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

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RUNNING TITLE: Structure and Function of the Splicing Factor Fyv6

KEYWORDS: yeast, spliceosome, pre-mRNA splicing, cryo-EM, snRNA, snRNP, structure, RNA-Seq

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 *fyv6Δ* 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 Prp22-dependent, 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 (**Fig. 1A**). First, the 5' splice site (5' SS) is cleaved by 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 $\geq$ 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

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

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

88 Here, we use a combination of transcriptomic, structural, biochemical, and genetic assays
89 to elucidate how Fyv6 controls 3' SS usage in yeast. RNA-seq analysis reveals widespread
90 activation of alternative, BP proximal nonconsensus 3' SS in the absence of Fyv6 consistent with
91 biochemical assays that show Fyv6 facilitates splicing of 3' SS located $\geq$ 21 nt from the BP. To
92 further elucidate Fyv6 function, we determined a 2.3 Å cryo-EM structure of the yeast P complex
93 spliceosome in which Fyv6 can be modeled. The structure reveals interactions between Fyv6 and
94 Prp22 as well as a domain in Syf1 that interacts with either Fyv6 or the 1st step factor Yju2. 3D
95 classification allowed us to identify two additional conformational states of P complex and
96 provides insights into the coupling of 3' SS active site docking and the presence of 2nd step factors.
97 We then used this structural data to dissect the functional domains of Fyv6, interpret the results
98 of a genetic screen for suppressors of *fyv6Δ*, and to probe Fyv6 and Prp22 interactions.
99 Combined, we propose a model in which Fyv6 is recruited to the spliceosome via interactions with
100 the NTC component Syf1 in order to promote usage of BP distal, and Prp22-dependent, 3' SS.

101 **RESULTS**

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

103 Our previous work showed that loss of *FYV6* causes changes in 3' SS usage in the *SUS1*
104 pre-mRNA. To probe for changes transcriptome-wide, we used nonsense mediated decay (NMD)-
105 deficient (*upf1Δ*, WT) yeast cells expressing or lacking Fyv6. By suppressing NMD, we hoped to
106 limit degradation of alternatively spliced mRNA isoforms generated in the absence of Fyv6
107 (Sayani et al., 2008). Consistent with results from *fyv6Δ* strains, the *fyv6Δ upf1Δ* double mutant
108 strain also showed reduced growth at 30°C and cold-sensitive (*cs*) and temperature-sensitive (*ts*)
109 phenotypes relative to a *upf1Δ* control (**Fig. 1B**). We then used RNA-Seq to analyze the isolated
110 RNAs from these strains. As expected, many more changes in splicing were observed in the *fyv6Δ*
111 *upf1Δ* strain relative to the *fyv6Δ* strain (**Supplemental Fig. S1A, B; Supplemental Tables S1,**
112 **S2**). This is consistent with many mRNA isoforms generated due to loss of Fyv6 being substrates

113 for NMD. Loss of Fyv6 resulted in a number of changes in gene expression with many non-intron
114 containing genes also being up- or down-regulated (**Supplemental Fig. S1C**).

115 We compared and classified changes in splicing between strains containing and lacking
116 *FYV6* under permissive growth conditions (30°C) or after the yeast had been shifted to a non-
117 permissive temperature (16 or 37°C) for 1 h (**Fig. 1C**). In this case, we only considered events
118 resulting in at least a 10% change in the percent spliced in (PSI) value when Fyv6 is deleted. At
119 all temperatures, we see changes in alternative 3' SS usage in the *fyv6Δ* strains relative to those
120 with *FYV6*. For example, we detected use of the alternative 3' SS in the first intron of the *SUS1*
121 transcript as we previously reported (not shown; Lipinski et al., 2023). We also saw increased use
122 of a cryptic, nonconsensus GAG 3' SS in the first, but not second, intron of the *YOS1* transcript
123 in the *fyv6Δ* data sets and confirmed this result by RT-PCR (**Supplemental Fig. S1D, E**). We
124 observed several cases of alternative 5' SS. However, the majority of these alternative 5' SS were
125 used with equal efficiencies in the WT and *fyv6Δ* strains (**Supplemental Fig. S1F**).

126 The strains temperature shifted to 16°C had the largest number of changes between
127 strains with and without Fyv6 and the strains shifted to 37°C the least. The temperature shift to
128 16° caused an increase in the numbers of both alternative 3' SS and retained intron events
129 detected (**Fig. 1C**). The changes in alternative 3' SS use at each temperature were mostly unique
130 with few events detected under all three conditions (**Fig. 1D**). Collectively, we observed that 61
131 different RNAs changed splicing patterns due to loss of Fyv6. For many of these mRNAs, we
132 detected multiple different 3' SS being used.

133 To investigate these splicing events more closely, we analyzed changes in usage of
134 alterative splice junctions by calculating the Fraction of Annotated Splicing (FAnS) in the presence
135 and absence of Fyv6 (Roy et al., 2023). FAnS ratios report the relative abundance of an alternative
136 splicing event in relation to the main spliced isoform. We calculated FAnS values for all exon
137 junction reads corresponding to usage of a canonical 5' SS and an alternative 3' SS for WT and

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

146 **Fyv6 Facilitates Usage of Branch Point Distal 3′ Splice Sites**

147 We next analyzed the features of the 3' SS impacted by Fyv6 loss. We used RNAs
148 collected from non-temperature shifted yeast grown at 30°C and sequenced these RNAs at higher
149 depth than those used for the temperature-shift experiment described in **Fig. 1** (~400 million vs.
150 200 million reads; **Supplemental Table S2**). We annotated each alternative 3' SS as being
151 upstream (5') or downstream (3') of the canonical 3' SS (Grate and Ares, 2002). This analysis
152 shows that the majority of the alternative 3' SS arising from Fyv6 deletion are upstream (**Fig. 2A**,
153 pink points above the diagonal). When these alternative 3' SS are mapped according to their
154 nucleotide distance from the canonical site, most of these are found within ~40 nt upstream (**Fig.**
155 **2B**, purple violin). Plotting these alternative 3' SS sites relative to their distance from the annotated
156 BP reveals that the highest density of sites is $\leq$ 20 nt from the BP for transcripts with the highest
157 FAnS ratios (**Fig. 2C**, purple violin). It is important to note that we did not map BP in Fyv6 deletion
158 strains, and it is possible that in some cases a shift in 3' SS usage could also coincide with a shift
159 in the BP. Indeed, a small number of alternative 3' SS detected in the Fyv6 data sets are located
160 upstream of the annotated BP, suggesting activation of an alternative BP and 3' SS. However, we
161 believe that the changes are predominantly due to usage of alternative 3' SS located between the
162 annotated BP and canonical 3' SS.

163 The above results suggest that Fyv6 is important for splicing of 3' SS located distant from
164 the BP. To test this directly and systematically, we created a series of ACT1-CUP1 reporters with
165 various BP to 3' SS distances (9-50 nt, **Fig. 2D**, **Supplemental Table S3**) based on sequences
166 used in previous studies of 3' SS selection (Brys and Schwer, 1996; Frank and Guthrie, 1992;
167 Schwer and Gross, 1998). We transformed these reporters into strains containing or lacking Fyv6
168 and without debranchase (Dbr1) to limit degradation of lariat intermediates. We then detected
169 RNA products by primer extension and calculated the 2nd step (exon ligation) efficiency for each
170 reporter (**Fig. 2E, F**). While loss of Fyv6 has minimal impact on exon ligation when BP to 3' SS
171 distances are short (9-15 nt), exon ligation efficiency decreases significantly at distances of 21 nt
172 or greater. In addition, we do not see evidence of cryptic BP usage in these assays. This result is
173 consistent with the RNA-seq analysis and supports a function for Fyv6 in facilitating splicing at
174 BP distal 3' SS.

175 Finally, we analyzed the sequence features of the alternative 3' SS activated by loss of
176 Fyv6 (**Fig. 2G, H**). For sites located upstream of the canonical 3' SS, we observed much greater
177 sequence variability relative to the canonical site. We detected increased use of highly variable 3'
178 SS including those with atypical HAU (H=A,C,U) and BG (B=C,U,G) motifs (**Fig. 2H**). Increased
179 use of variable 3' SS was also observed when the 2nd step factor Prp18 was deleted (Roy et al.,
180 2023). However, the subset of RNAs most impacted by splicing factor deletion appear to be
181 different. We were not able to detect changes in 3' SS usage for the *UBC12*, *MAF1*, *MUD1*,
182 *PHO85*, *SPT14*, or *YCL002C* transcripts in the *fyv6*Δ data sets as was reported for *prp18*Δ. Prp18
183 and Fyv6 likely have transcript-specific effects on splicing outcomes. Combined our results
184 strongly support Fyv6 as a 2nd step splicing factor that facilitates usage of consensus sites located
185 distal to the BP *in vivo*.

186 **A High-Resolution Spliceosome Structure Reveals Fyv6 Interactions**

187 Previous structures of the yeast P-complex spliceosome were solved with resolutions at
188 the core ranging from 3.3 Å to 3.7 Å (Bai et al., 2017; Liu et al., 2017; Wilkinson et al., 2017), and

189 with peripheral components such as the Prp22 helicase, U2 snRNP, and Prp19 Complex (NTC)
190 at much lower resolutions (5-10 Å) that precluded detailed investigation. To improve the
191 resolution, we purified P complex as previously described by stalling spliceosome disassembly
192 with a dominant negative mutant of Prp22 (S635A) defective in mRNA release (Schwer and
193 Meszaros, 2000; Wilkinson et al., 2017). However, by collecting a much larger cryo-EM dataset
194 on a more modern electron detector, we were able to resolve the structure to 2.3 Å within the
195 catalytic core, as well as visualize peripheral components at resolutions from 3.0 Å – 3.7 Å (**Fig.
196 3A, B; Supplemental Fig. S2-S5; Supplemental Table S4**). This is the highest resolution
197 spliceosome structure to date. The high-quality density at the active site shows the positions of
198 bases unambiguously, confirming the manner of the non-Watson-Crick base pairing that mediates
199 3' SS recognition (Liu et al., 2017; Wilkinson et al., 2017). Monovalent ions bound at the active
200 site were previously shown to be important during the 1st step of splicing (Wilkinson et al., 2021),
201 and our data indicate that the positions of these ions are preserved after exon ligation. Additionally,
202 the density also shows a metal ion, likely potassium, that bridges the base pair between +1G of
203 the 5' SS and -1G of the 3' SS (**Fig. 3C**).

