

## Supplementary Information for

# **Dynamics of the DEAD-box ATPase Prp5 RecA-like Domains Provide a Conformational Switch during Spliceosome Assembly**

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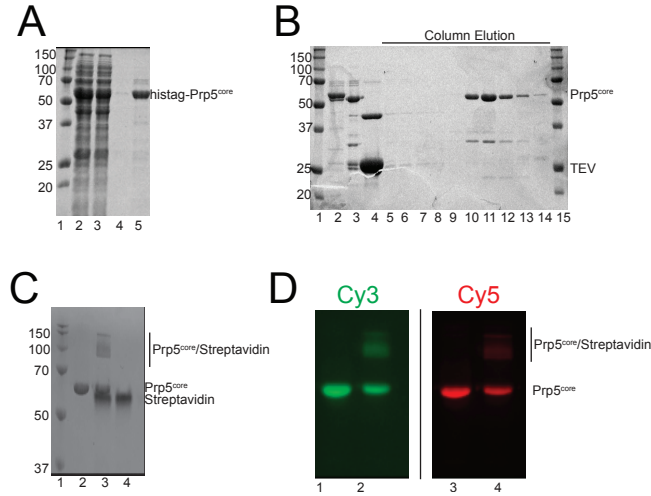
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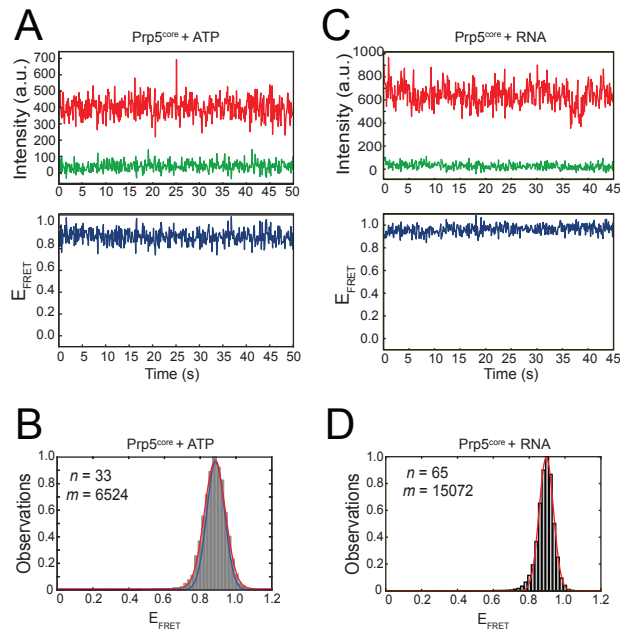
### **This PDF file includes:**

