allele in strains with (●) or without (○) Fyv6 present. Shown is a representative, single replicate of $N=3$.

Discussion

Here, we establish Fyv6 as a 2nd step splicing factor in yeast that promotes usage of BP-distal 3' SS. Structural, genetic, and biochemical studies reveal critical contacts and interactions between Fyv6 and the splicing machinery. From these data, we propose that Yju2/Syf1 interactions need to be disrupted for proper Fyv6 recruitment and that Fyv6 can enforce the Prp22-dependence of at least some splicing events. Combined, our data allow us to propose a model for Fyv6's role during the 2nd step (**Figure 8**). In this model, Fyv6 is recruited to the C* complex after Prp16-dependent release of Yju2, which exposes the binding site for Fyv6 on Syf1. Once bound, Fyv6 (possibly in concert with Slu7 and Prp18) facilitates docking of BP distal 3' SS into the active site. In the absence of Fyv6, the use of BP distal 3' SS becomes more difficult and BP proximal 3' SS are favored. Under these conditions, the requirement for Prp22 during exon ligation is reduced and Yju2 may also potentially rebind Syf1. One or both of these factors could contribute to formation of a lower fidelity spliceosome with relaxed sequence constraints for exon ligation.

Structural Insights into the Recruitment and Release of 2nd step Factors

A key principle of spliceosome structural biochemistry is the presence of mutually exclusive interactions that coordinate the stepwise and directional progression of splicing (Townsend et al., 2020). Our cryo-EM structure of the P complex spliceosome suggests another such interaction is involved in the 1st to 2nd-step transition during which Fyv6 displaces Yju2. Yju2 and Fyv6 occupy overlapping binding sites on the splicing factor Syf1 and, in fact, unassigned density in spliceosome P complexes due to Fyv6 was previously mis-interpreted as Yju2 due to their structural similarity (Wilkinson et al., 2017; Wilkinson et al., 2021). We propose that Prp16-promoted spliceosome remodeling results in release of Yju2 from the spliceosome C complex and permits mutually exclusive binding of Fyv6. Fyv6 remains bound in the P complex but then may dissociate to permit re-binding of Yju2, which was observed by cryo-EM to be a component of the yeast ILS complex (Wan et al., 2017). It should be noted, however, that intron-lariats only weakly co-immunoprecipitate with Yju2 (Y.-C. Liu et al., 2007) and that Yju2's presence in the cryo-EM data for ILS complexes could originate from the complexes being purified with a TAP-tag on Yju2 itself (Wan et al., 2017). Since Yju2 has also not been observed in metazoan ILS complexes, its role in spliceosome disassembly and the need for Fyv6-Yju2 exchange during the P to ILS transition should be studied further.

Previously, Chiang and Cheng noted that while the N-terminal half of Yju2 was able to restore 1st step activity to Yju2-depleted extracts, it is much more stably associated if a second polypeptide containing the C-terminal, Syf1-interacting half was included (Chiang and Cheng, 2013). It is possible that competition between Yju2 and Fyv6 for Syf1 binding can explain these results. If only the N-terminal half of Yju2 is present, Fyv6 could associate prematurely with Syf1 and result in destabilization of the 1st step conformation in favor of 2nd step interactions. This may then result in weaker binding for the N-terminal half of Yju2. Failure to properly recruit and exchange the Yju2/Fyv6 interaction on Syf1 may also be the origin of some of the yeast growth and splicing phenotypes we observe (**Figure 5**).

The need for Yju2/Fyv6 exchange hints at two distinct functions for Fyv6: a role in Yju2 displacement to prevent its re-binding during exon ligation and a role in stabilization of the 2nd step spliceosome conformation. These two functions can potentially result in the distinct *cs* and *ts* phenotypes in yeast. While more work is needed to dissect these phenotypes, it is possible that the *cs* phenotype results from increased stability of the Yju2/Syf1 interaction at lower temperatures

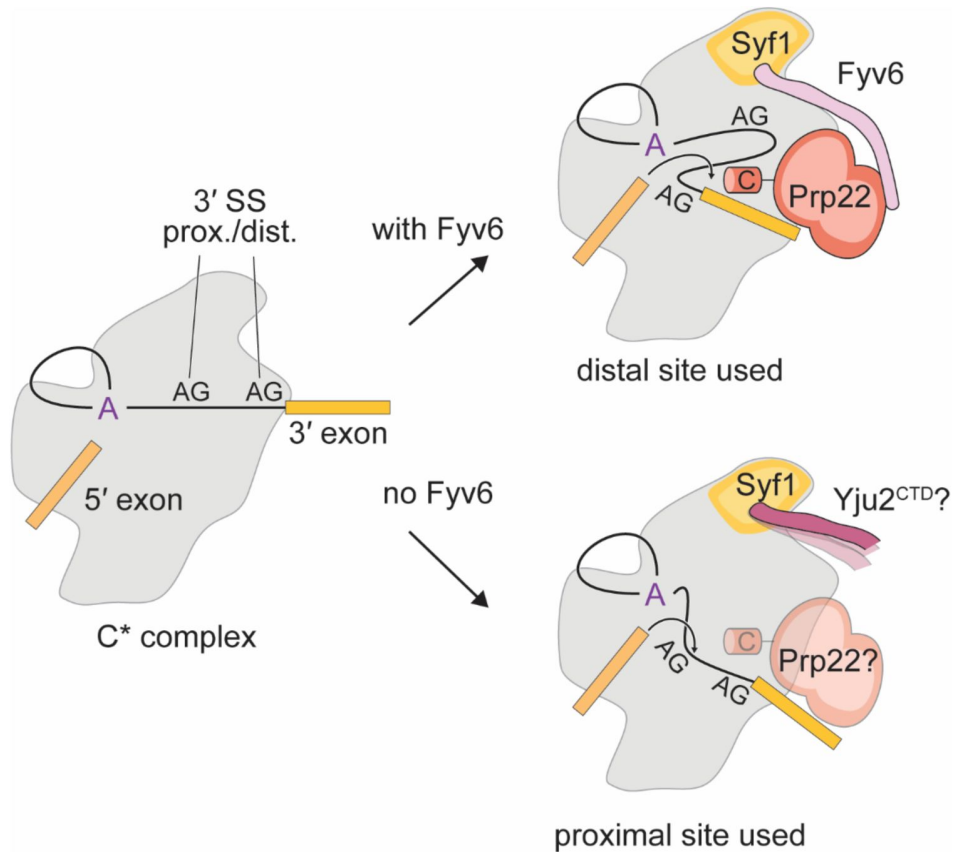


Figure 8.

Model for the role of Fyv6 during the 2nd step.

Fyv6 interacts with Syf1 and Prp22 and promotes usage of BP distal 3' SS. When Fyv6 is absent, BP proximal 3' SS are favored. It is possible that lack of Fyv6 also results in the presence of Yju2 during exon ligation and relaxing of the requirement for Prp22 at this step.

and the need for Fyv6 to act as a competitor of this interaction. The *ts* phenotype on the other hand may result from destabilization of the 2nd step conformation of the spliceosome active site due to loss of Fyv6. It is possible that loss of Fyv6 results in destabilization due to removal of a buttress that holds the RNase H-like domain of Prp8 in place (along with Prp18), changes in how the C-terminal domain of Prp22 interacts with the spliceosome active site, removal of an impediment to reversal of the U2 snRNA/BS duplex conformational change that occurs during the C to C* complex transition, or a combination of these factors.

The high quality of our cryo-EM data combined with 3D classification also provide new insights into the requirements for 3'SS docking into the active site and potentially for how the P complex spliceosome is disassembled into the ILS complex. It was previously speculated that the N-terminal domain of Slu7 may be critical for 3'SS docking by reducing the entropic cost of this process when the BP to 3'SS distance is long (Wilkinson et al., 2017). In our state II P complex structure, the 3'SS remains docked but this domain of Slu7 is not observed. This suggests that either Slu7 N-terminal domain interactions with Cwc22 and Prp8 are not essential for 3'SS docking into the active site for exon ligation and/or that these interactions are not essential for maintaining 3'SS docking within P complex once they form in C*.