204 The 2nd-step splicing factor Prp22 is resolved at 3.0 Å resolution, allowing atomic
205 interpretation for the first time (**Fig. 3B** and later figures). Bases 13 – 21 of the 3' exon are visible
206 between the two RecA domains of Prp22 consistent with biochemical footprinting data (Schwer,
207 2008). The S635A mutation used to stall P complex is also visible; however, the origin of defective
208 mRNA release due to this mutation is not apparent (Schwer and Meszaros, 2000). The loop
209 containing S635A is in a similar conformation in this structure and in the structure of WT human
210 Prp22 (Felisberto-Rodrigues et al., 2019) (**Supplemental Fig. S5**). Part of the extensive N-
211 terminal domain of Prp22 is resolved and constitutes a long helix that bridges between the Prp22
212 RecA2 domain and the C-terminal domain of Cwc22; this helix was unassigned in previous P-
213 complex structures (**Fig. 4B**).

214 To improve the resolution of the peripheral, flexible regions, we used a new data-driven
215 regularization strategy (Kimanius et al., 2023) that allows focused refinements of much smaller
216 domains than previously possible (**Supplemental Fig. S3, S5**). Combined with AlphaFold2-
217 assisted modelling, we were able to obtain more accurate models for the U2 snRNP, NTC, U5
218 Sm ring, and Cwc22 N-terminal domains. Our model for the NTC within the P complex is similar
219 to our previous model for the NTC within C and C^i complexes (Wilkinson et al., 2021) (**Fig. 4C**).
220 Notably however, we observed a star-shaped density coordinated by several conserved lysines
221 from Clf1 and Ntc20, which we were able to model as inositol hexakisphosphate (IP_6) (**Fig. 4D**).
222 The same density, at lower resolution, is visible in the C complex (Wilkinson et al., 2021). A
223 distinct, separate IP_6 molecule had previously been observed within all catalytic spliceosomes
224 between the B^{act} and P-complex stages coordinated by Prp8 and (for C* and P-complexes) Slu7
225 (**Supplemental Fig. S5**). We do not believe that the NTC-bound IP_6 is essential for splicing since
226 deletion of the Clf1 tetratricopeptide repeat that coordinates IP_6 does not result in growth or
227 splicing phenotypes in yeast and Ntc20 is a non-essential splicing factor (Chen et al, 2001; Chung
228 et al., 1999). Nonetheless, it is possible that IP_6 may regulate splicing in some manner via
229 interactions with both Prp8 and the NTC.

230 We were able to unambiguously assign three, connected long α-helices to Fyv6 (**Fig. 4A**).
231 These helices were visible in previous cryo-EM reconstructions of yeast C* and P complexes but
232 were either unassigned or misassigned. In the cases of misassigned densities, Fyv6 was
233 previously attributed to the C-terminal domain of the 1st step factor Yju2 (Wilkinson et al., 2017).
234 Yju2 also has an elongated helical architecture and adopts a similar position on C complex
235 (Wilkinson et al., 2021) and the post-P complex Intron Lariat Spliceosome (ILS) (Wan et al., 2017).
236 We closely inspected the density from previously determined spliceosome structures and
237 concluded that the Yju2 C-terminus is still a better fit than Fyv6 for densities in B* and C
238 complexes, whereas Fyv6 is a better fit than Yju2 for densities in C* and P complexes
239 (**Supplemental Fig. S6**). Therefore, this position of Fyv6 seems to be characteristic of a 2nd step

240 conformation of the spliceosome and consistent with the position of its distant human homolog
241 FAM192A in the human pre-C* complex (Zhan et al., 2022).

242 Fyv6 makes a multitude of interactions with essential splicing factors (**Fig. 3B; 4E, F, G**).
243 One conserved region of Fyv6 is the N-terminus with a FVSE motif (**Supplemental Fig. S7**). This
244 motif forms a 'hook' that interacts with a hydrophobic patch on the surface of the Prp22 RecA2
245 domain, where it also forms hydrogen bonds by β-sheet augmentation (**Fig. 4E**). The hook is
246 followed by three α-helices. The start of helix 2 sits on top of the intron between the BP and the
247 active site docked 3' SS, where it may act as a steric block to prevent 3' SS undocking and
248 promote exon ligation (**Fig. 4F**) (Liu et al., 2017). Helix 2 also contains a patch of conserved
249 arginines that form salt bridges with Asp317 and Glu318 of Slu7 (**Fig. 4F**). The linker between
250 helices 2 and 3 interacts with the NTC component Cef1, and helix 3 interacts with NTC component
251 Syf1 (**Fig. 4G**). The binding interface with Syf1 overlaps with the Syf1/Yju2 interaction in the C
252 complex (Wilkinson et al., 2021), suggesting these interactions are mutually exclusive (**Fig. 4G**).
253 Fyv6 thus provides a direct link between the Prp22 ATPase, NTC components, and other essential
254 splicing factors over a distance of ~100 Å.

255 By 3D classification, we were able to resolve a total of three states of the P-complex
256 spliceosome (**Fig. 3C-E**). State I, represented by 51% of particles, is the most complete and has
257 all the above-described factors visible, including a stably-docked 3' SS. State II, represented by
258 20% of particles, is similar to State I but lacks density for the N-terminal half of the 2nd step factor
259 Slu7. The endonuclease-like domain of Prp8, to which this domain of Slu7 binds, is
260 correspondingly shifted. Interestingly, this state retains strong density for the 3' SS, suggesting
261 the N-terminal regions of Slu7 are not involved in maintaining 3' SS docking after exon ligation.
262 Finally, State III, containing 29% of particles, entirely lacks density for the 2nd step factors Slu7,
263 Prp18, and Fyv6. Prp17 and the RNase H domain of Prp8 are still present but are weaker than in
264 States I and II, suggesting they are more flexible in State III. Most of Prp22 can still be observed

265 with weaker density except for the C-terminal domain that interacts with Prp8, which lacks density
266 entirely. Importantly, State III also lacks density for the 3' SS but has density for the 3' exon,
267 suggesting it is indeed post-catalytic and represents a state after loss of 2nd step factors and
268 undocking of the 3' SS from the catalytic core. The coincidence of loss of Fyv6 with loss of Prp18
269 and Slu7 reinforces the notion that these three factors act together and are important for 3' SS
270 docking and stabilization of Prp17, Prp22, and the RNase H domain of Prp8.

271 **The Fyv6/Syf1 Interaction is Critical for Suppressing *fyv6Δ* Phenotypes**

272 To determine the functionally critical regions and interactions of Fyv6, we used the cryo-
273 EM structure to design five truncation mutants (**Fig. 5A**). The first three truncations remove amino
274 acids from the N-terminus of Fyv6: deletion of the first 16 amino acids, which are not resolved in
275 the cryo-EM structure (Δ1-16); deletion of the first 23 amino acids, which removes the conserved
276 hook region of Fyv6 that interacts with the Prp22 RecA2 domain (Δ1-23); and deletion of amino
277 acids 1 to 51, which additionally removes the first α-helix and interactions with Prp22, Prp8, and
278 Slu7 (Δ1-51). The remaining two mutants are C-terminal truncations. In the first, amino acids 134
279 to 173, which do not have clear density in the cryo-EM structure and are not modeled but which
280 do contain a predicted nuclear localization signal (NLS), are deleted (Δ134-173) (Nguyen Ba et
281 al., 2009). In the second, amino acids 103 to 173 are deleted (Δ103-173), which additionally
282 removes the region of the protein that interacts with Syf1.

283 We tested each truncation mutant for its ability to suppress the *cs* and *ts* phenotypes of
284 the *fyv6Δ* strain when expressed from a plasmid (**Fig. 5B**). All of the N-terminal truncation
285 mutations could suppress the *cs* or *ts* phenotypes to some degree. Surprisingly, a strain with the
286 complete deletion of the conserved hook domain that interacts with Prp22 (Δ1-23) grew similarly
287 to the strain expressing WT Fyv6. We wondered if splicing changes could still be present even if
288 the growth phenotype was suppressed; so, we assayed *SUS1* isoform generation by RT-PCR.
289 We previously showed that loss of Fyv6 causes use of an alternative 3' SS in the first intron of

290 this pre-mRNA (Lipinski et al., 2023). The N-terminal truncation mutants also showed no evidence
291 for changes in *SUS1* splicing (**Fig. 5C**). The N-terminal region of Fyv6 composed of the hook
292 domain and the first α helix is not required for suppression of either the growth or splicing
293 phenotypes observed when Fyv6 is lost despite its conservation and interactions with critical
294 splicing factors (Prp8, Prp22, and Slu7).

295 For the C-terminal truncations, the Δ134-173 truncation was also able to suppress the *cs*
296 and *ts* phenotypes, indicating that the predicted NLS is also not essential (**Fig. 5B**). However, this
297 mutant did grow more poorly at 16°C than did the Δ1-16 and Δ1-23 truncations and did show
298 some evidence of alternative 3' SS usage in *SUS1* (**Fig. 5C**). The largest effects were seen when
299 the C-terminus was truncated further. The Δ103-173 truncation strain phenocopied the *fyv6Δ*
300 strain and grew poorly at 37°C and was dead at 23° or 16°C (**Fig. 5B**). Additionally, RT-PCR
301 showed increased use of the alternative 3' SS in *SUS1* with this truncation (**Fig. 5C**). To determine
302 if this phenotype could be due to loss of protein expression, we assayed expression of the epitope-
303 tagged proteins by western blot (**Supplemental Fig. S8A**). In all cases, we could detect protein;
304 however, the abundance of the N-terminally epitope-tagged Δ103-173 variant was much lower
305 than the others. Therefore, we also constructed a C-terminally epitope-tagged version of Δ103-
306 173 (**Supplemental Fig. S8B**). The C-terminally-tagged protein expressed at much higher levels
307 but also failed to suppress the *cs* and *ts* phenotypes. These data suggest that the C-terminal
308 region of Fyv6 that includes a portion of α helix 3 and the Syf1 interaction domain is critical for
309 function.

310 To obtain additional evidence for the importance of the Fyv6/Syf1 interaction, we created
311 a *syf1Δ* shuffle strain containing WT Fyv6 and expressed C-terminal Syf1 truncations (**Fig. 5D**).
312 We hypothesized that loss of the Fyv6-interacting domain on Syf1 could phenocopy *fyv6Δ*. We
313 observed just this effect. Loss of the Fyv6 interacting region in Syf1 Δ778-859 and Δ634-859
314 mutants resulted in either modest (Δ778-859) or strong (Δ634-859) *cs* and *ts* phenotypes. In

315 addition, these Syf1 truncation mutants also triggered use of the alternative 3' SS in *SUS1* (**Fig.**
316 **5F**). Interestingly, if the Δ778-859 and Δ817-859 Syf1 mutants are expressed in a *fyv6Δ*
317 background, it results in suppression of the *fyv6Δ ts* phenotype (**Fig. 5G; *c.f.* Fig. 5B, vector**).
318 However, the *SUS1* alternative 3' SS is still used (**Fig. 5H**). This indicates that *ts* phenotype
319 suppression and triggering usage of the alternative 3' SS in *SUS1* are separable processes.