Figs. S1 to S9

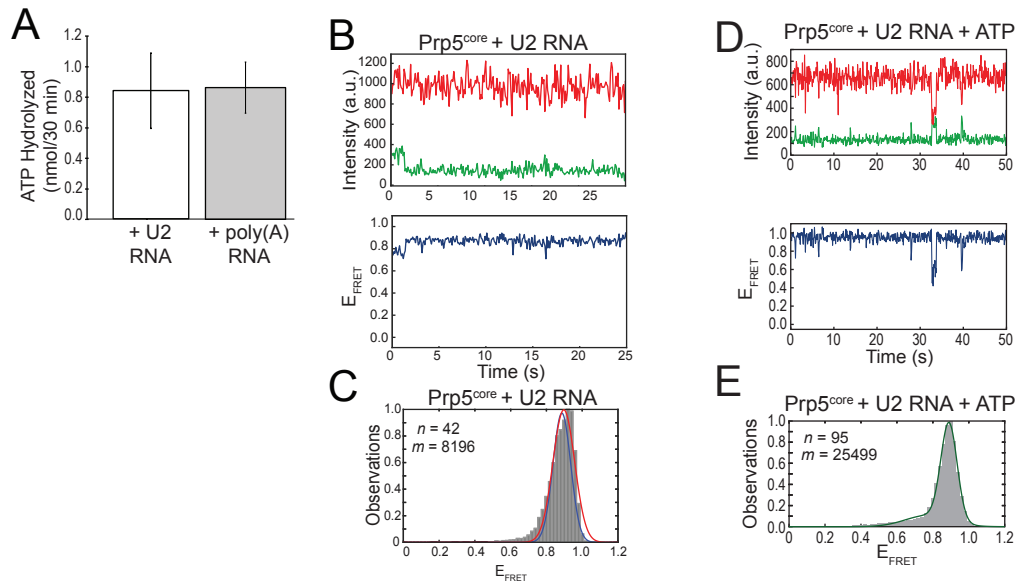
Tables S1 to S5



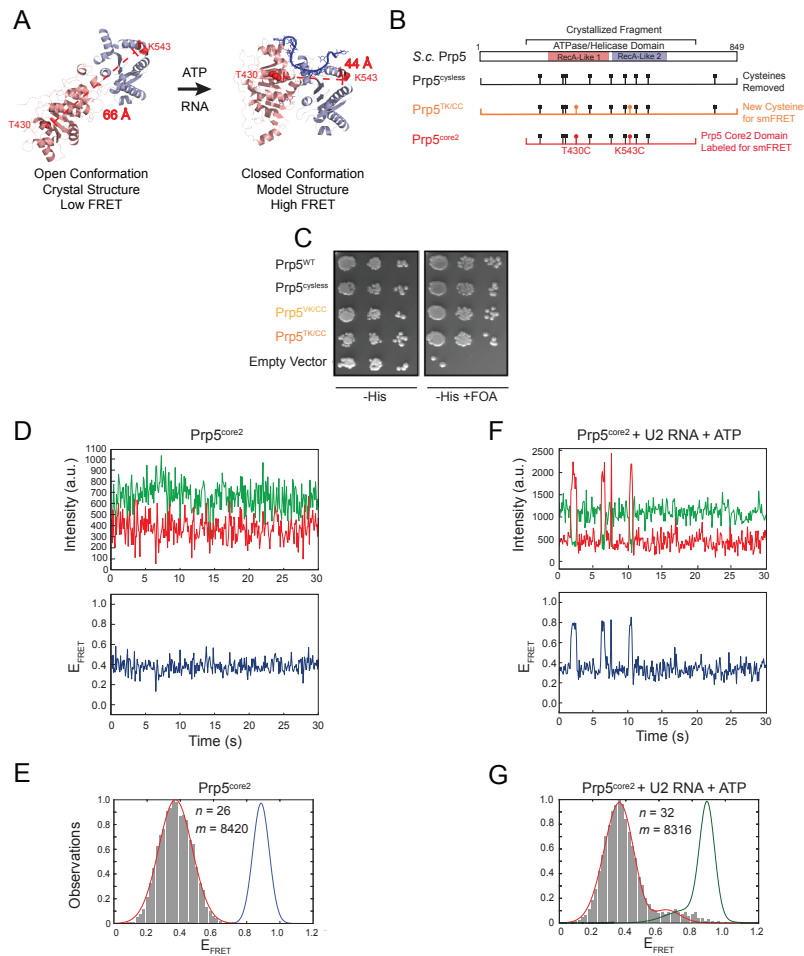
**Fig. S1. Purification and labeling of Prp5<sup>core</sup>.** (A) SDS-PAGE gel of histag-Prp5<sup>core</sup> before and after purification by Ni<sup>2+</sup>-NTA affinity chromatography. Lane 1: MW markers; Lane 2: soluble cell lysate; Lane 3: column flow-through; Lane 4: column wash; Lane 5: elution fraction. (B) SDS-PAGE gel of Prp5<sup>core</sup> before and after cleavage with TEV protease and ion exchange chromatography. Lane 1: MW markers; Lane 2: histag-Prp5<sup>core</sup>; Lane 3: Prp5<sup>core</sup> after TEV cleavage; Lane 4: TEV protease; Lanes 5-14: column elution fractions; lane 15: MW markers. (C) Gel-shift assay for Prp5<sup>core</sup> biotinylation. Lane 1: MW markers; Lane 2: purified Prp5<sup>core</sup>; Lane 3: Prp5<sup>core</sup> and streptavidin; Lane 4: streptavidin. (D) Fluorescence images of an SDS-PAGE gel of Cy3/Cy5 labeled Prp5<sup>core</sup>. Lanes 1 and 2 represent images of Cy3 fluorescence, and Lanes 3 and 4 represent the same gel imaged for Cy5 fluorescence. Lanes 1 and 3: Cy3/Cy5-Prp5<sup>core</sup>; Lanes 2 and 4: Cy3/Cy5-Prp5<sup>core</sup> + streptavidin.



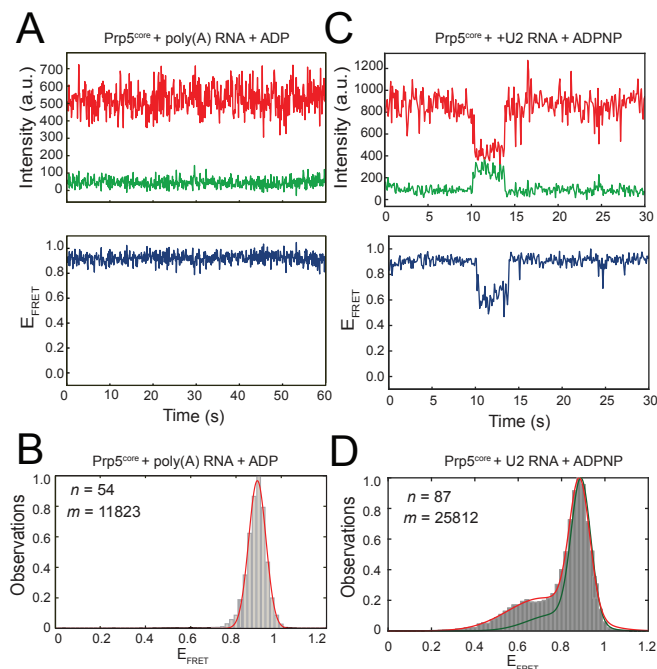
**Fig. S2. smFRET observations of Prp5<sup>core</sup> in the presence of ATP or RNA.** (A) Top: Cy3 (green) and Cy5 (red) fluorescence trajectories from a single molecule of Prp5<sup>core</sup> in the presence of ATP. Bottom: Calculated  $E_{\text{FRET}}$  from the Cy3/Cy5 fluorescence trajectories. (B) Histogram of  $E_{\text{FRET}}$  values obtained for Prp5<sup>core</sup> in the presence of ATP (grey boxes) and a fit of the data (red line). (C) Top: Cy3 (green) and Cy5 (red) fluorescence trajectories from a single molecule of Prp5<sup>core</sup> in the presence of poly(A)-RNA. Bottom: Calculated  $E_{\text{FRET}}$  from the Cy3/Cy5 fluorescence trajectories. (D) Histogram of  $E_{\text{FRET}}$  values obtained for Prp5<sup>core</sup> in the presence of poly(A)-RNA (grey boxes) and a fit of the data (red line). In (B) and (D), blue lines represent the fit of the histogram of  $E_{\text{FRET}}$  values obtained for Prp5<sup>core</sup> in the absence of ligands from Fig. 2C.  $n$  and  $m$  represent the number of individual molecules and  $E_{\text{FRET}}$  calculations, respectively, used to construct the histogram. Results from the data fits are listed in Table S4.



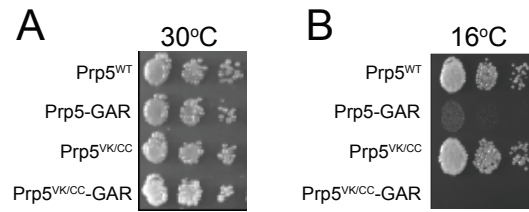
**Fig. S3. U2 RNA Stimulates Prp5<sup>core</sup> ATPase and Switching.** (A) Addition of U2 RNA (nt 1-90, 10 $\mu$ M) stimulates the ATPase activity of Prp5. (B) Top: Cy3 (green) and Cy5 (red) fluorescence trajectories from a single molecule of Prp5<sup>core</sup> in the presence of U2 RNA (1  $\mu$ M). Bottom: Calculated  $E_{\text{FRET}}$  from the Cy3/Cy5 fluorescence trajectories. (C) Histogram of  $E_{\text{FRET}}$  values obtained for Prp5<sup>core</sup> in the presence of U2 RNA (grey boxes) and a fit of the data (red line). (D) Data from a single molecule of Prp5<sup>core</sup> as in panel (B) except in the presence of U2 RNA (1  $\mu$ M) and ATP. (E) Histogram of  $E_{\text{FRET}}$  values obtained for Prp5<sup>core</sup> in the presence of U2 RNA and ATP as in panel (C) and a fit of the data (green line). In (C), the blue line represents the fit of the histogram of  $E_{\text{FRET}}$  values obtained for Prp5<sup>core</sup> in the absence of ligands from **Fig. 2C**.  $n$  and  $m$  represent the number of individual molecules and  $E_{\text{FRET}}$  data points, respectively, used to construct the histogram. Data fit results are listed in **Sup. Table S4**.