In the state III P complex structure, densities for the 2nd step factors Fyv6, Prp18, and Slu7 are entirely missing and the 3'SS is no longer docked into the active site. While it is possible that this may reflect decomposition of the P complex (or another intermediate) by a pathway unrelated to cellular spliceosome disassembly, the structure is suggestive that 3'SS undocking is coupled to 2nd step factor release. These steps may be necessary to help prepare the spliceosome for ILS complex formation since the 3'SS is undocked in all ILS structures obtained to date (Vorländer et al., 2024; Wan et al., 2017; Yan et al., 2015; Zhang et al., 2019). Prp22 is still present in this structure, and it is possible that state III represents a conformation occurring after exon ligation but prior to Prp22-dependent mRNA release. In this case, the state III structure implies that Prp22 ATPase activity is not needed for dissociation of Fyv6, Prp18, and Slu7. It is also interesting to note that the C-terminal domain of Prp22 can no longer be resolved in the state III structure. What exactly activates the Prp22 ATPase to promote proofreading or mRNA release is not known. It is conceivable that undocking of the 3'SS from the active site can be sensed by the Prp22 C-terminal domain and this contributes to ATPase activation. This model differs from that previously proposed for Prp22-enabled 3'SS sampling in which that ATPase activity of Prp22 is needed to undock the 3'SS (Semlow et al., 2016). However, sampling could involve multiple steps: ATP-independent 3'SS undocking and ATP-dependent entry into the sampling state. Whether or not the 2nd step factors must release or alter conformation to enable sampling is unknown. Regardless of the exact mechanism, it is important to recognize that the P complex structures determined here were all obtained using a Prp22 mutant defective in mRNA release. The chemical and kinetic competencies of these states will require future investigations.

Multiple, Non-Redundant 2nd Step Factors Promote Use of BP Distal 3'SS

The challenge of 3'SS selection involves not just selection of the correct nucleotide sequence (YAG) but also identifying which YAG sequence to use. Here, we define a function for Fyv6 in facilitating use of BP distal 3'SS, especially those located ≥ 21 nt away from the branch. This function is similar to those of other 2nd step factors important for efficient splicing when the BP-3'SS distance is ≥ 12 nt (Slu7, Prp18) or ≥ 21 nt (Prp22) (Brys and Schwer, 1996; Schwer and Gross, 1998; Zhang and Schwer, 1997). Human Slu7 has additionally been reported to be important for preventing suppression of the correct 3'SS in favor of up- or downstream alternative 3'SS (Chua and Reed, 1999). Our previous study using competing 3'SS in the ACT1-CUP1 intron did not provide evidence for suppression of the correct 3'SS in the absence of Fyv6, suggesting that this function is not shared between human Slu7 and yeast Fyv6. Nonetheless, it is important to recognize that the

splicing machinery employs multiple factors that enforce usage of BP distal 3'SS. These functions are not redundant as changes in 3'SS are still observed in the absence of Fyv6 but presence of Prp18 and vice versa (Roy et al., 2023 [↗](#)).

Intron architecture can also contribute to 3'SS selection since canonical “AG” 3'SS are depleted in humans in the region between the BP and annotated 3'SS (the AG exclusion zone, or AGEZ) (Corvelo et al., 2010 [↗](#); Gooding et al., 2006 [↗](#)). AG sites are also typically depleted in the corresponding region in yeast introns (data not shown). This would suggest that annotated 3'SS are also the most optimal sites located downstream of the BP. However, an important assumption of this model is that non-AG 3'SS can also be effectively proofread and not used. Deletion of either Prp18 or Fyv6 leads to activation of not just BP proximal 3'SS but also those with highly divergent sequences from the YAG consensus (Roy et al., 2023 [↗](#)). This suggests that an AGEZ alone is insufficient for correct 3'SS usage and that mechanisms to ensure preferential use of YAG sites are critical.

It is likely that Fyv6 and Prp18 enforce usage of YAG sites by common as well as factor-specific mechanisms. Both proteins stabilize the conformation of the Prp8 RNase H-like domain in P complex. Numerous splice site suppressor mutations map to this domain including those that enhance splicing of non-YAG 3'SS (Galej et al., 2013 [↗](#)). Fyv6 and Prp18 may be essential for establishing a “high fidelity” conformation of the RNase H-like domain that enforces use of canonical YAG 3'SS and preventing activation of cryptic sites within the AGEZ. In support of this, we note that in our state III P complex structure that lacks both Fyv6 and Prp18 and a stably docked 3'SS, the RNase H-like domain is also more flexible.

In addition to this possibility, Fyv6 and Prp18 may also contribute uniquely to 3'SS fidelity and selection. The conserved loop region of Prp18 inserts into the spliceosome active site and contacts the 3'SS. Partial deletion of this region of Prp18 phenocopies at least some of the changes in 3'SS selection when Prp18 is deleted entirely (Crotti et al., 2007 [↗](#); Roy et al., 2023 [↗](#)).

This indicates that even when the Prp8 RNaseH-like domain is still stabilized by Prp18 (and Fyv6) disruptions to 3'SS selection can still occur. Unlike Prp18, Fyv6 does not contact the spliceosome active site. However, it is the only 2nd step factor that contacts exon ligation fidelity factor Prp22. While these contacts are not essential for suppressing use of an alternative 3'in *SUS1*, a mutation in Prp22 can nonetheless suppress a *fyv61* phenotype and loss of Fyv6 relieves a Prp22 helicase mutant-dependent stall in *SUS1* splicing. These observations suggest that Fyv6 and Prp22 are functionally connected to one another.

Some 2nd Step Factors May Enforce Usage of Prp22 during Exon Ligation

We were particularly struck by the similarities in the BP-3'SS distances for the requirement of Prp22 during exon ligation and for promotion of BP distal 3'SS usage by Fyv6, both ≥ 21 nt (Schwer and Gross, 1998 [↗](#)). In addition, the alternative, BP proximal 3'SS that are activated when Fyv6 is deleted show much less sequence conservation relative to the annotated 3'SS and include sequences (GAG, BG) known to be proofread and rejected by Prp22. Together, this suggests that one function of Fyv6 is to enforce Prp22-dependence of exon ligation by stabilizing a spliceosome conformation that more easily enables usage of BP distal 3'SS (Figure 8 [↗](#)). As these sites require Prp22 for exon ligation, it is also likely that they are subject to Prp22-dependent proofreading, and this may enforce accurate exon ligation at the YAG 3'SS consensus. When Fyv6 is absent, many alternative, BP proximal 3'SS are activated. These sites could have a relaxed requirement for Prp22 and proofreading. While the Fyv6-dependence of Prp22 occupancy is beyond the scope of the current manuscript, loss of Prp18 (which also activates usage of non-canonical, BP-proximal 3'

SS) does result in lower occupancy of Prp22 on the *ACT1* intron (Strittmatter et al., 2021). This suggests that Prp22 occupancy, and possible involvement in the exon ligation step, can be influenced by 2nd step factors.

There is precedent for nonessential splicing factors enforcing use of a proofreading ATPase. Cus2 is not essential for pre-spliceosome formation (Yan et al., 1998). However, when Cus2 is present then ATPase activity of Prp5 is essential for disruption of the Hsh155/Cus2 interaction (Perriman et al., 2003; Talkish et al., 2019). In this case, Cus2 is not essential for Prp5 proofreading of BP selection, and the connection between the ATP hydrolysis activity of Prp5, removal of Cus2, and proofreading is unclear (Liang and Cheng, 2015; Talkish et al., 2019; Xu and Query, 2007). It is possible that enforcement of Prp5 ATPase activity by Cus2 is important for other aspects of pre-spliceosome formation *in vivo* and the impact of Prp5 and Cus2 on BP selection has not yet been studied transcriptome-wide using high-resolution, sequencing-based approaches. The C-terminal domain of the 1st step splicing factor Yju2 (the same domain that competes with Fyv6 for Syf1 binding) is also not essential for 5'SS cleavage. In the absence of this domain, the N-terminal domain of Yju2 is sufficient for promoting the 1st step reaction, and the spliceosome can transition to the 2nd step and carry out exon ligation without Prp16, albeit inefficiently (Chiang and Cheng, 2013). Importantly, addition of the C-terminal domain on a separate polypeptide reduces Prp16-independent activity. This suggests that the Yju2 C-terminal domain enforces the use of the Prp16 ATPase. As we propose with Fyv6 and Prp22, the C-terminal domain of Yju2 may not just help to promote the 1st to 2nd step transition by enforcing Prp16-dependence but could also facilitate Prp16-dependent proofreading during 5'SS cleavage (Koodathingal et al., 2010; Semlow et al., 2016). General operating principles of the yeast splicing machinery may not just include proofreading but also use of splicing factors that enforce usage of the proofreading ATPases.