320 As mentioned above, the Syf1/Fyv6 interaction is mutually exclusive with the Syf1/Yju2
321 interaction that occurs during 5' SS cleavage and is reformed in the ILS spliceosome. One
322 possible explanation for suppression of the *ts* phenotype in *fyv6Δ*/*syf1*(Δ778-859 or Δ817-859)
323 strains is that Fyv6 and Yju2 are competing with one another and binding of the wrong protein to
324 the wrong spliceosome complex results in growth and splicing defects. When the Yju2/Fyv6
325 binding site on Syf1 is removed, the observed phenotypes could be due to failure of Yju2 to be
326 properly recruited to the P complex spliceosome for displacement of Fyv6. However, recruitment
327 occurs correctly when Fyv6 is deleted since it no longer needs to be displaced. Similarly,
328 phenotypes due to loss of Fyv6 could arise from retention of Yju2 during exon ligation. A prediction
329 of this model is that failure to properly remove or recruit Yju2 and Fyv6 can result in similar
330 phenotypes. Further work will be needed to understand the binding dynamics of these factors.

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

332 To gain additional insights into Fyv6 function, we carried out a genetic screen to detect
333 spontaneously-arising suppressors that can correct temperature-dependent growth defects (**Fig.**
334 **6A**). This method has previously been used to illuminate many aspects of spliceosome
335 biochemistry and non-silent mutations within splicing factors are likely causative for suppression
336 (Montemayor et al. 2014; Kuhn and Brow, 2000; Brow, 2019).

337 We plated saturated *fyv6Δ* yeast cultures onto 20 different YPD plates and incubated them
338 at 37°C until colonies arose. Within several days, colonies of various sizes appeared and were
339 isolated. A subset of picked colonies were then tested for suppression of the *ts* phenotype. All of
340 these colonies were able to suppress growth defects at 37°C, and many also suppressed the *cs*

341 phenotype at 23°C (**Supplemental Fig. S9**). We then performed whole genome sequencing on
342 the largest of the picked colonies as these were expected to be the strongest suppressors. In 19
343 of 20 sequenced strains, we were able to identify at least one non-silent mutation in a splicing
344 factor (**Fig. 6B**, **Supplemental Table S5**). When we carried out a similar experiment aimed at
345 identifying *cs* suppressors, we were unable to identify any likely causative mutations in splicing
346 factors. Therefore, we focused our analysis on the *ts* suppressor mutations.

347 Collectively, the identified mutations tended to fall into two categories: gain of positive
348 charge or substitution with a bulkier side chain. Gain of positive charge mutations were found in
349 Prp8 (G1868R) as well as the second step factors Slu7 (A24R, and N28K), and Prp22 (I1133R).
350 Structurally, amino acids within Prp8 and Prp22 are proximal to RNA within the spliceosome and
351 addition of charged amino acid side chains may stabilize interactions with the phosphodiester
352 RNA backbone. The N-terminus of Slu7 containing A24 and N28 is not resolved in any
353 spliceosome structure to our knowledge and normally contains many negatively charged
354 residues. The majority of identified suppressors involve a mutation to a bulkier side chain including
355 several in Prp8 (S1594F/Y, V1862L), Prp17 (A251V, G386W), Cef1 (A37V), and Clf1 (T33I). The
356 increase in hydrophobic surface area provided by these mutations may help to stabilize
357 interactions with nearby proteins, compensating for structural destabilization due to the absence
358 of Fyv6.

359 We isolated one suppressor mutation within the U2 snRNA (A25G) near where the C-
360 terminal domain of Prp22 inserts towards the active site and located beneath the Fyv6 binding
361 site. It has previously been observed that mutation of this specific U2 snRNA nucleotide is not
362 deleterious to growth (McPheeters and Abelson, 1992), and is therefore well-tolerated by yeast.
363 The mechanism of suppression by this mutation is not clear; however, it is interesting to note that
364 we also observed suppressor mutations in the U2 snRNP component Rse1 (**Supplemental Table**
365 **S5**) which is released from the spliceosome prior to catalysis. The Rse1 mutations always
366 occurred coincident with mutations in other splicing factors. It is possible that suppressors in

367 factors present prior to or after exon ligation are able to suppress (or enhance suppression of)
368 *fyv6Δ* by changing how pre-mRNAs compete for a limited set of splicing factors or by reducing
369 blocks to pre-spliceosome assembly caused by accumulation of stalled complexes (Mendoza-
370 Ochoa et al., 2019; Munding et al., 2013). Together these data indicate that mutations in many
371 different splicing factors including second-step factors and the U2 snRNA can be isolated from
372 *fyv6Δ* suppressor strains.

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

374 We decided to study several of these mutations in greater detail and to confirm that they
375 were sufficient for *fyv6Δ ts* phenotype suppression. In the case of Cef1, we obtained several
376 mutants located near the interface between Cef1 and the 2nd step factor Prp17: A37V, M175I, and
377 Q193P (**Fig. 6C**). This interface has only been observed in structures of C* and P-complex
378 spliceosomes, suggesting its importance for exon ligation. In addition, it has previously been
379 reported that another mutant of Cef1 A37, A37P, is able to suppress splicing defects due to a BS
380 adenine to guanosine substitution (BS-G) which causes stalling after 5' SS cleavage (Query and
381 Konarska, 2012). Therefore, we wondered if other Cef1 mutants capable of suppressing defects
382 due to BS-G could also suppress *fyv6Δ* (Cef1 A37P, V36R, and S48R). We created *fyv6Δ* shuffle
383 strains for Cef1 and tested each mutant's ability to suppress *cs* and *ts* phenotypes (**Fig. 6D**). All
384 of these Cef1 mutants, both those isolated from our suppressor screen and those identified as
385 BS-G suppressors, could at least weakly suppress the *cs* phenotype at 23°C with our isolated
386 M175I mutant being a particularly strong suppressor. In addition, all of the mutants except Cef1
387 A37V could suppress the *ts* phenotype at 37°C to varying degrees. These results confirm that
388 single point mutants in Cef1 can suppress *fyv6Δ* growth phenotypes and that at least some BS-
389 G suppressors in Cef1 also suppress *fyv6Δ*. A common mechanism could be that these mutations
390 stabilize the exon ligation conformation of the spliceosome to favor the 2nd step either when a
391 BS-G is present or Fyv6 is absent.

392 In the case of Prp8, we tested five different suppressor mutations in *prp8* shuffle strains.
393 Identification of mutants in Prp8 that suppress *fyv6Δ* is not surprising since many different alleles
394 of Prp8 are known to modulate the conformational equilibrium between the 1st and 2nd steps.
395 We previously discovered that one Prp8 2nd step allele, P986T, could suppress a *fyv6Δ* growth
396 defect (Fica and Nagai, 2017; Lipinski et al., 2023; Liu et al., 2007; Query and Konarska, 2004).
397 We identified two suppressors within the Prp8 α-finger domain at position S1584 and
398 another three within the Rnase H-like domain (**Fig. 6E, Supplemental Table S5**) (Galej et al.
399 2013). S1584 is located adjacent to a residue that crosslinks with the BP (aa 1585) (Galej et al.,
400 2013). Proximity to the BP suggests that mutations at S1584 might destabilize RNA bound within
401 the active site after branching, alleviating the stall caused by *fyv6Δ* after the 1st step or facilitating
402 formation of the 2nd step conformation.
403 Suppressors of other splicing defects have been previously identified within the Prp8
404 RNase H-like domain, two of which are located at the same position as *fyv6Δ* suppressors.
405 V1862A/Y/D and T1982A were identified as suppressors of, respectively, the *U4-cs*1 allele (Kuhn
406 and Brow, 2000; Kuhn et al., 1999) or the 5' SS U2A mutant (Siatecka et al., 1999). V1862 is
407 located within the RNase H-like domain β-finger (**Fig. 6E**), while T1982 is located at the base of
408 the β-finger (**Fig. 6C**). We identified *fyv6Δ* suppressors at both V1862 (V1862L) and T1982
409 (T1982S) as well as a third suppressor (G1868R), also located in the β-finger. Several 2nd step
410 Prp8 alleles are also located proximal to G1868 (N1869D and V1870N) (Query and Konarska,
411 2004).
412 Four of the identified Prp8 suppressor mutations could partially suppress either or both of
413 the *cs* and *ts fyv6Δ* phenotypes (**Fig. 6F**). Prp8 S1574Y, V1862L, G1868R, and T1982S
414 suppressors were able to support growth at 37°C. Prp8 V1862L and T1982S also partially
415 suppressed the growth defects at 16 and 23°C. Even though we isolated the Prp8 S1584F mutant
416 in our screen and this substitution is very similar to S1584Y, we were not able observe
417 suppression due to this mutant in these strains. Mutations within the Prp8 β-finger are able to

418 compensate for loss of Fyv6 and these mutations are located near known Prp8 2nd step alleles.
419 As with Cef1 mutants, it is possible that *fyv6Δ* suppression is originating from stabilization of the
420 2nd step conformation.

421 Finally, given our observations with the Cef1 and Prp8 mutants, we wondered about the
422 generality of 2nd step splicing factor suppression. Several dominant mutants in the 2nd step factor
423 Prp18 were previously shown to suppress *ts* phenotypes due to various mutations in Slu7
424 (Aronova et al., 2007). We wondered if these Prp18 mutants would also suppress the *ts* (or *cs*)
425 phenotype due to Fyv6 loss. Indeed, the Prp18 V191A suppressor of *slu7* alleles was able to
426 improve growth at 37°C of *fyv6Δ* yeast (**Supplemental Fig. S10**). Together our results from the
427 suppressor analysis support a model in which a given 2nd step suppressor mutation may be able
428 to restore splicing activity lost by a variety of mechanisms.