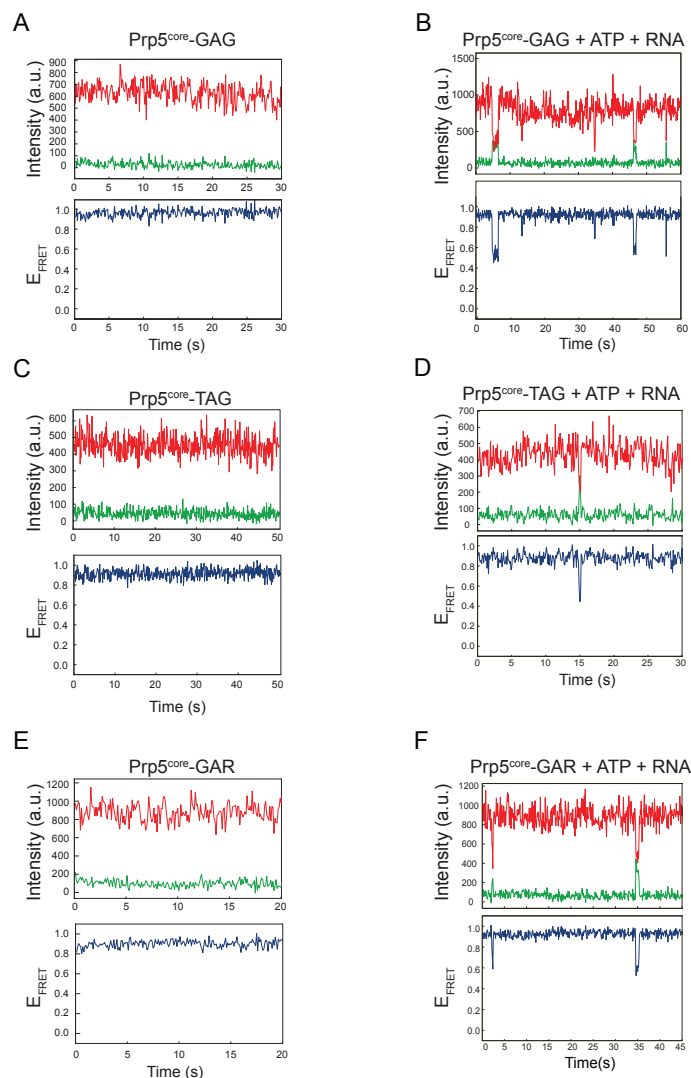
**Fig. S4. Data obtained with Prp5<sup>core2</sup>.** (A) Model for conformational changes of Prp5, similar to that shown in Fig. 1A, with predicted distances between sites of fluorophore incorporation in Prp5<sup>core2</sup> noted. (B) Illustration of mutations incorporated into the Prp5<sup>TK/CC</sup> and Prp5<sup>core2</sup> proteins, similar to that shown in Fig. 1B. (C) Prp5<sup>TK/CC</sup> is able to support yeast viability when grown on plates containing FOA. (D) Top: Cy3 (green) and Cy5 (red) fluorescence trajectories from a single molecule of Prp5<sup>core2</sup>. Bottom: Calculated  $E_{FRET}$  from the Cy3/Cy5 fluorescence trajectories. (E) Histogram of  $E_{FRET}$  values obtained for Prp5<sup>core2</sup> (grey boxes) and a fit of the data (red line). (F) Top: Cy3 (green) and Cy5 (red) fluorescence trajectories from a single molecule of Prp5<sup>core2</sup> in the presence of U2 RNA (1  $\mu$ M) and ATP. Bottom: Calculated  $E_{FRET}$  from the Cy3/Cy5 fluorescence trajectories. (G) Histogram of  $E_{FRET}$  values obtained for Prp5<sup>core2</sup> in the presence of RNA and ATP (grey boxes) and a fit of the data (red line). In (E) and (G), blue lines represent the fit of the histogram of  $E_{FRET}$  values obtained for Prp5<sup>core</sup> in the absence or presence of ligands from Sup. Figs. S3C, E.  $n$  and  $m$  represent the number of individual molecules and  $E_{FRET}$  calculations, respectively, used to construct the histogram. Results from the data fits are listed in Table S4.



**Fig. S5. smFRET observations of Prp5<sup>core</sup> in the presence of RNA and ADP or ADPNP.** (A) Top: Cy3 (green) and Cy5 (red) fluorescence trajectories from a single molecule of Prp5<sup>core</sup> in the presence of ADP and poly(A) RNA (310  $\mu$ M). Bottom: Calculated  $E_{\text{FRET}}$  from the Cy3/Cy5 fluorescence trajectories. (B) Histogram of  $E_{\text{FRET}}$  values obtained for Prp5<sup>core</sup> in the presence of poly(A) RNA and ADP (grey boxes) and a fit of the data (red line). (C) Top: Cy3 (green) and Cy5 (red) fluorescence trajectories from a single molecule of Prp5<sup>core</sup> in the presence of U2 RNA (1  $\mu$ M) and ADPNP. Bottom: Calculated  $E_{\text{FRET}}$  from the Cy3/Cy5 fluorescence trajectories. (D) Histogram of  $E_{\text{FRET}}$  values obtained for Prp5<sup>core</sup> in the presence of U2 RNA (1  $\mu$ M) and ADPNP (grey boxes) and a fit of the data (red line). The green line represents a fit of the histogram of  $E_{\text{FRET}}$  values obtained for Prp5<sup>core</sup> in the presence of RNA and ATP from **Sup. Fig. S3E**.  $n$  and  $m$  represent the number of individual molecules and  $E_{\text{FRET}}$  calculations, respectively, used to construct the histogram. Results from the data fit are listed in **Table S4**.