Materials and methods

Key Resources Table				
Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information

strain, strain backgr ound (<i>Sacch aromyc es cerevisi ae</i>)	Various S. cerevisia e strains			See Supplementary Table 6 for full list
recomb inant DNA reagent	Various plasmids			See Supplementary Table 7 for full list
Sequen ce- based reagent	Various oligonucl eotides			See Supplementary Table 8 for full list
antibod y	Anti- FLAG (Mouse monoclo nal)	Sigma-Aldrich	Cat #: F1804; RRID:A B_26204 4	WB (1:1000)
antibod y	Anti- actin (mouse monoclo nal)	Sigma-Aldrich	Cat #: MAB150 1; RRID:A B_22230 41	WB (1:5000)
antibod y	Anti- mouse IgG – HRP (Goat polyclon al)	Bio Rad	Cat #: 1706516 ; RRID:A B_11125 547	WB (1:8000)
peptide , recomb inant protein	Bovine Serum Albumi n	Sigma	Cat#: A7 906	

commercial assay or kit	Clarity Western ECL substrate	Bio Rad	Cat #: 1705060	
commercial assay or kit	Monarch RNA Cleanup Kit (50 ug)	New England Biolabs	Cat #: T2040	
commercial assay or kit	TURBO DNA-free kit	Thermo Fisher Scientific	Cat #: AM1907	
commercial assay or kit	TruSeq Stranded mRNA kit	Illumina	Cat #: 20020594	
commercial assay or kit	Master Pure Yeast RNA Purification kit	LGC Biosearch Technologies	Cat #: MPY03100	
commercial assay or kit	Access RT-PCR System	Promega	Cat #: A1250	
chemical compound, drug	Ni-NTA agarose	Qiagen	Cat #: 30210	
chemical compound, drug	Amylose resin	New England Biolabs	Cat #: E8022S	
chemical compound	Fluorescein-5-thiosem	Sigma-Aldrich	Cat #: 46985-100MG-F	

und, drug	icarbazi de			
softwar e, algorith m	RELIO N-5.0	Kimanius et al., 2024; doi: 10.1038/s41592 -024-02304-8	RRID:S CR_016 274	https://relion.readthedocs.io/en/latest/index.html
softwar e, algorith m	Topaz	Bepler et al., 2019; doi: 10.1038/s41592 -019-0575-8		https://cb.csail.mit.edu/topaz/
softwar e, algorith m	Coot	Casañal et al., 2020; doi: 10.1002/pro.379 1	RRID:S CR_014 222	https://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/
softwar e, algorith m	ISOLD E	Croll, 2018; doi: 10.1107/S20597 98318002425	RRID:S CR_025 577	https://tristanic.github.io/isolde/
softwar e, algorith m	PHENI X	Liebschner et al., 2019; doi: 10.1107/S20597 98319011471	RRID:S CR_014 224	https://phenix-online.org/
softwar e, algorith m	UCSF Chimer aX	Petterson et al., 2021; doi: 10.1002/pro.394 3	RRID:S CR_015 872	http://www.cgl.ucsf.edu/chimerax/
softwar e, algorith m	R Project for Statistic al Comput ing	R Foundation for Statistical Computing	RRID:S CR_001 905	https://www.r-project.org/
softwar e, algorith m	FASTQ C	Andrews, 2010	RRID:S CR_014 583	http://www.bioinformatics.babraham.ac.uk/projects/fastqc/
softwar e, algorith m	Fastp	Chen et al., 2018; doi:10.1093/bioi	RRID:S CR_016 962	https://github.com/OpenGene/fastp

algorithm		nformatics/bty560		
software, algorithm	STAR	Dobin et al., 2013; doi: 10.1093/bioinformatics/bts635	RRID:SCR_004463	https://github.com/alexdobin/STAR/releases
software, algorithm	SAMtools	Li et al., 2009; doi: 10.1093/bioinformatics/btp352	RRID:SCR_002105	https://www.htslib.org/
software, algorithm	Salmon	Patro et al., 2017; doi: 10.1038/nmeth.4197	RRID:SCR_017036	https://combine-lab.github.io/salmon/
software, algorithm	DeSeq2	Love et al., 2014; doi: 10.1186/s13059-014-0550-8	RRID:SCR_015687	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
software, algorithm	featureCounts	Liao et al., 2014; doi: 10.1093/bioinformatics/btt656	RRID:SCR_012919	https://bioconductor.org/packages/release/bioc/html/Rsubread.html
software, algorithm	SpliceWiz	Wong et al., 2024; doi: https://doi.org/10.1093/bib/bbad468		https://github.com/alexchwong/SpliceWiz
software, algorithm	ImageJ 1.53v	Schneider et al., 2012; doi:10.1038/nmeth.2089	RRID:SCR_003070	https://imagej.net/ij/
other	Cryo-EM grid	Quantifoil	Cat #: Q350CR2-2NM	
other	NuPAGE Bis-Tris mini protein gel	Invitrogen	Cat #: NP0322	

other	NuPAGE™ MES SDS Running Buffer	Invitrogen	Cat #: NP0002	
other	Standard Vitrobot Filter Paper, Ø55/20 mm, Grade 595	Ted Pella	Cat #: 47000-100	
other	Mixed cellulose ester filter paper	Whatman	WHA714 0104	
other	TRIzol	Thermo Fisher Scientific	Cat #: 15596018	
other	SuperScript III reverse transcriptase	Thermo Fisher Scientific	Cat #: 18080044	
other	Zymolyase	Zymo Research	Cat #: E1005	Used at 50 U/mL

Yeast growth and culture

Yeast transformation, plasmid shuffling/5-FOA selection, and growth were carried out using standard techniques and media (Trecó and Lundblad 1993 [↗](#); Sikorski and Boeke 1991 [↗](#)).

Cryo-electron microscopy

Purification of dominant-negative Prp22

Yeast Prp22 with a dominant-negative S635A mutation was cloned into pRS424 and pRS426 vectors after an N-terminal CBP-His-TEV tag (Galej et al., 2013 [↗](#)). The protein was expressed in 24 L of BCY123 yeast cells essentially as described (Galej et al., 2013 [↗](#)). Recombinant Prp22 was then purified first by Calmodulin-Sepharose in a buffer containing 50 mM Tris-HCl pH 8.5, 350 mM NaCl, 2 mM CaCl₂, 1 mM Mg acetate, 1 mM imidazole, 0.1% NP-40 substitute, 5 mM β-mercaptoethanol and eluted with 3 mM EGTA. The protein was dialyzed into 20 mM Tris-HCl pH 7.4, 350 mM NaCl, 10 mM imidazole, 0.1% NP-40 substitute and further purified over Ni-NTA resin at 500 mM NaCl and eluted with 200 mM imidazole. The eluted protein was treated with His-tagged TEV protease during dialysis against 20 mM HEPES-KOH pH 7.9, 300 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM DTT, before removal of the protease with Ni-NTA resin. The final protein was frozen in liquid nitrogen and stored at -80 °C.

Preparation and purification of P complex for cryo-EM

P complex was purified as described in (Wilkinson et al., 2017 [DOI](#)). Brr2-TAPS yeast were grown in a 120 L fermenter and splicing extract was prepared by the liquid nitrogen method essentially as previously described (Lin et al., 1985 [DOI](#)). A pre-mRNA substrate consisting of 20 nt 5'-exon, 95 nt intron, and 32 nt 3'-exon from the UBC4 pre-mRNA followed by 3xMS2 stem loops was generated by run-off in vitro transcription from an EcoRI-linearized plasmid template. The RNA product was labelled at the 3' end with fluorescein-5-thiosemicarbazide (Wu et al., 1996 [DOI](#)). A 180 mL in vitro splicing reaction was performed containing 60 mM potassium phosphate pH 7, 2 mM ATP, 2.5 mM MgCl₂, 3% w/v PEG8000, 3 nM pre-mRNA, 37.5 nM MS2-MBP protein, and 40% (v/v) splicing extract treated with dominant negative Prp22 S635A. Reactions were incubated for 30 min at 23°C, then incubated for 20 min with 5 mM of a DNA oligonucleotide complementary to the 3'-exon (oligo sequence 5'-ATGAAGTAGGTGGAT-3') to induce cleavage of the 3'-MS2 tag by the endogenous RNase H activity of the splicing extract. The reaction mixture was centrifuged through a 40% glycerol cushion in buffer A (20 mM HEPES, pH 7.9, 75 mM KCl, 0.25 mM EDTA). The cushion was collected and applied to amylose resin in the presence of 0.025% NP-40 substitute. After overnight incubation at 4°C the resin was washed and eluted with buffer A containing 5% glycerol, 0.01% NP-40 substitute and 12 mM maltose. Fractions containing spliceosomes were concentrated to 0.1 mg/mL then dialyzed against buffer A for 3 h.