429 **A Prp22-Dependent Splicing Stall is Relieved by Fyv6 Deletion**

430 The final *fyv6Δ* suppressor mutation that we studied was the Prp22 I1133R mutant. This
431 mutation is located within the C-terminal domain of Prp22 that enters the spliceosome active site
432 and is located proximal to U2 snRNA nucleotide A31 (**Fig. 7A**). Fyv6 bridges over this Prp22-U2
433 snRNA interaction. The I1133R mutation might stabilize Prp22/U2 snRNA contacts that can
434 compensate for Fyv6 loss and promote exon ligation. The Prp22 I1133R mutant is able to weakly
435 suppress both the *cs* and *ts* phenotypes in *fyv6Δ* yeast (**Fig. 7B, C**). Given its location in the C-
436 terminal domain, we wondered if it or *fyv6Δ* would show synthetic genetic interactions or epistasis
437 with mutations in the helicase domain. We tested two helicase domain mutations, R805A and
438 G810A, both of which are known to impact Prp22-dependent proofreading (Mayas et al., 2006).
439 The Prp22 R805A mutant is by itself *cs* (Schwer and Meszaros, 2000) and showed a synthetic
440 lethal interaction with *fyv6Δ* (**Fig. 7B, 7C**). This was only slightly rescued by simultaneously
441 introducing I1133R into Prp22. In contrast, Prp22 G810A mutant is viable in the presence and
442 absence of Fyv6 as well as in combination with the I1133R suppressor mutation (**Fig. 7B, 7C**).

443 Prp22 G810A by itself does not suppress the *ts fyv6Δ* phenotype; however, the Prp22
444 G810A/I1133R double mutant is a stronger suppressor of the *ts* phenotype than either mutant
445 alone. These additive genetic interactions suggest that the Prp22 *fyv6Δ* suppressor mutation
446 (I1133R) and the helicase domain mutations (R805A and G810A) impact distinct Prp22 functions.
447 These functions could correspond to Prp22's role in promoting exon ligation during the 2nd step
448 (the C-terminal domain mutation) and its role in promoting ATP-dependent mRNA release and
449 proofreading (the helicase domain mutations).

450 Recently, it was reported that the Prp22 G810A mutant could activate usage of some
451 alternative 3' SS that are also activated by Prp18 deletion (Roy et al., 2023). We therefore
452 wondered if any of the Prp22 mutants could activate alternative 3' SS also activated by Fyv6
453 deletion. We isolated RNA from these strains and analyzed *SUS1* splicing by RT-PCR (**Fig. 7D,**
454 **E**). None of the Prp22 mutants activated usage of the *SUS1* alternative 3' SS or enhanced usage
455 of this site beyond what is observed upon Fyv6 deletion. However, we noticed that the Prp22
456 helicase mutants, R805A and G810A, both caused an accumulation of unspliced pre-mRNA. This
457 is consistent with these mutations causing a block during pre-mRNA splicing and having a
458 different effect on splicing than Prp22 I1133R. Surprisingly, the block due to the G810A mutation
459 was relieved in the *fyv6Δ* strains and little pre-mRNA accumulation was observed in the absence
460 of Fyv6. This result suggests that Fyv6 could act as a negative regulator of splicing of the SUS1
461 transcript when a Prp22 helicase domain mutant is present. Removing Fyv6 restores splicing by
462 activation of the *SUS1* BP proximal 3' SS.

463 To further explore the connections between Fyv6 and Prp22 fidelity, we used the ACT1-
464 CUP1 assay in which yeast growth in the presence of increasing concentrations of Cu^{2+} is
465 proportional to the extent of splicing of a reporter RNA (**Fig. 7F, G**; **Supplemental Fig. S11, S12**).
466 Since the Prp22 R805A mutant grew poorly, we only assayed the Prp22 G810A, I1133R, and
467 G810A/I1133R (double) mutants in the presence and absence of Fyv6. Consistent with previous

468 results, we found that *fyv6Δ* is deleterious for splicing of the WT ACT1-CUP1 reporter containing
469 consensus splice sites. Copper tolerance was improved when *fyv6Δ* was combined with Prp22
470 I1133R. Unexpectedly, Prp22 I1133R was actually deleterious when Fyv6 was present for this
471 reporter. If this Prp22 mutant and Fyv6 both promote the 2nd step, then their combination may
472 have an additive, negative effect on splicing due to over-stabilization of this spliceosome
473 conformation.

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

486 **DISCUSSION**

487 Here, we establish Fyv6 as a 2nd step splicing factor in yeast that promotes usage of BP-
488 distal 3' SS. Structural, genetic, and biochemical studies reveal critical contacts and interactions
489 between Fyv6 and the splicing machinery. From these data, we propose that Yju2/Syf1
490 interactions need to be disrupted for proper Fyv6 recruitment and that Fyv6 can enforce the
491 Prp22-dependence of at least some splicing events. Combined, our data allow us to propose a
492 model for Fyv6's role during the 2nd step (**Fig. 7H**). 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 dissociates to permit re-binding of Yju2 in the ILS complex.

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

518 exchange the Yju2/Fyv6 interaction on Syf1 may also be the origin of some of the yeast growth
519 and splicing phenotypes we observe (**Fig. 5**).

520 The need for Yju2/Fyv6 exchange hints at two distinct functions for Fyv6: a role in Yju2
521 displacement to prevent its re-binding during exon ligation and a role in stabilization of the 2nd
522 step spliceosome conformation. These two functions can potentially result in the distinct *cs* and
523 *ts* phenotypes in yeast. While more work is needed to dissect these phenotypes, it is possible that
524 the *cs* phenotype results from increased stability of the Yju2/Syf1 interaction at lower
525 temperatures and the need for Fyv6 to act as a competitor of this interaction. The *ts* phenotype
526 on the other hand may result from destabilization of the 2nd step conformation of the spliceosome
527 active site due to loss of Fyv6. It is possible that loss of Fyv6 results in destabilization due to
528 removal of a buttress that holds the RNase H-like domain of Prp8 in place (along with Prp18),
529 changes in how the C-terminal domain of Prp22 interacts with the spliceosome active site,
530 removal of an impediment to reversal of the U2 snRNA/BS duplex conformational change that
531 occurs during the C to C* complex transition, or a combination of these factors.

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

541 In the state III P complex structure, densities for the 2nd step factors Fyv6, Prp18, and Slu7
542 are entirely missing and the 3' SS is no longer docked into the active site. This result suggests
543 that 3' SS undocking is coupled to 2nd step factor release. Prp22 is still present in this structure,

544 and it is possible that state III represents a conformation occurring after exon ligation but prior to
545 Prp22-dependent mRNA release. In this case, the state III structure implies that Prp22 ATPase
546 activity is not needed for dissociation of Fyv6, Prp18, and Slu7. It is also interesting to note that
547 the C-terminal domain of Prp22 can no longer be resolved in the state III structure. What exactly
548 activates the Prp22 ATPase to promote proofreading or mRNA release is not known. It is
549 conceivable that undocking of the 3' SS from the active site can be sensed by the Prp22 C-
550 terminal domain and this contributes to ATPase activation. This model differs from that previously
551 proposed for Prp22-enabled 3' SS sampling in which that ATPase activity of Prp22 is needed to
552 undock the 3' SS (Semlow et al., 2016). However, sampling could involve multiple steps: ATP-
553 independent 3' SS undocking and ATP-dependent entry into the sampling state. Whether or not
554 the 2nd step factors must release or alter conformation to enable sampling is unknown. Regardless
555 of the exact mechanism, it is important to recognize that the P complex structures determined
556 here were all obtained using a Prp22 mutant defective in mRNA release. The chemical and kinetic
557 competencies of these states will require future investigations.

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

559 The challenge of 3' SS selection involves not just selection of the correct nucleotide
560 sequence (YAG) but also identifying which YAG sequence to use. Here, we define a function for
561 Fyv6 in facilitating use of BP distal 3' SS, especially those located $\geq$ 21 nt away from the branch.
562 This function is similar to those of other 2nd step factors important for efficient splicing when the
563 BP-3' SS distance is $\geq$12 nt (Slu7, Prp18) or $\geq$21 nt (Prp22) (Brys and Schwer, 1996; Schwer and
564 Gross, 1998; Zhang and Schwer, 1997). Human Slu7 has additionally been reported to be
565 important for preventing suppression of the correct 3' SS in favor of up- or downstream alternative
566 3' SS (Chua and Reed, 1999). Our previous study using competing 3' SS in the ACT1-CUP1
567 intron did not provide evidence for suppression of the correct 3' SS in the absence of Fyv6,
568 suggesting that this function is not shared between human Slu7 and yeast Fyv6. Nonetheless, it
569 is important to recognize that the splicing machinery employs multiple factors that enforce usage

570 of BP distal 3' SS. These functions are not redundant as changes in 3' SS are still observed in the
571 absence of Fyv6 but presence of Prp18 and vice versa (Roy et al., 2023).

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

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

590 In addition to this possibility, Fyv6 and Prp18 may also contribute uniquely to 3' SS fidelity
591 and selection. The conserved loop region of Prp18 inserts into the spliceosome active site and
592 contacts the 3' SS. Partial deletion of this region of Prp18 phenocopies at least some of the
593 changes in 3' SS selection when Prp18 is deleted entirely (Crotti et al., 2007; Roy et al., 2023).
594 This indicates that even when the Prp8 RNaseH-like domain is still stabilized by Prp18 (and Fyv6)
595 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 *fyv6Δ* 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 (**Fig. 7H**). 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,

622 removal of Cus2, and proofreading is unclear (Liang and Cheng, 2015; Talkish et al., 2019; Xu
623 and Query, 2007). It is possible that enforcement of Prp5 ATPase activity by Cus2 is important for
624 other aspects of pre-spliceosome formation *in vivo* and the impact of Prp5 and Cus2 on BP
625 selection has not yet been studied transcriptome-wide using high-resolution, sequencing-based
626 approaches. The C-terminal domain of the 1st step splicing factor Yju2 (the same domain that
627 competes with Fyv6 for Syf1 binding) is also not essential for 5' SS cleavage. In the absence of
628 this domain, the N-terminal domain of Yju2 is sufficient for promoting the 1st step reaction, and
629 the spliceosome can transition to the 2nd step and carry out exon ligation without Prp16, albeit
630 inefficiently (Chiang and Cheng, 2013). Importantly, addition of the C-terminal domain on a
631 separate polypeptide reduces Prp16-independent activity. This suggests that the Yju2 C-terminal
632 domain enforces the use of the Prp16 ATPase. As we propose with Fyv6 and Prp22, the C-
633 terminal domain of Yju2 may not just help to promote the 1st to 2nd step transition by enforcing
634 Prp16-dependence but could also facilitate Prp16-dependent proofreading during 5' SS cleavage
635 (Koodathingal et al., 2010; Semlow et al., 2016). General operating principles of the yeast splicing
636 machinery may not just include proofreading but also use of splicing factors that enforce usage
637 of the proofreading ATPases.
638

639 **MATERIALS AND METHODS**

640 The yeast strains and plasmids used in this study are described in **Supplemental Tables**
641 **S6 and S7**. Yeast transformation, plasmid shuffling/5-FOA selection, and growth were carried out
642 using standard techniques and media (Treco and Lundblad 1993; Sikorski and Boeke 1991).