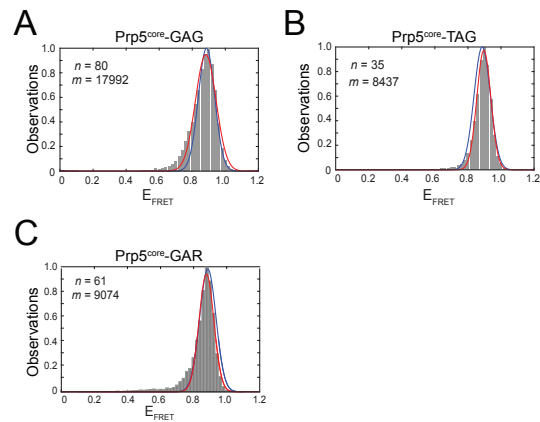


**Fig. S6. Cold sensitivity of strains containing the Prp5-GAR mutation.** (A) Growth of strains containing either the “WT” (native SAT-motif) or the GAR mutation of Prp5 or Prp5<sup>VK/CC</sup> after 2 days at 30°C on YPD/agar. (B) Growth of strains containing either the “WT” (native SAT-motif) or the GAR mutation of Prp5 or Prp5<sup>VK/CC</sup> after 7 days at 16°C on YPD/agar.

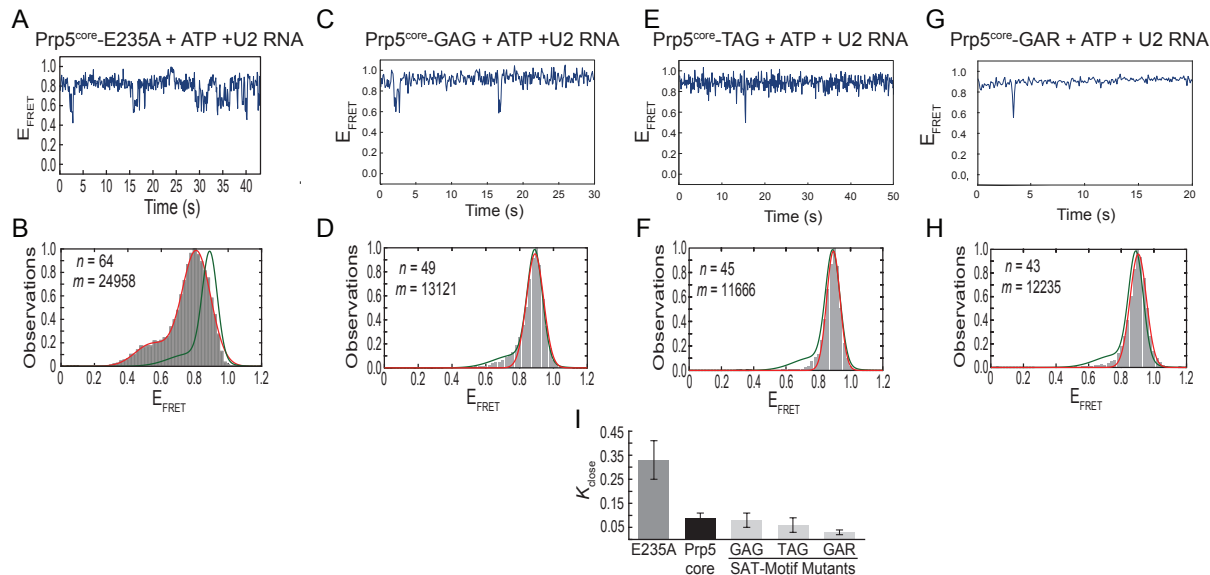


**Fig. S7. smFRET observations of Prp5<sup>core</sup> containing SAT-motif mutations.** (A) Top: Cy3 (green) and Cy5 (red) fluorescence trajectories from a single molecule of Prp5<sup>core</sup>-GAG. Bottom: Calculated  $E_{\text{FRET}}$  from the Cy3/Cy5 fluorescence trajectories. (B) Top: Cy3 (green) and Cy5 (red) fluorescence trajectories from a single molecule of Prp5<sup>core</sup>-GAG in the presence of ATP and poly(A)-RNA. Bottom: Calculated  $E_{\text{FRET}}$  from the Cy3/Cy5 fluorescence trajectories. (C) Top: Cy3 (green) and Cy5 (red) fluorescence trajectories from a single molecule of Prp5<sup>core</sup>-TAG. Bottom: Calculated  $E_{\text{FRET}}$  from the Cy3/Cy5 fluorescence trajectories. (D) Top: Cy3 (green) and Cy5 (red) fluorescence trajectories from a single molecule of Prp5<sup>core</sup>-TAG in the presence of ATP and poly(A)-RNA. Bottom: Calculated  $E_{\text{FRET}}$  from the Cy3/Cy5 fluorescence trajectories. (E) Top: Cy3 (green) and Cy5 (red) fluorescence trajectories from a single molecule of Prp5<sup>core</sup>-GAR. Bottom: Calculated  $E_{\text{FRET}}$  from the Cy3/Cy5 fluorescence trajectories. (F) Top: Cy3 (green) and Cy5 (red) fluorescence trajectories from a single molecule of Prp5<sup>core</sup>-GAR in the presence of ATP and poly(A)-RNA. Bottom: Calculated  $E_{\text{FRET}}$  from the Cy3/Cy5 fluorescence trajectories.





**Fig. S8. Histograms of calculated  $E_{\text{FRET}}$  values obtained from Prp5<sup>core</sup> containing SAT-motif mutations.** (A) Histogram of  $E_{\text{FRET}}$  values obtained for Prp5<sup>core</sup>-GAG (grey boxes) and a fit of the data (red line). (B) Histogram of  $E_{\text{FRET}}$  values obtained for Prp5<sup>core</sup>-TAG (grey boxes) and a fit of the data (red line). (C) Histogram of  $E_{\text{FRET}}$  values obtained for Prp5<sup>core</sup>-GAR (grey boxes) and a fit of the data (red line). Results from the data fits are listed in **Table S4**.