For cryo-EM grid preparation, a freshly glow-discharged Cu300 R2/2 holey carbon grid with a 2-nm layer of amorphous carbon (Quantifoil) was mounted in the chamber of a Vitrobot Mark IV (Thermo Fisher Scientific) maintained at 4°C and 100% humidity. Spliceosomes (3 µL) were applied and after 60 s was manually blotted using Ø55 grade 595 filter paper (Ted Pella), followed by application of another 3 µL of spliceosomes, waiting 60 s, before blotting again and plunging into liquid ethane.

Cryo-EM data collection

Cryo-EM data were collected as two datasets from two grids made from the same sample. Dataset 1 was acquired using a Thermo Scientific Titan Krios cryo-TEM (LMB Krios 1) and dataset 2 was acquired using a Thermo Scientific Titan Krios G3i cryo-TEM (LMB Krios 3). Both used a K3 direct detector (Gatan) operated in super-resolution mode with 2-fold binning, and an energy filter with slit width of 20 eV. Micrographs were collected automatically using EPU in AFIS mode, yielding a total of 51,113 movies at 81,000x or 130,000x magnification with a real nominal pixel size of 0.93 Å or 0.669 Å, with defocus ranging from -1.3 µm to -3.1 µm and a total fluence per micrograph of 40 e-/Å², fractionated into 40 frames.

Cryo-EM data processing

All cryo-EM data were processed using RELION-5.0 (Kimanius et al., 2024 [DOI](#)). Both datasets were initially processed separately. Movies were corrected for motion using the RELION implementation of MotionCor2, with 4x4 patches and dose-weighting. CTF parameters were estimated using CTFFIND-4.1. Particle picking was done using Topaz with the general model (Bepler et al., 2019 [DOI](#)), yielding 1,820,457 particles. 3D classification was performed on 4-fold (dataset 1) or 5-fold (dataset 2) binned particles using a P-complex reference map (EMD-10140), and 299,741 (dataset 1) or 599,123 (dataset 2) were selected and refined to 2.99 Å or 2.78 Å resolution respectively. After Bayesian Polishing and CTF refinement (per-particle defocus, anisotropic magnification, beam tilt, trefoil, and 4th order aberrations) the resolution improved to 2.67 Å or 2.32 Å respectively. To improve the general density quality, 3D classification without alignment was performed with a soft mask around the whole complex with T=15. Particles with good density, without selecting for Fyv6 occupancy, were selected (151,443 and 257,501 for datasets 1 and 2) and refined to 2.66 Å or 2.27 Å respectively. The particles were then merged, refined, and then refined for anisotropic magnification. The overall scaling factors from the

“anisotropic” magnification matrix was then used to calculate the corrected pixel size for dataset 1 as 0.94057 Å, keeping dataset 2 constant at 0.669 Å per pixel. Defocus values for dataset 1 were then scaled by the square of 0.94057 / 0.93, with an empirical constant correction of -10.06 Å (Wilkinson et al., 2019 [↗](#)). Finally, another round of per-particle defocus refinement and anisotropic magnification refinement was performed to reduce errors in defocus correction and dataset scaling. The combined particles refined to 2.24 Å resolution. Resolution is reported using the gold-standard Fourier Shell Correlation with 0.143 cutoff.

The refined particles after merging were subjected to 3D classification without alignment, with a soft mask around the entire spliceosome, with T=15. The resulting four classes were used to define States I, II and III. To improve the densities for the periphery of the spliceosome that displayed local flexibility, focused refinements were used with Blush regularization in RELION-5 to reduce overfitting (Kimanius et al., 2024 [↗](#)). The Prp22 density was improved using a soft-mask just around the Prp22 helicase domain for refinement of particles from States I and II, yielding a 2.96 Å map. Densities for the U2 snRNP, NTC, U5 Sm ring, and Cwc22 N-terminal domain were each improved first by 3D classification without alignment of particles from all three states, selecting particles with strong density (72,869 for the U2 snRNP, 260,409 for the NTC, 113,561 for the U5 Sm ring, 201,984 for Cwc22-NTD), performing signal subtraction to remove density for the rest of the spliceosome (recentering on the mask center of mass and re-boxing to 300 pixels, or 192 pixels for Cwc22-NTD), and finally 3D refinement starting with 1.8 degree local angular searches, giving 3.42 Å for the U2 snRNP, 3.72 Å for the NTC, 3.06 Å for the U5 Sm, and 3.57 Å for Cwc22-NTD. In all cases, control refinements without Blush regularization did not produce interpretable maps (figure 3-figure supplement 4).

Model building

The model was built using Coot (Casañal et al., 2020 [↗](#)) and ISOLDE (Croll, 2018 [↗](#)) starting with PDB 6EXN (Wilkinson et al., 2017 [↗](#)) as an initial model for the core and PDB 7B9V (Wilkinson et al., 2021 [↗](#)) as an initial model for the periphery. The core was improved by manual fixing in Coot, while the peripheral U2 snRNP and NTC were improved by applying torsion and distance restraints to reference models produced by AlphaFold2. The model was refined using PHENIX `real_space_refine` (Liebschner et al., 2019 [↗](#)), just performing one macro-cycle. Figures were generated using UCSF ChimeraX.

Plasmid cloning

Fyv6 plasmids were made by PCR amplification from gDNA of the *FYV6* coding sequence plus ~250 nt upstream and downstream, restriction digest with NotI and SalI, and ligation into pRS414 or pRS416. An N-terminal 1x FLAG tag was added onto Fyv6 in the plasmids by PCR mutagenesis. Fyv6 truncations were cloned by Genewiz (Azenta Life Sciences) from pAAH1572. Restriction digest and ligation were used to place Fyv6 constructs into the pRS413 backbone.

Syf1 plasmids were made by PCR amplification from gDNA of the *SYF1* coding sequence plus ~275 nt upstream and downstream, restriction digest, and ligation into pRS414 or pRS416.

Cef1 mutant plasmids, Prp22 I1133R mutant plasmid, Prp8 mutant plasmids, ACT1-CUP1 BP-3'SS distance reporters, and Syf1 truncation plasmids were prepared by PCR-based site directed mutagenesis of pAAH1611, pAAH1042, pAAH1440, pAAH0470, and pAAH1625 respectively. All plasmids were fully sequenced to confirm their nucleotide sequence.

Yeast strain creation

The *DBR1* and *FYV6* genes were deleted by replacement with a nourseothricin resistance cassette (NatMX4) or hygromycin resistance cassette (hphMX4), respectively, through homologous recombination (see supplementary table 7; Goldstein and McCusker, 1999 [↗](#)). The *SYF1* and *CEF1*

genes were deleted by replacement with a kanamycin resistance cassette (KanMX) through homologous recombination in strains transformed with pRS416-Syf1 or pRS316-Cef1 respectively. Gene deletion was confirmed by colony PCR and/or genomic DNA extraction from the strains and PCR amplification of the corresponding genomic locus.

RNA isolation

Yeast were grown in YPD media until OD₆₀₀ 0.5–0.8. For RNA-sequencing of temperature-shifted samples, cultures were shifted to either 37°C or 16°C or remained at 30°C and grown for 1 hr. A 15 mL volume of culture was harvested via vacuum filtration with mixed cellulose ester filter paper (Whatman, 47 mm diameter) in a Buchner funnel. Filter papers were separated into 5 mL tubes and flash frozen. Filters were stored at –80°C until RNA isolation. Filters were washed with 1 mL TRIzol to collect cells. For all other experiments involving RNA isolation, 10 OD₆₀₀ units of OD₆₀₀ 0.5–0.8 yeast were harvested via centrifugation.

Cell walls were disrupted by incubation with Zymolyase (50 U/mL, Zymo Research) in Y1 buffer (1 M sorbitol, 100 mM EDTA pH 8.0, 13 mM β-mercaptoethanol) (primer extension, RNA-seq of non-temperature-shifted samples) or vortexing with silica disruption beads in TRIzol (RT-PCR, RNA-seq of temperature-shifted samples). Total RNA was then isolated using TRIzol reagent (Thermo Fisher Scientific) according to manufacturer's instructions. Samples were cleaned using a 50 µg Monarch RNA Cleanup Kit (New England Biolabs) and treated with TURBO DNase (Thermo Fisher Scientific) to remove residual contaminating DNA. For RNA-sequencing, quality of RNA samples was assessed prior to library preparation by NanoDrop (concentration, A₂₆₀/A₂₈₀, and A₂₆₀/A₂₃₀), Qubit (RNA High Sensitivity and RNA IQ), and TapeStation (RNA ScreenTape Analysis). High quality samples showing minimal rRNA degradation with RIN >7 and A₂₆₀/A₂₈₀ ratios of ~2.0 were selected for library preparation.