643 **Cryo-electron microscopy**

644 <u>Purification of dominant-negative Prp22</u>

645 Yeast Prp22 with a dominant-negative S635A mutation was cloned into pRS424 and
646 pRS426 vectors after an N-terminal CBP-His-TEV tag (Galej et al., 2013). The protein was

647 expressed in 24 L of BCY123 yeast cells essentially as described (Galej et al., 2013).
648 Recombinant Prp22 was then purified first by Calmodulin-Sepharose in a buffer containing 50
649 mM Tris-HCl pH 8.5, 350 mM NaCl, 2 mM $CaCl_2$, 1 mM Mg acetate, 1 mM imidazole, 0.1% NP-
650 40 substitute, 5 mM β-mercaptoethanol and eluted with 3 mM EGTA. The protein was dialyzed
651 into 20 mM Tris-HCl pH 7.4, 350 mM NaCl, 10 mM imidazole, 0.1% NP-40 substitute and further
652 purified over Ni-NTA resin at 500 mM NaCl and eluted with 200 mM imidazole. The eluted protein
653 was treated with His-tagged TEV protease during dialysis against 20 mM HEPES-KOH pH 7.9,
654 300 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM DTT, before removal of the protease with Ni-
655 NTA resin. The final protein was frozen in liquid nitrogen and stored at -80 °C.

656 Preparation and purification of P complex for cryo-EM

657 P complex was purified as described in (Wilkinson et al., 2017). Brr2-TAPS yeast were
658 grown in a 120 L fermenter and splicing extract was prepared by the liquid nitrogen method
659 essentially as previously described (Lin et al., 1985). A pre-mRNA substrate consisting of 20 nt
660 5'-exon, 95 nt intron, and 32 nt 3'-exon from the UBC4 pre-mRNA followed by 3xMS2 stem loops
661 was generated by run-off in vitro transcription from an EcoRI-linearized plasmid template. The
662 RNA product was labelled at the 3' end with fluorescein-5-thiosemicarbazide (Wu et al., 1996). A
663 180 mL in vitro splicing reaction was performed containing 60 mM potassium phosphate pH 7, 2
664 mM ATP, 2.5 mM $MgCl_2$, 3% w/v PEG8000, 3 nM pre-mRNA, 37.5 nM MS2-MBP protein, and
665 40% (v/v) splicing extract treated with dominant negative Prp22 S635A. Reactions were incubated
666 for 30 min at 23°C, then incubated for 20 min with 5 mM of a DNA oligonucleotide complementary
667 to the 3'-exon (oligo sequence 5'-ATGAAGTAGGTGGAT-3') to induce cleavage of the 3'-MS2 tag
668 by the endogenous RNase H activity of the splicing extract. The reaction mixture was centrifuged
669 through a 40% glycerol cushion in buffer A (20 mM HEPES, pH 7.9, 75 mM KCl, 0.25 mM EDTA).
670 The cushion was collected and applied to amylose resin in the presence of 0.025% NP-40
671 substitute. After overnight incubation at 4°C the resin was washed and eluted with buffer A

672 containing 5% glycerol, 0.01% NP-40 substitute and 12 mM maltose. Fractions containing
673 spliceosomes were concentrated to 0.1 mg/mL then dialyzed against buffer A for 3 h.
674 For cryo-EM grid preparation, a freshly glow-discharged Cu300 R2/2 holey carbon grid
675 with a 2-nm layer of amorphous carbon (Quantifoil) was mounted in the chamber of a Vitrobot
676 Mark IV (Thermo Fisher Scientific) maintained at 4°C and 100% humidity. Spliceosomes (3 µL)
677 were applied and after 60 s was manually blotted using Ø55 grade 595 filter paper (Ted Pella),
678 followed by application of another 3 µL of spliceosomes, waiting 60 s, before blotting again and
679 plunging into liquid ethane.

680 Cryo-EM data collection

681 Cryo-EM data were collected as two datasets from two grids made from the same sample.
682 Dataset 1 was acquired using a Thermo Scientific Titan Krios cryo-TEM (LMB Krios 1) and dataset
683 2 was acquired using a Thermo Scientific Titan Krios G3i cryo-TEM (LMB Krios 3). Both used a
684 K3 direct detector (Gatan) operated in super-resolution mode with 2-fold binning, and an energy
685 filter with slit width of 20 eV. Micrographs were collected automatically using EPU in AFIS mode,
686 yielding a total of 51,113 movies at 81,000x or 130,000x magnification with a real nominal pixel
687 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
688 micrograph of 40 $e^-/Å^2$, fractionated into 40 frames.

689 Cryo-EM data processing

690 All cryo-EM data were processed using RELION-5.0 (Kimanius et al., 2023). Both datasets
691 were initially processed separately. Movies were corrected for motion using the RELION
692 implementation of MotionCor2, with 4x4 patches and dose-weighting. CTF parameters were
693 estimated using CTFFIND-4.1. Particle picking was done using Topaz with the general model
694 (Bepler et al., 2019), yielding 1,820,457 particles. 3D classification was performed on 4-fold
695 (dataset 1) or 5-fold (dataset 2) binned particles using a P-complex reference map (EMD-10140),
696 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., 2023). 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

723 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
724 for Cwc22-NTD. In all cases, control refinements without Blush regularization did not produce
725 interpretable maps (**Supplemental Fig. S5).**

726 Model building

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

734 **Plasmid cloning**

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

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

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

746 **Yeast strain creation**

747 The *DBR1* and *FYV6* genes were deleted by replacement with a nourseothricin resistance
748 cassette (NatMX4) or hygromycin resistance cassette (hphMX4), respectively, through
749 homologous recombination (see **Supplemental Table S7**; Goldstein and McCusker, 1999). The
750 *SYF1* and *CEF1* genes were deleted by replacement with a kanamycin resistance cassette
751 (KanMX) through homologous recombination in strains transformed with pRS416-Syf1 or
752 pRS316-Cef1 respectively. Gene deletion was confirmed by colony PCR and/or genomic DNA
753 extraction from the strains and PCR amplification of the corresponding genomic locus.

754 **RNA isolation**

755 Yeast were grown in YPD media until OD_{600} 0.5-0.8. For RNA-sequencing of temperature-
756 shifted samples, cultures were shifted to either 37°C or 16°C or remained at 30°C and grown for
757 1 hr. A 15 mL volume of culture was harvested via vacuum filtration with mixed cellulose ester
758 filter paper (Whatman, 47 mm diameter) in a Buchner funnel. Filter papers were separated into 5
759 mL tubes and flash frozen. Filters were stored at –80°C until RNA isolation. Filters were washed
760 with 1 mL TRIzol to collect cells. For all other experiments involving RNA isolation, 10 OD_{600} units
761 of OD_{600} 0.5-0.8 yeast were harvested via centrifugation.

762 Cell walls were disrupted by incubation with Zymolyase (50 U/mL, Zymo Research) in Y1
763 buffer (1 M sorbitol, 100 mM EDTA pH 8.0, 13 mM β-mercaptoethanol) (primer extension, RNA-
764 seq of non-temperature-shifted samples) or vortexing with silica disruption beads in TRIzol (RT-
765 PCR, RNA-seq of temperature-shifted samples). Total RNA was then isolated using TRIzol
766 reagent (Thermo Fisher Scientific) according to manufacturer's instructions. Samples were
767 cleaned using a 50 µg Monarch RNA Cleanup Kit (New England Biolabs) and treated with TURBO
768 DNase (Thermo Fisher Scientific) to remove residual contaminating DNA. For RNA-sequencing,
769 quality of RNA samples was assessed prior to library preparation by NanoDrop (concentration,
770 A_{260}/A_{280}, and A_{260}/A_{230}), Qubit (RNA High Sensitivity and RNA IQ), and TapeStation (RNA
771 ScreenTape Analysis). High quality samples showing minimal rRNA degradation with RIN >7 and
772 A260/A280 ratios of ~2.0 were selected for library preparation.

773 **Library preparation and RNA sequencing**

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

778 **Bioinformatic analysis of RNA-seq datasets**

779 FASTQC was used for quality control of reads pre- and post- trimming (Andrews, 2010).
780 Trimming of reads was accomplished using fastp (Chen et al., 2018) prior to mapping to the
781 SacCer3 genome (Ensembl, R64-1-1) with STAR (Dobin et al., 2013). All samples were aligned
782 with STAR in a first pass. Novel junctions from all samples (WT and *fyv6Δ*) were combined and
783 filtered for likely false-positive junctions (see Supplemental Methods). STAR was run in a second
784 pass with the additional input of the filtered set of novel junctions. Indexing of bam files was
785 accomplished with SAMtools (Li et al., 2009). Quantitation of read counts aligned to the SacCer3
786 transcriptome was accomplished with Salmon (Patro et al., 2017). Differential gene expression
787 analysis was conducted with DeSeq2 (Love et al., 2014). Junction reads within genes were
788 counted with featureCounts (Liao et al., 2014) and used to calculate fraction of Annotation Splicing
789 (FAnS) based on methods in Roy et al., 2023 (see Supplemental Methods). Canonical branch
790 points were used as defined in the Ares Intron Database (Grate and Ares, 2002). Differential
791 splicing analysis was conducted with SpliceWiz (Wong et al., 2024).

792 **Primer extension**

793 RNA was isolated using the protocol above. IR700 dye conjugated probes (Integrated DNA
794 Technologies) were used for primer extension with SuperScript III (Thermo Fisher) of the ACT1-
795 CUP1 reporters (2 pmol yAC6: /5IRD700/GGCACTCATGACCTTC) and U6 snRNA (0.4 pmol
796 yU6: /5IRD700/GAACTGCTGATCATGTCTG) (Carrocci et al., 2017; van der Feltz et al., 2021).
797 Primer extension products were visualized on a 7% (w/v) denaturing polyacrylamide gel run at 35

798 W for 80 min at RT. Gels were imaged with an Amersham Typhoon NIR laser scanner (Cytiva),
799 and band intensities were quantified with Image J (version 1.53v, 2022).