**Fig. S9. Prp5 Mutations Also Change Dynamics in the Presence of U2 RNA** (A) Calculated  $E_{FRET}$  from a single molecule of Prp5<sup>core</sup>-E235A in the presence of ATP and U2 RNA (1  $\mu$ M). (B) Histogram of  $E_{FRET}$  values obtained for Prp5<sup>core</sup>-E235A in the presence of ATP and U2 RNA (grey boxes) and a fit of the data (red line). (C) Calculated  $E_{FRET}$  from a single molecule of Prp5<sup>core</sup>-GAG in the presence of ATP and U2 RNA (1  $\mu$ M). (D) Histogram of  $E_{FRET}$  values obtained for Prp5<sup>core</sup>-GAG in the presence of ATP and U2 RNA (grey boxes) and a fit of the data (red line). (E) Calculated  $E_{FRET}$  from a single molecule of Prp5<sup>core</sup>-TAG in the presence of ATP and U2 RNA (1  $\mu$ M). (F) Histogram of  $E_{FRET}$  values obtained for Prp5<sup>core</sup>-TAG in the presence of ATP and U2 RNA (grey boxes) and a fit of the data (red line). (G) Calculated  $E_{FRET}$  from a single molecule of Prp5<sup>core</sup>-GAR in the presence of ATP and U2 RNA (1  $\mu$ M). (H) Histogram of  $E_{FRET}$  values obtained for Prp5<sup>core</sup>-TAG in the presence of ATP and U2 RNA (grey boxes) and a fit of the data (red line). (I) Calculated  $K_{close}$  values obtained for Prp5<sup>core</sup> and the indicated mutants with the U2 RNA. Calculated lifetimes and  $K_{close}$  values are listed in **Sup. Table S5**

**Supplemental Table S1. Plasmids used in this study.**

| <b>#</b> | <b>Plasmid Name</b>           | <b>Plasmid Description</b>   | <b>Reference</b>  |
|----------|-------------------------------|--|---|
| pAAH0692 | pRS413 Prp5                   | CEN6/ARSH4 HIS3 plasmid with Prp5 <sup>WT</sup>  | Carrocci, T.J., Zoerner, D.M., Paulson, J.C., and Hoskins, A.A. SF3b1 mutations associated with myelodysplastic syndromes alter the fidelity of branchsite selection in yeast. <i>Nucleic Acids Research</i> , v45 (2017), 4837-4852. |
| pAAH0709 | pRS413 Prp5 E235A             | CEN6/ARSH4 HIS3 plasmid with Prp5-E235A  | Carrocci, T.J., Zoerner, D.M., Paulson, J.C., and Hoskins, A.A. SF3b1 mutations associated with myelodysplastic syndromes alter the fidelity of branchsite selection in yeast. <i>Nucleic Acids Research</i> , v45 (2017), 4837-4852. |
| pAAH0711 | pRS413 Prp5 TAG               | CEN6/ARSH4 HIS3 plasmid with Prp5-TAG  | Carrocci, T.J., Zoerner, D.M., Paulson, J.C., and Hoskins, A.A. SF3b1 mutations associated with myelodysplastic syndromes alter the fidelity of branchsite selection in yeast. <i>Nucleic Acids Research</i> , v45 (2017), 4837-4852. |
| pAAH0777 | Prp5 GAR                      | CEN TRP1 plasmid with Prp5-GAR   | Xu, Y.Z. and Query, C.C. Competition between the ATPase Prp5 and branch region-U2 snRNA pairing modulates the fidelity of spliceosome assembly. <i>Mol. Cell</i> , v28 (2007), 838-49.  |
| pAAH0828 | Prp5 GAG                      | CEN TRP1 plasmid with Prp5-GAG   | Xu, Y.Z. and Query, C.C. Competition between the ATPase Prp5 and branch region-U2 snRNA pairing modulates the fidelity of spliceosome assembly. <i>Mol. Cell</i> , v28 (2007), 838-49.  |
| pAAH0846 | Prp5 <sup>core</sup>          | This is the plasmid that contains Prp5 <sup>core</sup> cloned into the NdeI/BamHI sites of pet28bTEV/KanR with an AP biotinylation site. |   |
| pAAH0848 | RS413 Prp5 <sup>cysless</sup> | CEN6/ARSH4 HIS3 plasmid with Prp5 <sup>cysless</sup>   |   |
| pAAH0849 | RS413 Prp5 <sup>VK/CC</sup>   | CEN6/ARSH4 HIS3 plasmid with Prp5 <sup>VK/CC</sup>   |   |
| pAAH0884 | RS413 Prp5 <sup>TK/CC</sup>   | CEN6/ARSH4 HIS3 plasmid with Prp5 <sup>TK/CC</sup>   |   |

|          |                                    |   |  |
|----------|------------------------------------|---|--|
| pAAH0889 | RS413 Prp5 <sup>VK/CC</sup> -E235A | CEN6/ARSH4 HIS3 plasmid with Prp5 <sup>VK/CC</sup> -E235A   |  |
| pAAH0890 | RS413 Prp5 <sup>VK/CC</sup> -TAG   | CEN6/ARSH4 HIS3 plasmid with Prp5 <sup>VK/CC</sup> -TAG   |  |
| pAAH0897 | Prp5 <sup>core</sup> -E235A        | This is the plasmid that contains Prp5 <sup>core</sup> -E235A cloned into the NdeI/BamHI sites of pet28bTEV/KanR with an AP biotinylation site. |  |
| pAAH0898 | Prp5 <sup>core</sup> -TAG          | This is the plasmid that contains Prp5 <sup>core</sup> -TAG cloned into the NdeI/BamHI sites of pet28bTEV/KanR with an AP biotinylation site.   |  |
| pAAH0902 | Prp5 <sup>core2</sup>              | This is the plasmid that contains Prp5 <sup>core2</sup> cloned into the NdeI/BamHI sites of pet28bTEV/KanR with an AP biotinylation site.       |  |
| pAAH0990 | RS413 Prp5 <sup>VK/CC</sup> -GAG   | CEN6/ARSH4 HIS3 plasmid with Prp5 <sup>VK/CC</sup> -GAG   |  |
| pAAH0991 | RS413 Prp5 <sup>VK/CC</sup> -GAR   | CEN6/ARSH4 HIS3 plasmid with Prp5 <sup>VK/CC</sup> -GAR   |  |
| pAAH0992 | Prp5 <sup>core</sup> -GAG          | This is the plasmid that contains Prp5 <sup>core</sup> -  |  |

|          |                           |  |  |
|----------|---------------------------|--|--|
|          |                           | GAG cloned into the NdeI/BamHI sites of pet28bTEV/KanR with an AP biotinylation site.  |  |
| pAAH0993 | Prp5 <sup>core</sup> -GAR | This is the plasmid that contains Prp5 <sup>core</sup> -GAR cloned into the NdeI/BamHI sites of pet28bTEV/KanR with an AP biotinylation site.            |  |
| pAAH0947 | BirA                      | <i>E. coli</i> biotin ligase subcloned into the BamHI and XhoI sites of pGEX1-4. Used for <i>in vivo</i> biotinylation of Prp5 <sup>core</sup> proteins. |  |

Plasmids encoding ACT1-CUP1 reporter genes have been previously described by Carrocci, T.J., *et al.*, [Nucleic Acids Research, v45 (2017), 4837-4852].