Library preparation and RNA sequencing

Library preparation was conducted by the University of Wisconsin-Madison Biotechnology Center Gene Expression Center. mRNA was selected from total RNA samples through polyA enrichment with the TruSeq Stranded mRNA kit (Illumina). Sequencing was performed with an NovaSeq6000 sequencer (Illumina).

Bioinformatic analysis of RNA-seq datasets

FASTQC was used for quality control of reads pre-and post-trimming (Andrews, 2010 [DOI](#)). Trimming of reads was accomplished using fastp (Chen et al., 2018 [DOI](#)) prior to mapping to the SacCer3 genome (Ensembl, R64-1-1) with STAR (Dobin et al., 2013 [DOI](#)). All samples were aligned with STAR in a first pass. Novel junctions from all samples (WT and *fjv6Δ*) were combined and filtered for likely false-positive junctions (see Supplemental Methods). STAR was run in a second pass with the additional input of the filtered set of novel junctions. Indexing of bam files was accomplished with SAMtools (Li et al., 2009 [DOI](#)). Quantitation of read counts aligned to the SacCer3 transcriptome was accomplished with Salmon (Patro et al., 2017 [DOI](#)). Differential gene expression analysis was conducted with DeSeq2 (Love et al., 2014 [DOI](#)). Junction reads within genes were counted with featureCounts (Liao et al., 2014 [DOI](#)) and used to calculate fraction of Annotation Splicing (FANs) based on methods in Roy et al., 2023 [DOI](#) (see Supplemental Methods). Canonical branch points were used as defined in the Ares Intron Database (Grate and Ares, 2002 [DOI](#)). Differential splicing analysis was conducted with SpliceWiz (Wong et al., 2024 [DOI](#)).

Primer extension

RNA was isolated using the protocol above. IR700 dye conjugated probes (Integrated DNA Technologies) were used for primer extension with SuperScript III (Thermo Fisher) of the ACT1-CUP1 reporters (2 pmol yAC6: /5IRD700/GGCACTCATGACCTTC) and U6 snRNA (0.4 pmol yU6: /5IRD700/GAACTGCTGATCATGTCTG) (Carrocci et al., 2017 [DOI](#); van der Feltz et al., 2021 [DOI](#)). Primer

extension products were visualized on a 7% (w/v) denaturing polyacrylamide gel run at 35 W for 80 min at RT. Gels were imaged with an Amersham Typhoon NIR laser scanner (Cytiva), and band intensities were quantified with Image J (version 1.53v, 2022).

RT-PCR

Yeast cultures were inoculated from cultures grown to stationary phase overnight and grown until $OD_{600} = 0.7-0.9$. RNA was isolated and depleted of contaminating DNA using the MasterPure Yeast RNA Purification Kit (LGC Biosearch Technologies) protocol as previously described (Carrocci et al., 2017) (RT-PCR shown in Fig. 4C) or using the RNA isolation protocol above (all others).

RT-PCR reactions were set up using the Access RT-PCR system (Promega Corporation) following the kit procedure with 100 ng of input RNA per 25 μ L reaction. Primers to amplify *SUS1* were SUS1-exon1 5'-TGGATACTGCGCAATTAAAGAGTC-3' and SUS1-exon3 5'-TCATTGTGTATCTACAATCTCTTCAAG-3'. Reaction products were separated on 2% (w/v) agarose-TBE gels and imaged using ethidium bromide fluorescence.

Selection of *fyv6Δ* suppressors

Strain yAAH3353 was previously described (Lipinski et al. 2023). Twenty single colonies of yAAH3353 were selected from a YPD plate and used to inoculate 20 x 5mL YPD liquid culture. Cultures were grown for 48 h at 30°C. A 1 mL volume of culture was spun down, resuspended in 200 μ L YPD liquid medium, and spread on a YPD plate containing 40 mg/L adenine hemisulfate (to prevent *ade2* reversion). Duplicate plates for each overnight were placed in incubators at either 18°C or 37°C. Many colonies of various sizes appeared on each plate by 9 days for 37°C and 12 days for 18°C. Colonies were picked after that time and annotated first by growth temperature, then by overnight number, and finally roughly by colony size with 01 generally being the largest colony on a plate (ex: 370101 from a plate grown at 37°C inoculated from overnight 01 and the largest colony selected from the plate). Picked colonies were grown overnight in 5mL YPD at 30°C and frozen stocks were made. Anywhere from 0 to ~15 colonies were picked per plate, covering the variety of colony sizes that arose.

Sequencing of yeast genomic DNA

Yeast were grown to saturated stationary phase in 5mL YPD and ~10 OD units were sent as a frozen yeast pellet to Azenta or SeqCoast for genomic DNA extraction, library, preparation, and sequencing with 30x/400 mbp coverage.

Bioinformatic analysis of WGS data

Azenta and SeqCoast utilized the SacCer3 genome (Ensembl, R64-1-1) for variant calling and provided a list of >6,000 SNPs. Since the strain is not a perfect match for this reference, non-unique SNPs were filtered from each dataset by taking the union of two sets with each set obtained from colonies isolated on different plates (to reduce the chance of computationally eliminating the same variant SNP). Filtering reduced the dataset to <100 unique variants per colony. Few unique SNPs were within protein-or snRNA-coding genes. Of SNPs within protein-coding regions, single amino acid changes were frequently found within splicing factors (see Supplemental Table S4). Datasets were reexamined upon identification of suppressor mutations for suppressor mutation duplication that were potentially filtered out during comparison of SNP datasets.

Yeast temperature growth assays

Yeast were grown to stationary phase overnight in the appropriate liquid medium indicated in the figure legends. The cultures were first diluted to OD_{600} 0.5, serially diluted 1:10, 1:100, and 1:1000, and stamped onto agar plates containing the appropriate growth medium. The plates were

incubated at 16°, 23°, 30°, and 37°C, and plates were imaged after the number of days indicated in each figure.

ACT1-CUP1 copper tolerance assays

Yeast strains expressing ACT1-CUP1 reporters were grown to stationary phase in -Leu DO media, diluted to OD₆₀₀ = 0.5 in 10% (v/v) glycerol, and spotted onto -Leu DO plates containing 0 to 2.5 mM CuSO₄ (Lesser and Guthrie 1993 [↗](#); Carrocci et al., 2018 [↗](#)). Plates were scored and imaged after 48 h of growth at 30°C for WT strains and after 72 h of growth at 30°C for *fyv6Δ* strains due to differential growth between strains.

Western blotting

Total soluble protein was isolated by TCA precipitation from 10 ODs of an overnight yeast culture. Proteins were separated with a NuPAGE 4 to 12% Bis-Tris pre-cast gel (Invitrogen) using 1X NuPAGE MES running buffer (Invitrogen) and transferred to a PVDF membrane (0.2 μm pore size) at 30V for 120 min at 4°C using 1x NuPAGE Transfer Buffer (Invitrogen) with 20% (v/v) methanol in a XCell II Blot Module (Invitrogen). The membrane was blocked with 3% (w/v) BSA (Sigma, A7906) in 1x TBST for 1h at room temperature with gentle shaking. All antibodies were diluted in 1x TBST with 3% (w/v) BSA. The primary antibodies anti-FLAG (Millipore Sigma, F1804; diluted 1:1000) and anti-actin (Sigma Aldrich, MAB1501; diluted 1:5000) were incubated overnight at 4°C. The membrane was then washed with 1x TBST before incubation with goat anti-mouse IgG HRP conjugate secondary antibody (Biorad, #1706516; diluted 1:8000) for 2h at room temperature. The membrane was then washed with 1x TBST before developing with Clarity Western ECL substrate (Biorad, #1705060) with a 20s exposure for anti-actin and a 100s exposure for anti-FLAG.

Data and materials availability

RNA-seq data has been uploaded to NCBI SRA with BioProject ID PRJNA1113593. R code for calculation of FAnS values can be found at the following DOI: 10.5281/zenodo.13798743. The cryo-EM maps and atomic model of the P complex (state I) have been deposited in the Electron Microscopy Data Bank (<https://www.ebi.ac.uk/pdbe/emdb/> [↗](#)) and in the Protein Data Bank (<https://www.rcsb.org> [↗](#)). The data are available under the following accession codes: P complex state I (EMD-47157, EMD-47160, EMD-47161, EMD-47162, EMD47163, EMD-47164; PDB 9DTR), state II (EMD-47158), and state III (EMD-47159). Raw cryo-EM data is available from EMPIAR (EMPIAR-12330).