800 **RT-PCR**

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

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

811 **Selection of *fyv6Δ* suppressors**

812 Strain yAAH3353 was previously described (Lipinski et al. 2023). Twenty single colonies
813 of yAAH3353 were selected from a YPD plate and used to inoculate 20 x 5mL YPD liquid culture.
814 Cultures were grown for 48 h at 30°C. A 1 mL volume of culture was spun down, resuspended in
815 200 µL YPD liquid medium, and spread on a YPD plate containing 40 mg/L adenine hemisulfate
816 (to prevent ade2 reversion). Duplicate plates for each overnight were placed in incubators at either
817 18°C or 37°C. Many colonies of various sizes appeared on each plate by 9 days for 37°C and 12
818 days for 18°C. Colonies were picked after that time and annotated first by growth temperature,
819 then by overnight number, and finally roughly by colony size with 01 generally being the largest
820 colony on a plate (ex: 370101 from a plate grown at 37°C inoculated from overnight 01 and the
821 largest colony selected from the plate). Picked colonies were grown overnight in 5mL YPD at 30°C
822 and frozen stocks were made. Anywhere from 0 to ~15 colonies were picked per plate, covering
823 the variety of colony sizes that arose.

824 **Sequencing of yeast genomic DNA**

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

828 **Bioinformatic analysis of WGS data**

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

839 **Yeast temperature growth assays**

840 Yeast were grown to stationary phase overnight in the appropriate liquid medium indicated
841 in the figure legends. The cultures were first diluted to OD_{600} 0.5, serially diluted 1:10, 1:100, and
842 1:1000, and stamped onto agar plates containing the appropriate growth medium. The plates
843 were incubated at 16°, 23°, 30°, and 37°C, and plates were imaged after the number of days
844 indicated in each figure.

845 **ACT1-CUP1 copper tolerance assays**

846 Yeast strains expressing ACT1-CUP1 reporters were grown to stationary phase in -Leu
847 DO media, diluted to OD_{600} = 0.5 in 10% (v/v) glycerol, and spotted onto -Leu DO plates containing
848 0 to 2.5 mM $CuSO_4$ (Lesser and Guthrie 1993; Carrocci et al., 2018). Plates were scored and

849 imaged after 48 h of growth at 30°C for WT strains and after 72 h of growth at 30°C for *fyv6Δ*
850 strains due to differential growth between strains.
851
852
853 **ACKNOWLEDGEMENTS**

854 We thank David Brow for his advice on the suppressor screen. We thank Justin Mabin for
855 his advice on RNA-sequencing data analysis. MEW is grateful to Feng Zhang for funding and
856 support, and to the labs of Kiyoshi Nagai and Kelly Nguyen for reagents and support. The authors
857 utilized the University of Wisconsin – Madison Biotechnology Center Gene Expression Center
858 (Research Resource Identifier – RRID:SCR_017757) for preparation of NGS libraries for RNA-
859 sequencing and the Biochemistry Computational Research Facility (BCRF) for analysis of RNA-
860 sequencing datasets. This work was supported by grants from the National Institutes of Health
861 (R35 GM136261 to AAH), NIH Biotechnology Training Program (T32 GM135066) and National
862 Science Foundation Graduate Research Fellowship Program (Grant No. DGE-1747503)
863 fellowships to KAS, a Helen Hay Whitney Postdoctoral Fellowship (to MEW). This study was
864 supported by the MRC Laboratory of Molecular Biology electron microscopy facility.
865

866 **CONTRIBUTIONS**

867 AAH, KAS, KAL, and MEW conceptualized the project; MEW collected and analyzed the cryo-EM
868 data; KAL and NJZ prepared RNA for sequencing; KAL, NJZ, and AG analyzed the RNA-
869 sequencing data; AAH, KAS, NJZ, and KAL created yeast strains; KAS, KAL, NJZ, and AG
870 performed genetic and biochemical assays and analyzed data; KAS, KAL, MEW, and AAH wrote
871 the manuscript.
872

873 **DATA AND MATERIALS AVAILABILITY**

874 The cryo-EM maps will be deposited in the Electron Microscopy Data Bank and the atomic model
875 to the Protein Data Bank upon manuscript submission. Raw cryo-EM data will be available from
876 EMPIAR.
877

878 **COMPETING INTEREST STATEMENT**

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

881
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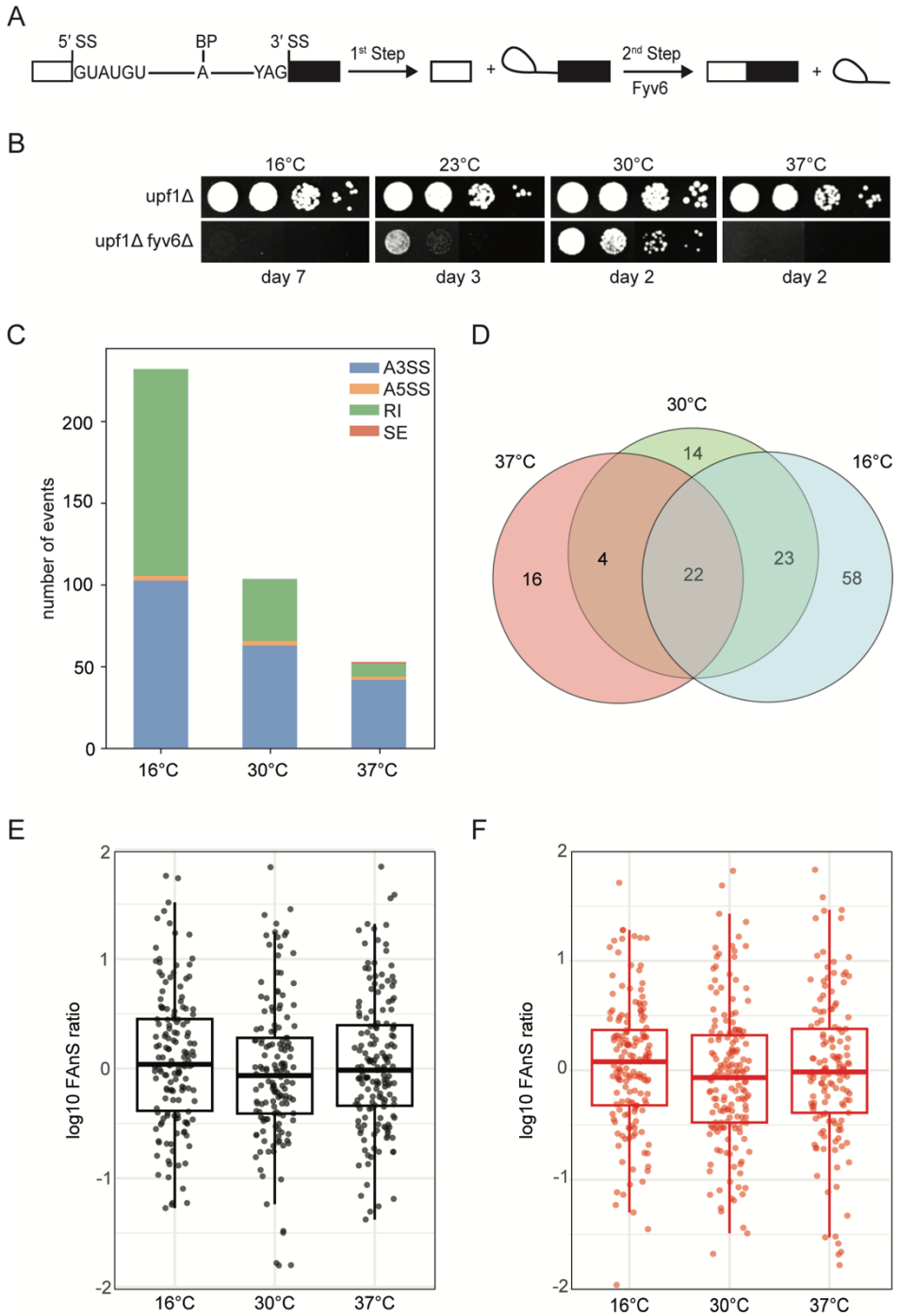

Figure 1. Widespread splicing changes due to loss of Fyv6. A) Pre-mRNA splicing reaction steps and products. **B)** Growth of *upf1Δ* and *fyv6Δupf1Δ* yeast at 16, 23, 30, and 37°C. Images were taken at the indicated days. **C)** Number of alternative splicing events observed in a *fyv6Δupf1Δ* strain relative to a strain containing FYV6 after a temperature shift to 16, 30, and 37°C. **D)** Venn diagram of shared alternative splicing events at 16 (blue), 30 (green), and 37°C (red). **E, F)** Box plots of the $\log_{10}$ of the ratios of *fyv6Δ upf1Δ* to WT (*upf1Δ*) FAnS for non-ribosomal protein genes (non-RPGs, panel E) and RPGs (panel F).

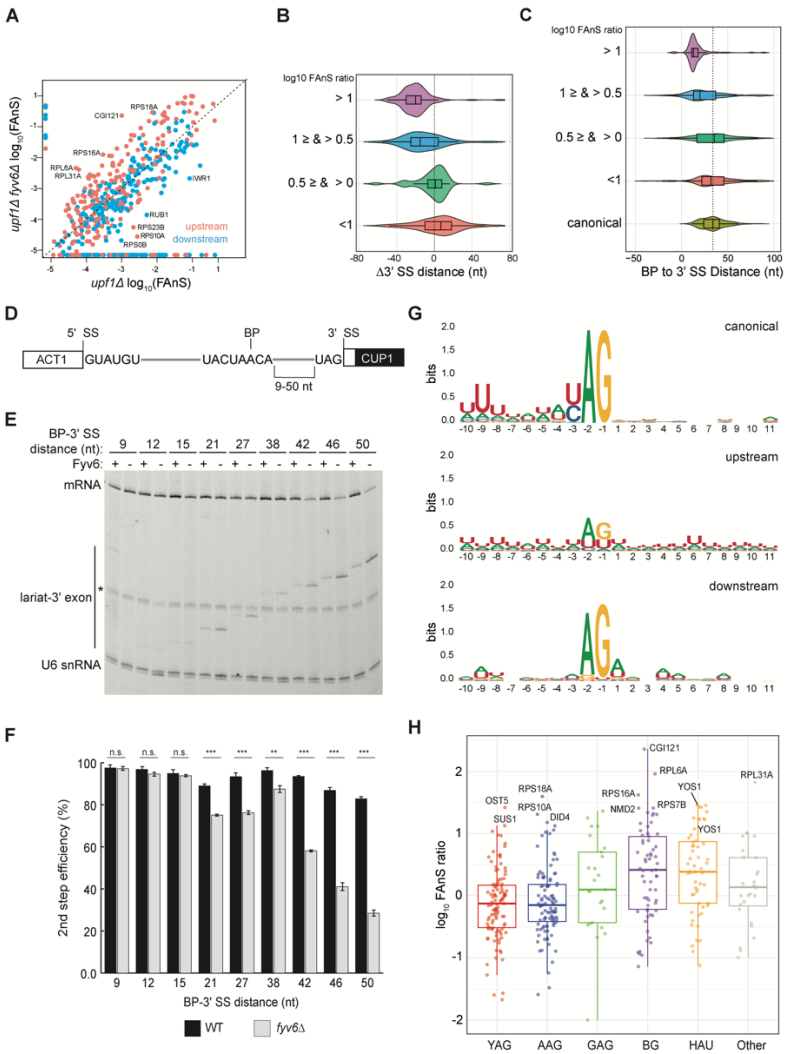

Figure 2: Loss of Fyv6 activates BP proximal, non-consensus 3' SS. A) Plot of the $\log_{10}$ of FAnS values for changes in alternative 3' SS usage in *fyv6Δ upf1Δ* and *upf1Δ* strains. Points are colored based on whether they are upstream (pink) or downstream (blue) to the canonical 3' SS. **B)** Violin plots of the ratios of *fyv6Δupf1Δ* and *upf1Δ* FAnS values based on the distances between the alternative and canonical 3' SS. FAnS ratios >0 are indicative of the site being upregulated in *fyv6Δ*. **C)** Violin plots of the ratios of *fyv6Δupf1Δ* and *upf1Δ* 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 *fyv6Δ*. **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 (*fyv6Δ*). 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 ±SD. Means between WT and *fyv6Δ* 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.