**Supplemental Table S2. Yeast strains used in these studies.**

| <u>Name</u> | <u>Genotype</u>   | <u>Description</u>  |
|-------------|---|---|
| yAAH1972    | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2 prp5Δ::loxP trp1 + pAAH0990 + pAAH0470</i> | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying Prp5 <sup>VK/CC</sup> -GAG on a pRS413 (HIS/CEN) plasmid along with a second plasmid containing the ACT1-CUP1 reporter gene (LEU2).         |
| yAAH1973    | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2 prp5Δ::loxP trp1 + pAAH0990 + pAAH0439</i> | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying Prp5 <sup>VK/CC</sup> -GAG on a pRS413 (HIS/CEN) plasmid along with a second plasmid containing the U257C ACT1-CUP1 reporter gene (LEU2).   |
| yAAH1974    | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2 prp5Δ::loxP trp1 + pAAH0990 + pAAH0524</i> | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying Prp5 <sup>VK/CC</sup> -GAG on a pRS413 (HIS/CEN) plasmid along with a second plasmid containing the A258U ACT1-CUP1 reporter gene (LEU2).   |
| yAAH1975    | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2 prp5Δ::loxP trp1 + pAAH0889 + pAAH0470</i> | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying Prp5 <sup>VK/CC</sup> -E235A on a pRS413 (HIS/CEN) plasmid along with a second plasmid containing the ACT1-CUP1 reporter gene (LEU2).       |
| yAAH1976    | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2 prp5Δ::loxP trp1 + pAAH0889 + pAAH0439</i> | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying Prp5 <sup>VK/CC</sup> -E235A on a pRS413 (HIS/CEN) plasmid along with a second plasmid containing the U257C ACT1-CUP1 reporter gene (LEU2). |
| yAAH1977    | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2 prp5Δ::loxP trp1 + pAAH0889 + pAAH0524</i> | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying Prp5 <sup>VK/CC</sup> -E235A on a pRS413 (HIS/CEN) plasmid along with a second plasmid containing the A258U ACT1-CUP1 reporter gene (LEU2). |
| yAAH1978    | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2 prp5Δ::loxP trp1 + pAAH0709 + pAAH0470</i> | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying Prp5-E235A on a pRS413 (HIS/CEN) plasmid along with a second plasmid containing the ACT1-CUP1 reporter gene (LEU2).                         |
| yAAH1979    | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2 prp5Δ::loxP trp1 + pAAH0709 + pAAH0439</i> | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying Prp5-E235A on a pRS413 (HIS/CEN) plasmid along with a second plasmid containing the U257C ACT1-CUP1 reporter gene (LEU2).                   |
| yAAH1980    | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2 prp5Δ::loxP trp1 + pAAH0709 + pAAH0524</i> | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying Prp5-E235A on a pRS413 (HIS/CEN) plasmid along with a second plasmid containing the A258U ACT1-CUP1 reporter gene (LEU2).                   |
| yAAH1981    | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2 prp5Δ::loxP trp1 + pAAH0828+ pAAH0470</i>  | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying Prp5-GAG on a pRS413 (HIS/CEN) plasmid along with a second plasmid containing the ACT1-CUP1 reporter gene (LEU2).                           |
| yAAH1982    | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2 prp5Δ::loxP trp1 + pAAH0828+ pAAH0439</i>  | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying Prp5-GAG on a pRS413 (HIS/CEN) plasmid along with a second plasmid containing the U257C ACT1-CUP1 reporter gene (LEU2).                     |
| yAAH1983    | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2 prp5Δ::loxP trp1 + pAAH0828+ pAAH0524</i>  | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying Prp5-GAG on a pRS413 (HIS/CEN) plasmid along with a second plasmid containing the A258U ACT1-CUP1 reporter gene (LEU2).                     |