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Additional information

Contributions

AAH, KAS, KAL, and MEW conceptualized the project; MEW collected and analyzed the cryo-EM data; KAL and NJZ prepared RNA for sequencing; KAL, NJZ, and AFG analyzed the RNA-sequencing data; AAH, KAS, NJZ, and KAL created yeast strains; KAS, KAL, NJZ, and AFG performed genetic and biochemical assays and analyzed data; KAS, KAL, MEW, and AAH wrote the manuscript.

Competing interest statement

AAH is a member of the scientific advisory board and carrying out sponsored research for Remix Therapeutics.

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Author information

Katherine A Senn*

Department of Biochemistry, University of Wisconsin-Madison, Madison, USA
ORCID iD: [0000-0003-2172-7113](https://orcid.org/0000-0003-2172-7113)

*Co-first authors

Karli A Lipinski*

Department of Chemistry, University of Wisconsin-Madison, Madison, USA
ORCID iD: [0000-0002-4271-1586](https://orcid.org/0000-0002-4271-1586)

*Co-first authors

Natalie J Zeps

Department of Biochemistry, University of Wisconsin-Madison, Madison, USA

Amory F Griffin

Department of Biochemistry, University of Wisconsin-Madison, Madison, USA
ORCID iD: [0009-0004-0976-3749](https://orcid.org/0009-0004-0976-3749)

Max E Wilkinson

MRC Laboratory of Molecular Biology, Cambridge, UK

For correspondence: mwilkin@mit.edu

Aaron A Hoskins

Department of Biochemistry, University of Wisconsin-Madison, Madison, USA, Department of Chemistry, University of Wisconsin-Madison, Madison, USA

ORCID iD: [0000-0002-9777-519X](https://orcid.org/0000-0002-9777-519X)

For correspondence: ahoskins@wisc.edu

Editors

Reviewing Editor

Olivia Rissland

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Reviewer #1 (Public Review):

Summary:

A key challenge at the second chemical step of splicing is the identification of the 3' splice site of an intron. This requires recruitment of factors dedicated to the second chemical step of splicing and exclusion of factors dedicated to the first chemical step of splicing. Through the highest resolution cryoEM structure of the spliceosome to-date, the authors show the binding site for Fyv6, a factor dedicated to the second chemical step of splicing, is mutually exclusive with the binding site for a distinct factor dedicated to the first chemical step of splicing, highlighting that splicing factors bind to the spliceosome at a specific stage not only by recognizing features specific to that stage but also by competing with factors that bind at other stages. The authors further reveal that Fyv6 functions at the second chemical step to promote selection of 3' splice sites distal to a branch point and thereby discriminate against proximal, suboptimal 3' splice site. Lastly, the authors show by cryoEM that Fyv6 physically interacts with the RNA helicase Prp22 and by genetics Fyv6 functionally interacts with this factor, implicating Fyv6 in 3'SS proofreading and mRNA release from the spliceosome. The evidence for this study is robust, with the inclusion of genomics, reporter assays, genetics, and cryoEM. Further, the data overall justify the conclusions, which will be of broad interest.

Strengths:

- (1) The resolution of the cryoEM structure of Fyv6-bound spliceosomes at the second chemical step of splicing is exceptional (2.3 Angstroms at the catalytic core; 3.0-3.7 Angstroms at the periphery), providing the best view of this spliceosomal intermediate in particular and the core of the spliceosome in general.
- (2) The authors observe by cryoEM three distinct states of this spliceosome, each distinguished from the next by progressive loss of protein factors and/or RNA residues. The authors appropriately refrain from overinterpreting these states as reflecting distinct states in the splicing cycle, as too many cryoEM studies are prone to do, and instead interpret these observations to suggest interdependencies of binding. For example, when Fyv6, Slu7, and

- Prp18 are not observed, neither are the first and second residues of the intron, which otherwise interact, suggesting an interdependence between 3' splice site docking on the 5' splice site and binding of these second step factors to the spliceosome.
- (3) Conclusions are supported from multiple angles.
- (4) The interaction between Fyv6 and Syf1, revealed by the cryoEM structure, was shown to account for the temperature-sensitive phenotypes of a *fyv6* deletion, through a truncation analysis.
- (5) Splicing changes were observed in vivo both by indirect copper reporter assays and directly by RT-PCR.
- (6) Changes observed by RNA-seq are validated by RT-PCR.
- (7) The authors go beyond simply observing a general shift to proximal 3'SS usage in the *fyv6* deletion by RNA-seq by experimentally varying branch point to 3' splice site distance experimentally in a reporter and demonstrating in a controlled system that Fyv6 promotes distal 3' splice sites.
- (8) The importance of the Fyv6-Syf1 interaction for 3'SS recognition is demonstrated by truncations of both Fyv6 and of Syf1.
- (9) In general, the study was executed thoroughly and presented clearly.

Comments on revisions:

The authors have satisfactorily addressed the comments.

<https://doi.org/10.7554/eLife.100449.2.sa3>

Reviewer #2 (Public Review):

In this manuscript, Senn, Lipinski, and colleagues report on the structure and function of the conserved spliceosomal protein Fyv6. Pre-mRNA splicing is a critical gene expression step that occurs in two steps, branching and exon ligation. Fyv6 had been recently identified by the Hoskins' lab as a factor that aids exon ligation (Lipinski et al., 2023), yet the mechanistic basis for Fyv6 function was less clear. Here, the authors combine yeast genetics, transcriptomics, biochemical assays, and structural biology to reveal the function of Fyv6. Specifically, they describe that Fyv6 promotes the usage of distal 3'SSs by stabilizing a network of interactions that include the RNA helicase PRP22 and the spliceosome subunit SYF1. They discuss a generalizable mechanism for splice site proofreading by spliceosomal RNA helicases that could be modulated by other, regulatory splicing factors.

This is a very high quality study, which expertly combines various approaches to provide new insights into the regulation of 3'SS choice, docking, and undocking. The cryo-EM data is also of excellent quality, which substantially extends on previous yeast P complex structures. This is also supported by the authors use of the latest data analysis tools (Relion-5, AlphaFold2 multimer predictions, Modelangelo). The authors re-evaluate published EM densities of yeast spliceosome complexes (B*, C,C*,P) for the presence or absence of Fyv6, substantiate Fyv6 as a 2nd step specific factor, confirm it as the homolog of the human protein FAM192A, and provide a model for how Fyv6 may fit into the splicing pathway. The biochemical experiments on probing the splicing effects of BP to 3'SS distances after Fyv6 KO, genetic experiments to probe Fyv6 and Syf1 domains, and the suppressor screening add substantially to the study and are well executed. The manuscript is clearly written and we particularly appreciated the nuanced discussions, for example for an alternative model by which Prp22 influences 3'SS undocking. The research findings will be of great interest to the pre-mRNA splicing community.

Comments on revisions:

I'm satisfied with the changes.

Reviewer #3 (Public Review):

In this manuscript the authors expand their initial identification of Fyv6 as a protein involved in the second step of pre-mRNA splicing to investigate the transcriptome-wide impact of Fyv6 on splicing and gain a deeper understanding of the mechanism of Fyv6 action.

They first use deep sequencing of transcripts in cells depleted of Fyv6 together with Upf1 (to limit loss of mis-spliced transcripts) to identify broad changes in the transcriptome due to loss of Fyv6. This includes both changes in overall gene expression, that are not deeply discussed, as well as alterations in choice of 3' splice sites - which is the focus of the rest of the manuscript

They next provide the highest resolution structure of the post-catalytic spliceosome to date; providing unparalleled insight into details of the active site and peripheral components that haven't been well characterized previously.

Using this structure they identify functionally critical interactions of Fyv6 with Syf1 but not Prp22, Prp8 and Slu7. Finally, a suppressor screen additionally provides extensive new information regarding functional interactions between these second step factors.

Overall this manuscript reports new and essential information regarding molecular interactions within the spliceosome that determine the use of the 3' splice site. It would be helpful, especially to the non-expert, to summarize these in a table, figure or schematic in the discussion.

Comments on revisions:

I'm satisfied with the changes made in the revision.

<https://doi.org/10.7554/eLife.100449.2.sa1>

Author response:

The following is the authors' response to the original reviews.

eLife Assessment

This important study addresses how 3' splice site choice is modulated by the conserved spliceosome-associated protein Fyv6. The authors provide compelling evidence Fyv6 functions to enable selection of 3' splice sites distal to a branch point and in doing so antagonizes more proximal, suboptimal 3' splice sites. The study would be improved through a more nuanced discussion of alternative possibilities and models, for instance in discussing the phenotypic impact of Fyv6 deletion.

We thank the editors and reviewers for their supportive comments and assessment of this manuscript. We have improved the discussion at several points as suggested by the reviewers to include discussion of alternative possibilities.