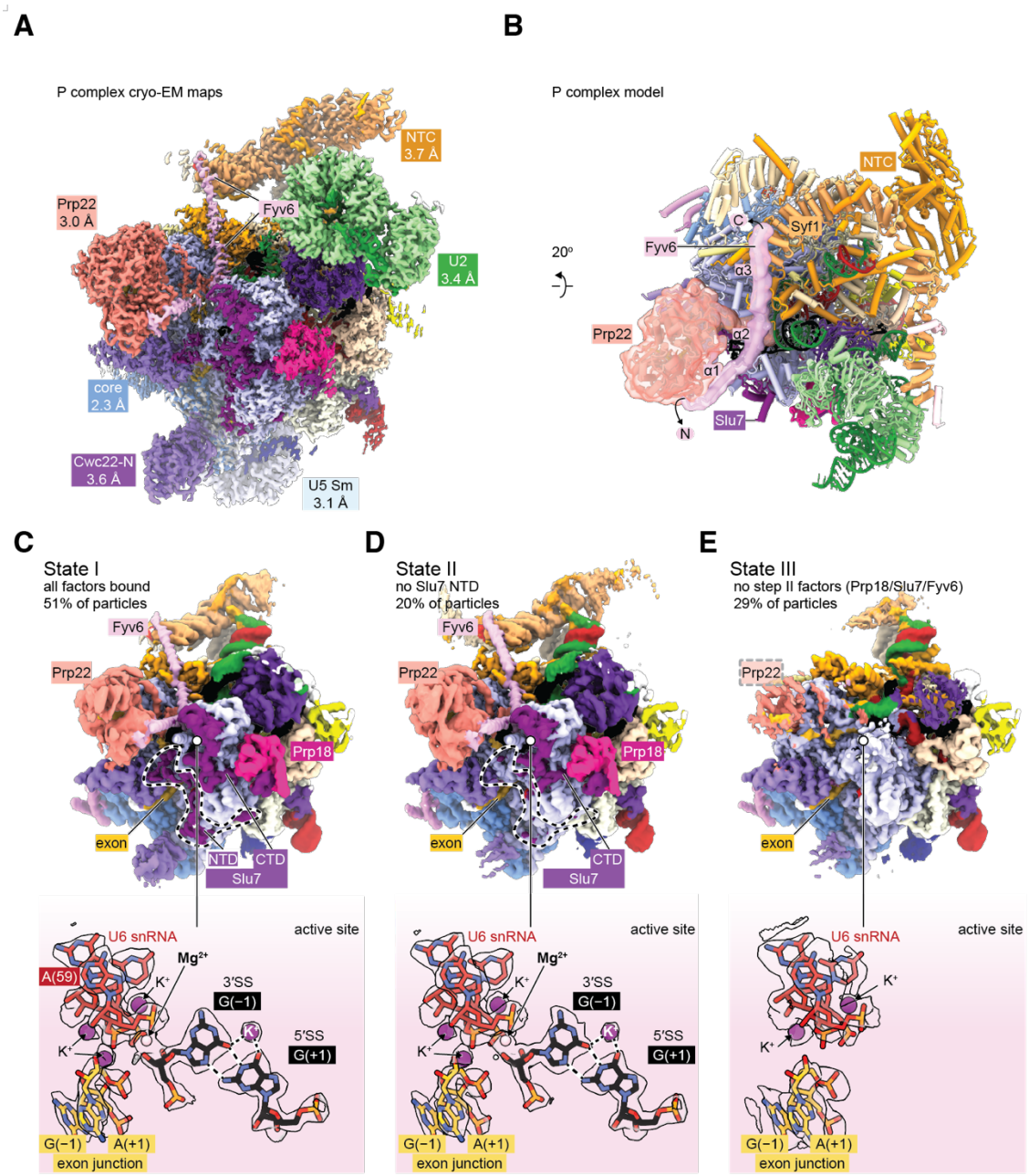

Figure 3. Cryo-EM structure of the yeast P complex spliceosome at 2.3 Å resolution. A) A composite density map for P complex showing focused refinements of the Prp22, NTC, U2 snRNP, U5 Sm ring, and Cwc22 N-terminal domain regions. **B)** Overall model for the P complex spliceosome. **C)** Cryo-EM density 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**).

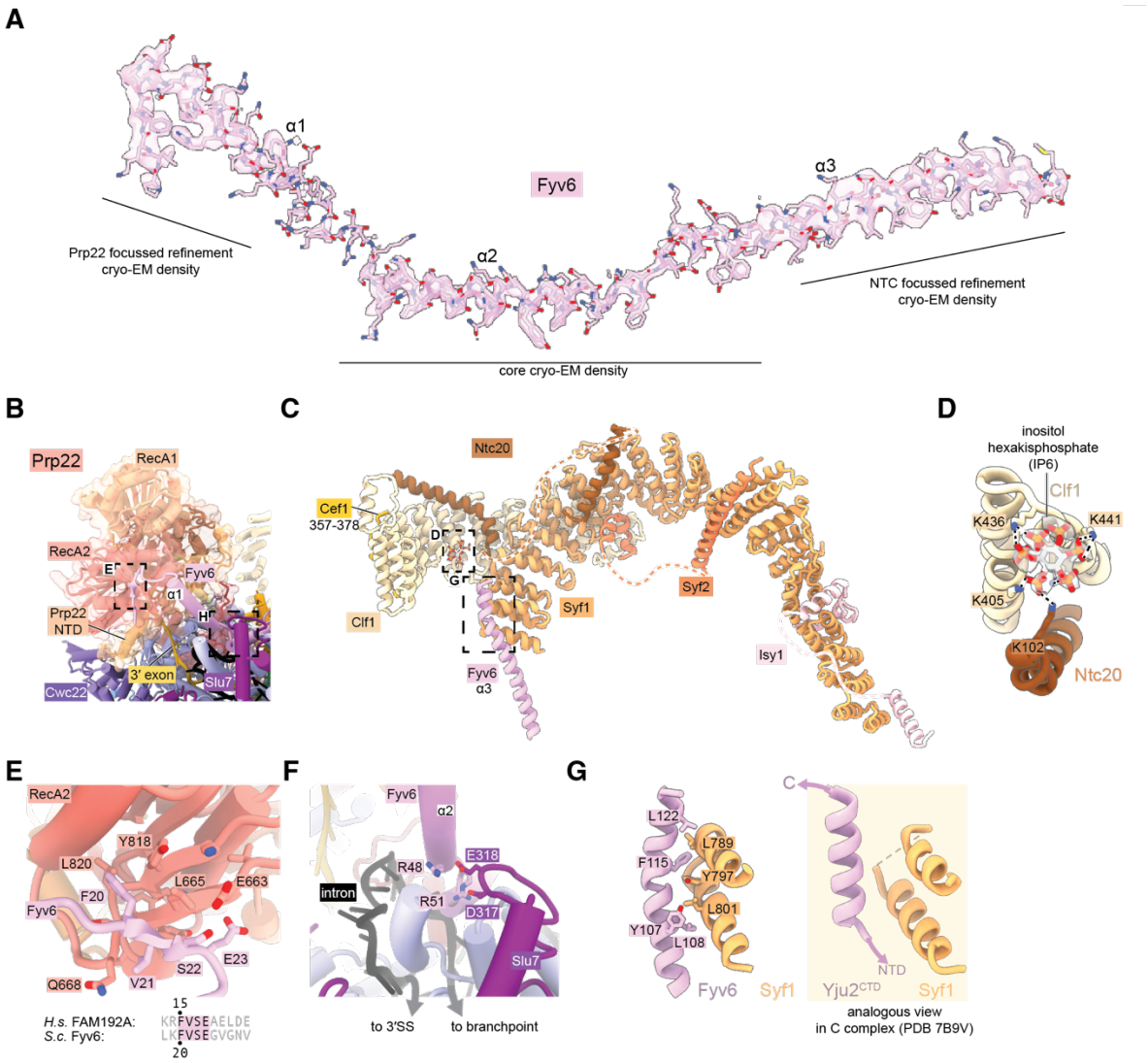

Figure 4. Fyv6 and its interactors in the P complex cryo-EM structure. A) Cryo-EM density segmented around Fyv6. **B)** Structure of Prp22. **C)** Structure of the NTC within P complex. **D)** IP_6 site and cryo-EM density in NTC. **E)** Interaction of the hook domain of Fyv6 with the Prp22 RecA2 domain. **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).