|          |   |   |
|----------|---|---|
| yAAH1984 | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2 prp5Δ::loxP trp1+ pAAH0692+ pAAH0470</i>   | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying Prp5 on a pRS413 (HIS/CEN) plasmid along with a second plasmid containing the ACT1-CUP1 reporter gene (LEU2).                             |
| yAAH1985 | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2 prp5Δ::loxP trp1 + pAAH0692+ pAAH0524</i>  | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying Prp5 on a pRS413 (HIS/CEN) plasmid along with a second plasmid containing the A258U ACT1-CUP1 reporter gene (LEU2).                       |
| yAAH1986 | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2 prp5Δ::loxP trp1 + pAAH0849+ pAAH0470</i>  | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying Prp5 <sup>VK/CC</sup> on a pRS413 (HIS/CEN) plasmid along with a second plasmid containing the ACT1-CUP1 reporter gene (LEU2).            |
| yAAH1987 | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2 prp5Δ::loxP trp1 + pAAH0849+ pAAH0439</i>  | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying Prp5 <sup>VK/CC</sup> on a pRS413 (HIS/CEN) plasmid along with a second plasmid containing the U257C ACT1-CUP1 reporter gene (LEU2).      |
| yAAH1988 | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2 prp5Δ::loxP trp1 + pAAH0849+ pAAH0524</i>  | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying Prp5 <sup>VK/CC</sup> on a pRS413 (HIS/CEN) plasmid along with a second plasmid containing the A258U ACT1-CUP1 reporter gene (LEU2).      |
| yAAH2056 | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2 prp5Δ::loxP trp1 + pAAH0849</i>            | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying Prp5 <sup>VK/CC</sup> on a pRS413 (HIS/CEN) plasmid.  |
| yAAH2057 | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2 prp5Δ::loxP trp1 + pAAH00692</i>           | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying Prp5 on a pRS413 (HIS/CEN) plasmid.   |
| yAAH2058 | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2 prp5Δ::loxP trp1 + pAAH0848</i>            | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying Prp5-cysless on a pRS413 (HIS/CEN) plasmid.   |
| yAAH2059 | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2 prp5Δ::loxP trp1 + pAAH0134</i>            | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying the empty pRS413 (HIS/CEN) plasmid.   |
| yAAH2060 | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2 prp5Δ::loxP trp1 + pAAH0884</i>            | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying Prp5 <sup>TK/CC</sup> on a pRS413 (HIS/CEN) plasmid.  |
| yAAH2061 | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2 prp5Δ::loxP trp1 + pAAH0890 + pAAH0470</i> | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying Prp5 <sup>VK/CC</sup> -TAG on a pRS413 (HIS/CEN) plasmid along with a second plasmid containing the ACT1-CUP1 reporter gene (LEU2).       |
| yAAH2062 | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2 prp5Δ::loxP trp1 + pAAH0890 + pAAH0439</i> | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying Prp5 <sup>VK/CC</sup> -TAG on a pRS413 (HIS/CEN) plasmid along with a second plasmid containing the U257C ACT1-CUP1 reporter gene (LEU2). |
| yAAH2063 | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2 prp5Δ::loxP trp1 + pAAH0890+ pAAH0524</i>  | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying Prp5 <sup>VK/CC</sup> -TAG on a pRS413 (HIS/CEN) plasmid along with a second plasmid containing the A258U ACT1-CUP1 reporter gene (LEU2). |
| yAAH2064 | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2 prp5Δ::loxP trp1 + pAAH0711 + pAAH0470</i> | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying Prp5-TAG on a pRS413 (HIS/CEN) plasmid along with a second plasmid containing the ACT1-CUP1 reporter gene (LEU2).                         |
| yAAH2065 | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2 prp5Δ::loxP trp1 + pAAH0711 + pAAH0439</i> | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying Prp5-TAG on a pRS413 (HIS/CEN) plasmid along with a second plasmid containing the U257C ACT1-CUP1 reporter gene (LEU2).                   |
| yAAH2066 | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2 prp5Δ::loxP trp1 + pAAH0711+ pAAH0524</i>  | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying Prp5-TAG on a pRS413 (HIS/CEN) plasmid along with a second plasmid containing the A258U ACT1-CUP1 reporter gene (LEU2).                   |

|          |  |   |
|----------|--|---|
| yAAH2054 | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2<br/>prp5Δ::loxP trp1 + pAAH0777</i> | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying Prp5-GAR on a pRS413 (HIS/CEN) plasmid.                   |
| yAAH2055 | <i>MATa, ade2 cup1Δ::ura3 his3 leu2 lys2<br/>prp5Δ::loxP trp1 + pAAH0991</i> | Cu <sup>2+</sup> -sensitive, Prp5 shuffle strain carrying Prp5 <sup>VK/CC</sup> -GAR on a pRS413 (HIS/CEN) plasmid. |



**Supplemental Table S3. Amino acid sequences of proteins used in these studies.** For Prp5, the SAT-motif is highlighted in **red**, E235 is highlighted in **blue**, and the AP site for biotinylation is highlighted in **green**. Cysteine sites for smFRET fluorophore incorporation are shown in **yellow**.