Public Reviews:***Reviewer #1 (Public Review):***

Summary:

A key challenge at the second chemical step of splicing is the identification of the 3' splice site of an intron. This requires recruitment of factors dedicated to the second chemical step of splicing and exclusion of factors dedicated to the first chemical step of splicing. Through the highest resolution cryoEM structure of the spliceosome to-date, the authors show the binding site for Fyv6, a factor dedicated to the second chemical step of splicing, is mutually exclusive with the binding site for a distinct factor dedicated to the first chemical step of splicing, highlighting that splicing factors bind to the spliceosome at a specific stage not only by recognizing features specific to that stage but also by competing with factors that bind at other stages. The authors further reveal that Fyv6 functions at the second chemical step to promote selection of 3' splice sites distal to a branch point and thereby discriminate against proximal, suboptimal 3' splice site. Lastly, the authors show by cryoEM that Fyv6 physically interacts with the RNA helicase Prp22 and by genetics Fyv6 functionally interacts with this factor, implicating Fyv6 in 3'SS proofreading and mRNA release from the spliceosome. The evidence for this study is robust, with the inclusion of genomics, reporter assays, genetics, and cryoEM. Further, the data overall justify the conclusions, which will be of broad interest.

Strengths:

- (1) The resolution of the cryoEM structure of Fyv6-bound spliceosomes at the second chemical step of splicing is exceptional (2.3 Angstroms at the catalytic core; 3.0-3.7 Angstroms at the periphery), providing the best view of this spliceosomal intermediate in particular and the core of the spliceosome in general.
- (2) The authors observe by cryoEM three distinct states of this spliceosome, each distinguished from the next by progressive loss of protein factors and/or RNA residues. The authors appropriately refrain from overinterpreting these states as reflecting distinct states in the splicing cycle, as too many cryoEM studies are prone to do, and instead interpret these observations to suggest interdependencies of binding. For example, when Fyv6, Slu7, and Prp18 are not observed, neither are the first and second residues of the intron, which otherwise interact, suggesting an interdependence between 3' splice site docking on the 5' splice site and binding of these second step factors to the spliceosome.
- (3) Conclusions are supported from multiple angles.
- (4) The interaction between Fyv6 and Syf1, revealed by the cryoEM structure, was shown to account for the temperature-sensitive phenotypes of a *fyv6* deletion, through a truncation analysis.
- (5) Splicing changes were observed in vivo both by indirect copper reporter assays and directly by RT-PCR.
- (6) Changes observed by RNA-seq are validated by RT-PCR.
- (7) The authors go beyond simply observing a general shift to proximal 3'SS usage in the *fyv6* deletion by RNA-seq by experimentally varying branch point to 3' splice site distance experimentally in a reporter and demonstrating in a controlled system that Fyv6 promotes distal 3' splice sites.
- (8) The importance of the Fyv6-Syf1 interaction for 3'SS recognition is demonstrated by truncations of both Fyv6 and of Syf1.
- (9) In general, the study was executed thoroughly and presented clearly.

We thank the reviewer for their recognition of the strengths of our multi-faceted approach that led to highly supported conclusions.

Weaknesses:

(1) Despite the authors restraint in interpreting the three states of the spliceosome observed by cryoEM as sequential intermediates along the splicing pathway, it would be helpful to the general reader to explicitly acknowledge the alternative possibility that the difference states simply reflect decomposition from one intermediate during isolation of the complex (i.e., the loss of protein is an in vitro artifact, if an informative one).

We thank the reviewer for noticing our restraint in interpreting these structures, and we agree that the scenario described by the reviewer is a possibility. We have now explicitly mentioned this in the Discussion on lines 755-757.

(2) The authors acknowledge that for prp8 suppressors of the fyv6 deletion, suppression may be indirect, as originally proposed by the Query and Konarska labs - that is, that defects in the second step conformation of the spliceosome can be indirectly suppressed by compensating, destabilizing mutations in the first step spliceosome. Whereas some of the other suppressors of the fyv6 deletion can be interpreted as impacting directly the second step spliceosome (e.g., because the gene product is only present in the second step conformation), it seems that many more suppressors beyond prp8 mutants, especially those corresponding to bulky substitutions, which would more likely destabilize than stabilize, could similarly act indirectly by destabilization of first step conformation. The authors should acknowledge this where appropriate (e.g., for factors like Prp8 that are present in both first and second step conformations).

We agree that this is also a possibility and have now included this on lines 480-486.

Reviewer #2 (Public Review):

In this manuscript, Senn, Lipinski, and colleagues report on the structure and function of the conserved spliceosomal protein Fyv6. Pre-mRNA splicing is a critical gene expression step that occurs in two steps, branching and exon ligation. Fyv6 had been recently identified by the Hoskins' lab as a factor that aids exon ligation (Lipinski et al., 2023), yet the mechanistic basis for Fyv6 function was less clear. Here, the authors combine yeast genetics, transcriptomics, biochemical assays, and structural biology to reveal the function of Fyv6. Specifically, they describe that Fyv6 promotes the usage of distal 3'SSs by stabilizing a network of interactions that include the RNA helicase PRP22 and the spliceosome subunit SYF1. They discuss a generalizable mechanism for splice site proofreading by spliceosomal RNA helicases that could be modulated by other, regulatory splicing factors.

This is a very high quality study, which expertly combines various approaches to provide new insights into the regulation of 3'SS choice, docking, and undocking. The cryo-EM data is also of excellent quality, which substantially extends on previous yeast P complex structures. This is also supported by the authors use of the latest data analysis tools (Relion-5, AlphaFold2 multimer predictions, Modelangelo). The authors re-evaluate published EM densities of yeast spliceosome complexes (B, C,C*,P) for the presence or absence of Fyv6, substantiate Fyv6 as a 2nd step specific factor, confirm it as the homolog of the human protein FAM192A, and provide a model for how Fyv6 may fit into the splicing pathway. The biochemical experiments on probing the splicing effects of BP to 3'SS distances after Fyv6 KO, genetic experiments to probe Fyv6 and Syf1 domains, and the suppressor screening add substantially to the study and are well executed. The manuscript is clearly written and we particularly appreciated the nuanced discussions, for example for an alternative model by which Prp22 influences 3'SS undocking. The research findings will be of great interest to the pre-mRNA splicing community.*

We thank the reviewer for their positive comments on our manuscript.

We have only few comments to improve an already strong manuscript.

Comments:

(1) Can the authors comment on how they justify K⁺ ion positions in their models (e.g. the K⁺ ion bridging G-1 and G+1 nucleotides)? How do they discriminate e.g. in the 'G-1 and G+1' case K⁺ from water?

The assignment of K⁺ at this position is justified by both longer coordination distances and relatively high cryo-EM density compared to structured water molecules in the same vicinity. We have added a panel to figure3-figure supplement 4C to show the density for the G-1/G+1 bridging K⁺ ion and to show the adjacent density for putative water molecules which coordinate the ion. The K⁺ ion density is larger and has stronger signal than the adjacent water molecules. The coordination distances are also longer than would be expected for a Mg²⁺. For these reasons and because K⁺ was present in the purification buffer, we modelled the density as K⁺.

(2) The authors comment on Yju2 and Fyv6 assignments in all yeast structures except for the ILS. Can the authors comment on if they have also looked into the assignment of Yju2 in the yeast ILS structure in the same manner? While it is possible that Fyv6 could dissociate and Yju2 reassociate at the P to ILS transition, this would merit a closer look given that in the yeast P complex Yju2 had been misassigned previously.

We thank the reviewer for pointing out this very interesting topic! We have used ModelAngelo to analyze the *S. cerevisiae* ILS structure for support of density assignment as Yju2 (and not Fyv6). This analysis supports the assignment as Yju2 in this structure and we have no evidence to doubt its presence in those particular purified spliceosomes. We have updated Figure 4- figure supplement 1B accordingly.

That being said, we do think that this issue should be studied more carefully in the future. The *S. cerevisiae* ILS structure (5Y88) was determined by purifying spliceosome complexes with a TAP-tag on Yju2. So the conclusion that Yju2 is part of the ILS spliceosome involves some circular logic: Yju2 is part of ILS spliceosome complexes because it is present in ILS complexes purified with Yju2. We also note that Yju2 was absent in ILS complexes recently determined from metazoans by the Plaschka group. We have added some additional nuance to the Discussion to raise this important mechanistic point at lines 711-718.