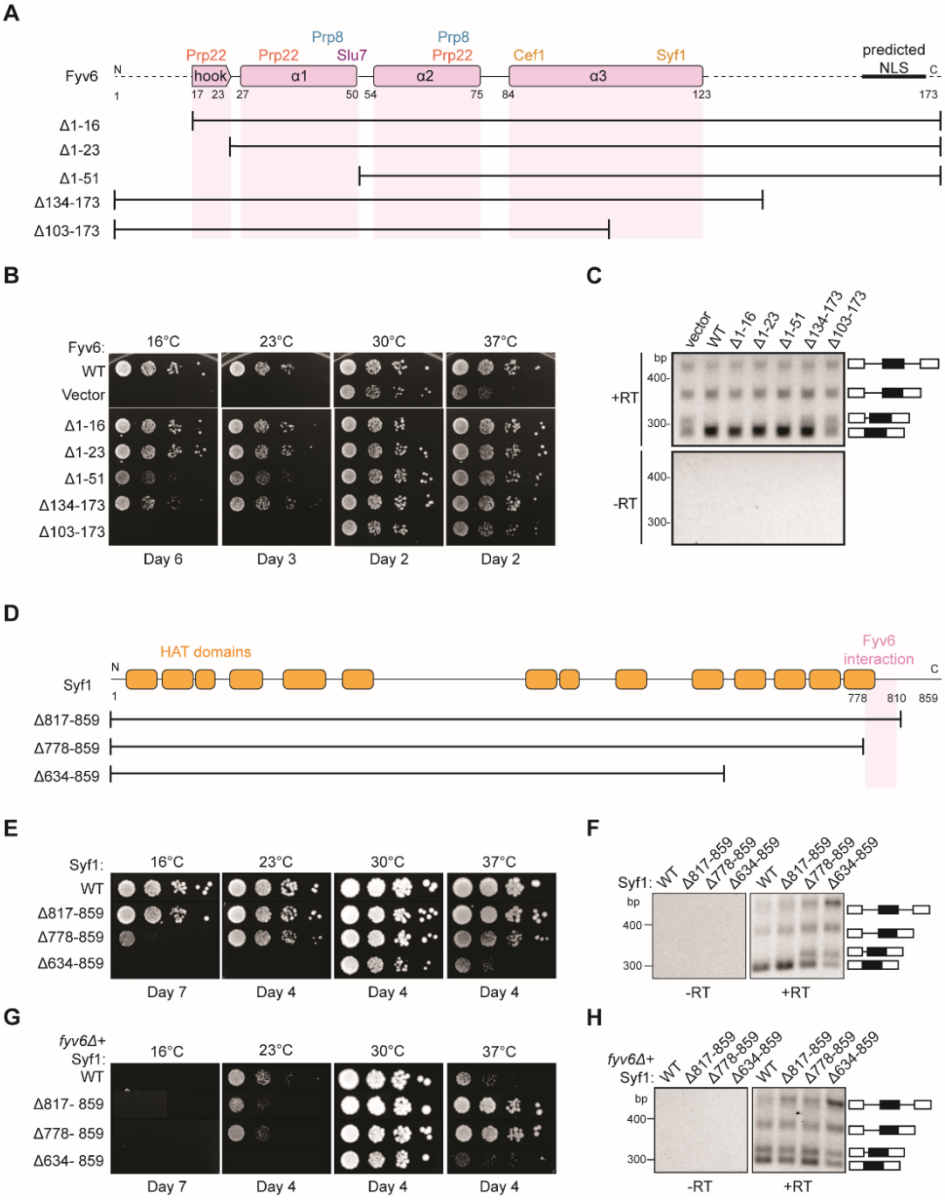

Figure 5. Structure-based analysis of Fyv6 domain function. A) Diagram of Fyv6 protein structure, protein interactors, and protein truncations. NLS= nuclear localization signal **B)** Spot dilution assay of strains with Fyv6 truncations on –Trp DO plates at different temperatures. Plates were imaged 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.

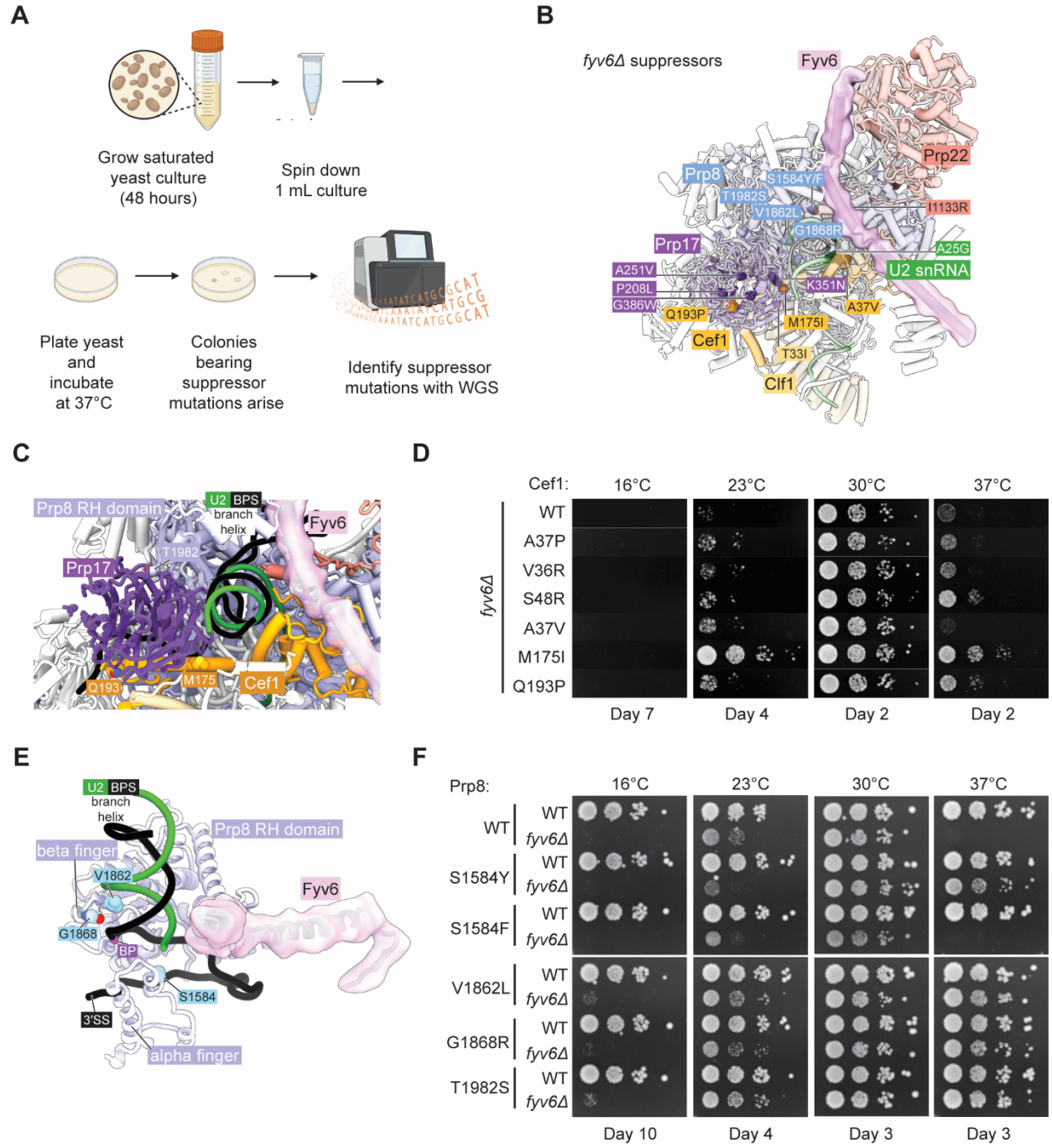

Figure 6. Identification of novel suppressors of *fyv6Δ* temperature sensitivity. A) Workflow for the suppressor screen. **B)** P complex spliceosome structure with *fyv6Δ* suppressor mutations labelled. (See **Supplemental Table S5**) **C)** Close-up view of Cef1 and Prp8 suppressor mutations in P complex. **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.

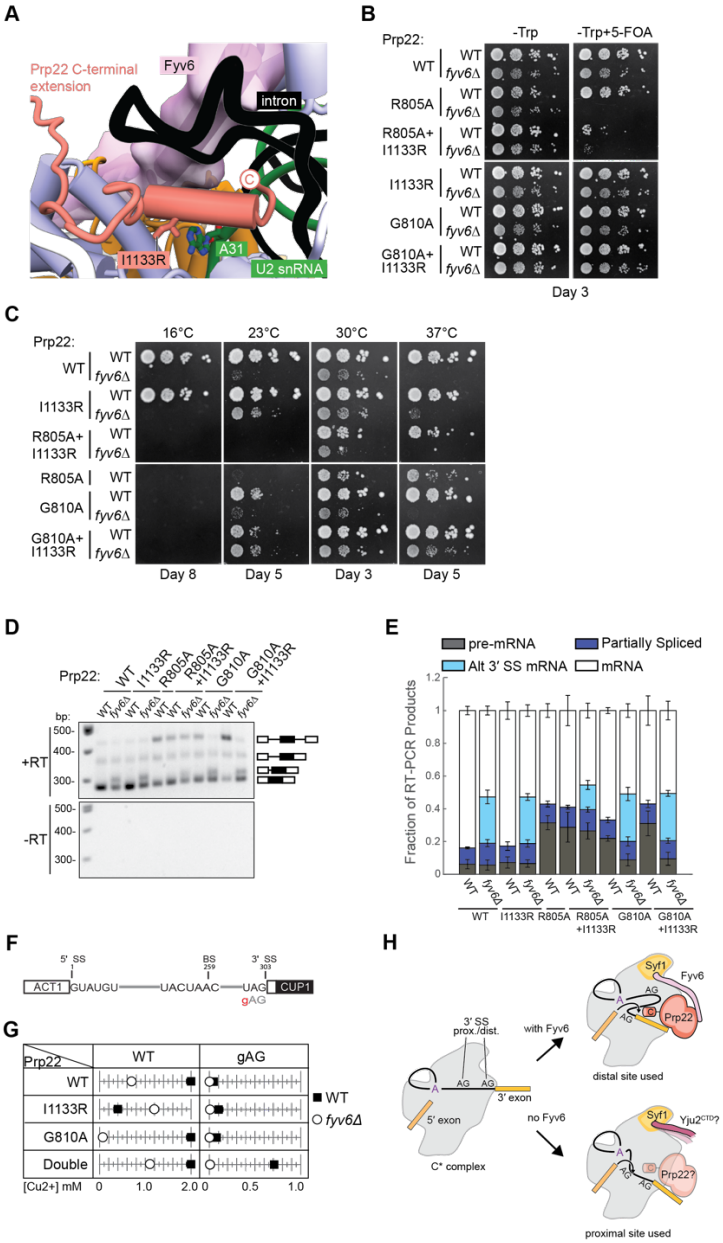

Figure 7. Genetic interactions between Prp22 and Fyv6. A) Location of the Prp22 I1133R *fyv6Δ* suppressor in the P complex structure. **B)** Spot dilution growth assay with Prp22 mutants in WT or *fyv6Δ* backgrounds grown on –Trp DO or –Trp+5-FOA plates. Plates were imaged after 3 days at 30°C. **C)** 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 ± 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. **H)** 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.