(3) For accessibility to a general reader, figures 1c, d, e, 2a, b, would benefit from additional headings or labels, to immediately convey what is being displayed. It is also not clear to us if Fig 1e might fit better in the supplement and be instead replaced by Supplementary Figure 1a (wt), b (delta upf1), and a new c (delta fyv6) and new d (delta upf1, delta fyv6). This may allow the reader to better follow the rationale of the authors' use of the Fyv6/Upf1 double deletion.

We thank the reviewer for the suggestion and have updated Figures 1 C-E to include additional information in the headings and labels. We have not changed the labels in Figures 2A, B but have added additional clarifying language to the legend.

In terms of rearranging the figures, we thank the reviewer for the suggestion but have decided that the figures are best left in their current ordering.

(4) The authors carefully interpret the various suppressor mutants, yet to a general reader the authors may wish to focus this section on only the most critical mutants for a

better flow of the text.

We thank the reviewer for this suggestion. While this section of the manuscript does contain (to quote Reviewer #3) “extensive new information regarding functional interactions”, it was a bit long. We have reduced this section of the manuscript by ~200 words for a more focused presentation for general readers.

Reviewer #3 (Public Review):

In this manuscript the authors expand their initial identification of Fyv6 as a protein involved in the second step of pre-mRNA splicing to investigate the transcriptome-wide impact of Fyv6 on splicing and gain a deeper understanding of the mechanism of Fyv6 action.

They first use deep sequencing of transcripts in cells depleted of Fyv6 together with Upf1 (to limit loss of mis-spliced transcripts) to identify broad changes in the transcriptome due to loss of Fyv6. This includes both changes in overall gene expression, that are not deeply discussed, as well as alterations in choice of 3' splice sites - which is the focus of the rest of the manuscript

They next provide the highest resolution structure of the post-catalytic spliceosome to date; providing unparalleled insight into details of the active site and peripheral components that haven't been well characterized previously.

Using this structure they identify functionally critical interactions of Fyv6 with Syf1 but not Prp22, Prp8 and Slu7. Finally, a suppressor screen additionally provides extensive new information regarding functional interactions between these second step factors.

Overall this manuscript reports new and essential information regarding molecular interactions within the spliceosome that determine the use of the 3' splice site. It would be helpful, especially to the non-expert, to summarize these in a table, figure or schematic in the discussion.

We thank the reviewer for the positive comments and suggestions. We did include a summary figure in panel 7H. However, it was a bit buried. To highlight the summary figure more clearly, we have moved panel 7H to its own figure (Fig. 8).

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

(1) The resolution of some panels is poor, nearly illegible (e.g., Supp Fig 1A, B).

The resolution of panels in supplemental figure 1 has been increased. However, this may be an artifact of the PDF conversion process. We will pay attention to this during the publication process.

(2) Panel S6B: 6HYU is a structure of DHX8, not DDX8

We have corrected DDX8 to DHX8 in Supplemental Fig. S6D and associated figure legend.

(3) The result that Syf1 truncations can suppress the Fyv6 deletion is impressive. The subsequent discussion seems muddled. A discussion of Fyv6 binding at the first step, instead of Yju2, doesn't seem relevant here (though worthy of consideration in the discussion), given that the starting mutation is the Fyv6 deletion. Further, conjuring rebinding of Yju2 based on the data in the paper seems unnecessarily speculative

(assumes that biochemical state III is on pathway), unless I am unaware of some other evidence for such rebinding. Instead, a simpler explanation would seem to be that in the absence of Fyv6, Syf1 inappropriately binds Yju2 instead at the second step and that deletion of the common Fyv6/Yju2 binding site on Syf1 suppresses this defect. In this case, the ts phenotype of the Fyv6 deletion would result from inappropriate binding of Yju2, and the splicing defect would be due to loss of Fyv6 activity. Alternatively, especially considering the work of the labs of Query and Konarska, the authors should consider the possibility that i) the Fyv6 deletion destabilizes the second step conformation, shifting an equilibrium to the first step conformation, and that ii) the Syf1 truncation destabilizes binding of Yju2, thereby restoring the equilibrium. In this case the ts phenotype of the Fyv6 deletion is due to a disturbed equilibrium and the splicing defect is due to the failure of Fyv6 to function at the second step.

We believe the reviewer is specifically referencing the final paragraph of this Results section (the paragraph that comes just before the section “Mutations in many different splicing factors...”). In retrospect, we agree that our discussion was convoluted. In particular, we emphasized rebinding of Yju2 based on its presence in the cryo-EM structure of the yeast ILS complex. However, given some uncertainties about whether or not Yju2 is a *bona fide* ILS component (as discussed above). We don't think it is appropriate to over-emphasize rebinding of Yju2 and have decided to incorporate the elegant mechanisms proposed by the reviewer. This paragraph has now been edited accordingly (lines 386-395).

(4) The authors imply they have performed biochemical studies, which I think is misleading. Of course, RT-PCR and primer extension assays for example are performed in vitro, but these are an analysis of RNA events that occurred in vivo. In my view a higher threshold should be used for defining "biochemistry". To me "biochemistry" would imply that the authors have, for example, investigated 3' splice site usage in splicing extracts of the fyv6 deletion or engaged in an analysis of the Syf1-Fyv6 interaction involving the expression of the interacting domains in bacteria followed by a binding analysis in the test tube.

We disagree with the reviewer on this point. Biochemistry is defined as the “branch of sciences concerned with the chemical substances, reactions, and physico chemical processes which occur within living organisms; biological or physical chemistry.” (Oxford English Dictionary). Biochemical studies are not defined by whether or not they take place *in vitro*, *in vivo*, or even *in silico*. Indeed, much of the history of biochemistry (especially in studies of metabolism, for example) involved experiments occurring *in vivo* that reported on the molecular properties and mechanisms of biological processes. We think many of our experiments fall into this category including our structure/function analysis of splicing factors and the use of the ACT1-CUP1 reporter substrate.

(5) The monovalents are shown; inositol phosphate is shown; is the binding of Prp22 to RNA shown?

We have added a panel to Figure 3-figure supplement 4D showing density for the 3' exon within Prp22.

(6) The authors invoke undocking of the 3'SS in the P complex. Where is the 3'SS in the ILS? The author's model predicts: undocked.

In all ILS structures to date, the 3' SS is undocked, in agreement with this prediction. We have now noted this observation in line 760.

(7) Would be helpful to show fyv6 deletion in Fig 1b.

We have included growth data for an additional *fyv6* deletion strain (in a *cup1Δ* background) in Figure 1b. The results are quite similar to the *upf1_Δ* background except with slightly worse growth at 23°C.

Reviewer #2 (Recommendations For The Authors):

Minor comments

(1) Fig.3b is the arrow indicating the right rotation?

This typo has been fixed.

(2) Fig.4b, panel H is annotated, which should read 'F'.

This typo has been fixed.

(3) Line 178: "Finally, we analyzed the sequence features of the alternative 3'SS activated by loss of Fyv6." We would suggest 'used after' instead of 'activated by'.

We have replaced 'activated by' with 'with increased use after'.

(4) In Line 544, the authors speculate on a *Slu7* requirement for 3'SS docking and on 3'SS docking maintenance. In the results section (Line 265) they however only mention the latter possibility. These statements should be consistent.

We thank the reviewer for pointing this out. We have added a reference to docking maintenance to the results section at line 325.

(5) Line 476: "Unexpectedly, Prp22 I1133R was actually deleterious when Fyv6 was present for this reporter." We suggest removing "actually".

We have removed 'actually'.

(6) The authors describe the observed changes in splicing events in absolute numbers (e.g. in Fig 1c). To better assess for the reader whether these numbers reflect large or small effects of Fyv6 in defining mRNA isoforms, it would be more useful to state these as percent changes of total events or to provide a reference number for how many introns are spliced in S.c. See for example the statements in Lines 132 and 145.

We have added a percentage at line 138 that indicates ~20% of introns in yeast showed splicing changes.

Reviewer #3 (Recommendations For The Authors):

Do the authors have a proposed explanation for the observed DGE in non-intron containing genes in the Fyv6 depleted cells?

The simplest explanation is that this is an indirect effect due to splicing changes occurring in other genes (such as transcription factors, ribosomal protein genes, etc.). It is possible that this can be further dissected in the future using shorter-term knockdown of Fyv6 using Anchors Away or AID-tagging. However, that is beyond the scope of the current manuscript, and we do not wish to comment on these non-intron containing genes further at present.

Figure 2A - What is going on with the events that show no FAnS value under one condition (i.e. are up against the X or Y axis)? These are of interest as most on the Y- axis are blue.

The events along one of the axes denote alternative splice sites that are only detected under one condition (either when Fyv6 is present or when it is absent). At this stage, we do not wish to interpret these events further since most have a relatively low number of reads overall.

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