| Name                        | Primary Sequence  |
|-----------------------------|---|
| Prp5                        | METIDSKQNINRESLLEERRKKLAKWKQKKAQFDAQKEHQTSRNDIVTNSLEGGQTTEKFTERQERVKEELRKRKNEFRKSDPEVSVKPSKSKKSKRSKVKKKISDFDSDDDDSEIGVSF<br>RSKEHIQKAPEDHNEKDPDEFMTSLKEEKMSNSKGMYDRGDIILDVEDQLFELGGTDEDEDVNTDNSNIAKIAKLAKKRVKQIYYSPEELEPFQKNFYIESETVSSMSEMEVEE LRL<br>SLDNIKIKGTGPKPVTKWSQLGLSTD TMLITEKLHFGSLTPIQSALPAIMSGRDVIGISKTGSGKTSYLLPRLRQVKAQRPLSKHETGPMGLILAPTRELALQIHEEVTKFTTEAD<br>TSIRSVCTGGSEMKKQITDLKRGTEIVVATPGRFIDILTLNDGKLLSTKRITFVVMDEADRLFDLGFEPQITQIMKTVRPDKQCVLFSATFPNKLRSFAVRVLHSPISITINSKGMVN<br>ENVKQKFRIVHSEDEKFDNLVQLIHERSEFFDEVQSENDGQSSDVEEVDAKAIFVSSQNIADFISSKLLNAGIVTMAIHAGKPYQERLMNLEKFKREKNSILLCTEVL SRGLNVPEVS<br>LVI IYNAVKTFAQYVHTTGRTARGSRSGTAITLLHDELHSGAYILSKAMRDEEIKALDPLQAKELQEMSAK FESGMKKGKFRLSKGFGGKGLNENIKSKREEAQNKDLELKKNDKRSDDL<br>EKKISNPREGHDSVSESSALIPRLNYELFKESTDGSIFYAKVYINDLPQIVRWEATKNTTLFLIKHETGCSITNKGKGYFPEGKEPKNENDEPKLYLLIEGQDEKDIQLSIELLEQKVK<br>EGVVKAASLSLKSTKY |
| Prp5 <sup>VK/CC</sup>       | METIDSKQNINRESLLEERRKKLAKWKQKKAQFDAQKEHQTSRNDIVTNSLEGGQTTEKFTERQERVKEELRKRKNEFRKSDPEVSVKPSKSKKSKRSKVKKKISDFDSDDDDSEIGVSF<br>RSKEHIQKAPEDHNEKDPDEFMTSLKEEKMSNSKGMYDRGDIILDVEDQLFELGGTDEDEDVNTDNSNIAKIAKLAKKRVKQIYYSPEELEPFQKNFYIESETVSSMSEMEVEE LRL<br>SLDNIKIKGTGPKPVTKWSQLGLSTD TMLITEKLHFGSLTPIQSALPAIMSGRDVIGISKTGSGKTSYLLPRLRQVKAQRPLSKHETGPMGLILAPTRELALQIHEEVTKFTTEAD<br>TSIRSVVVTGGSEMKKQITDLKRGTEIVVATPGRFIDILTLNDGKLLSTKRITFVVMDEADRLFDLGFEPQITQIMKTVRPDKQTVLFSATFPNKLRSFAVRVLHSPISITINSKGMVN<br>ENVKQKFRIVHSEDEKFDNLVQLIHERSEFFDEVQSENDGQSSDVEEVDAKAIFVSSQNIADFISSKLLNAGIVTMAIHAGKPYQERLMNLEKFKREKNSILLATEVLSRGLNVPEVS<br>LVI IYNAVKTFAQYVHTTGRTARGSRSGTAITLLHDELHSGAYILSKAMRDEEIKALDPLQAKELQEMSAK FESGMKKGKFRLSKGFGGKGLNENIKSKREEAQNKDLELKKNDKRSDDL<br>EKKISNPREGHDSVSESSALIPRLNYELFKESTDGSIFYAKVYINDLPQIVRWEATKNTTLFLIKHETGASITNKGKGYFPEGKEPKNENDEPKLYLLIEGQDEKDIQLSIELLEQKVK<br>EGVVKAASLSLKSTKY |
| Prp5 <sup>TK/CC</sup>       | METIDSKQNINRESLLEERRKKLAKWKQKKAQFDAQKEHQTSRNDIVTNSLEGGQTTEKFTERQERVKEELRKRKNEFRKSDPEVSVKPSKSKKSKRSKVKKKISDFDSDDDDSEIGVSF<br>RSKEHIQKAPEDHNEKDPDEFMTSLKEEKMSNSKGMYDRGDIILDVEDQLFELGGTDEDEDVNTDNSNIAKIAKLAKKRVKQIYYSPEELEPFQKNFYIESETVSSMSEMEVEE LRL<br>SLDNIKIKGTGPKPVTKWSQLGLSTD TMLITEKLHFGSLTPIQSALPAIMSGRDVIGISKTGSGKTSYLLPRLRQVKAQRPLSKHETGPMGLILAPTRELALQIHEEVTKFTTEAD<br>TSIRSVVVTGGSEMKKQITDLKRGTEIVVATPGRFIDILTLNDGKLLSTKRITFVVMDEADRLFDLGFEPQITQIMKTVRPDKQTVLFSATFPNKLRSFAVRVLHSPISITINSKGMVN<br>ENVKQKFRIVHSEDEKFDNLVQLIHERSEFFDEVQSENDGQSSDVEEVDAKAIFVSSQNIADFISSKLLNAGIVTMAIHAGKPYQERLMNLEKFKREKNSILLATEVLSRGLNVPEVS<br>LVI IYNAVKTFAQYVHTTGRTARGSRSGTAITLLHDELHSGAYILSKAMRDEEIKALDPLQAKELQEMSAK FESGMKKGKFRLSKGFGGKGLNENIKSKREEAQNKDLELKKNDKRSDDL<br>EKKISNPREGHDSVSESSALIPRLNYELFKESTDGSIFYAKVYINDLPQIVRWEATKNTTLFLIKHETGASITNKGKGYFPEGKEPKNENDEPKLYLLIEGQDEKDIQLSIELLEQKVK<br>EGVVKAASLSLKSTKY |
| Prp5 <sup>VK/CC</sup> E235A | METIDSKQNINRESLLEERRKKLAKWKQKKAQFDAQKEHQTSRNDIVTNSLEGGQTTEKFTERQERVKEELRKRKNEFRKSDPEVSVKPSKSKKSKRSKVKKKISDFDSDDDDSEIGVSF<br>RSKEHIQKAPEDHNEKDPDEFMTSLKEEKMSNSKGMYDRGDIILDVEDQLFELGGTDEDEDVNTDNSNIAKIAKLAKKRVKQIYYSPEELEPFQKNFYIESETVSSMSEMEVEE LRL<br>SLDNIKIKGTGPKPVTKWSQLGLSTD TMLITEKLHFGSLTPIQSALPAIMSGRDVIGISKTGSGKTSYLLPRLRQVKAQRPLSKHETGPMGLILAPTRELALQIHEEVTKFTTEAD<br>TSIRSVVVTGGSEMKKQITDLKRGTEIVVATPGRFIDILTLNDGKLLSTKRITFVVMDEADRLFDLGFEPQITQIMKTVRPDKQTVLFSATFPNKLRSFAVRVLHSPISITINSKGMVN<br>ENVKQKFRIVHSEDEKFDNLVQLIHERSEFFDEVQSENDGQSSDVEEVDAKAIFVSSQNIADFISSKLLNAGIVTMAIHAGKPYQERLMNLEKFKREKNSILLATEVLSRGLNVPEVS<br>LVI IYNAVKTFAQYVHTTGRTARGSRSGTAITLLHDELHSGAYILSKAMRDEEIKALDPLQAKELQEMSAK FESGMKKGKFRLSKGFGGKGLNENIKSKREEAQNKDLELKKNDKRSDDL<br>EKKISNPREGHDSVSESSALIPRLNYELFKESTDGSIFYAKVYINDLPQIVRWEATKNTTLFLIKHETGASITNKGKGYFPEGKEPKNENDEPKLYLLIEGQDEKDIQLSIELLEQKVK<br>EGVVKAASLSLKSTKY |
| Prp5 <sup>VK/CC</sup> TAG   | METIDSKQNINRESLLEERRKKLAKWKQKKAQFDAQKEHQTSRNDIVTNSLEGGQTTEKFTERQERVKEELRKRKNEFRKSDPEVSVKPSKSKKSKRSKVKKKISDFDSDDDDSEIGVSF<br>RSKEHIQKAPEDHNEKDPDEFMTSLKEEKMSNSKGMYDRGDIILDVEDQLFELGGTDEDEDVNTDNSNIAKIAKLAKKRVKQIYYSPEELEPFQKNFYIESETVSSMSEMEVEE LRL<br>SLDNIKIKGTGPKPVTKWSQLGLSTD TMLITEKLHFGSLTPIQSALPAIMSGRDVIGISKTGSGKTSYLLPRLRQVKAQRPLSKHETGPMGLILAPTRELALQIHEEVTKFTTEAD<br>TSIRSVVVTGGSEMKKQITDLKRGTEIVVATPGRFIDILTLNDGKLLSTKRITFVVMDEADRLFDLGFEPQITQIMKTVRPDKQTVLFSATFPNKLRSFAVRVLHSPISITINSKGMVN<br>ENVKQKFRIVHSEDEKFDNLVQLIHERSEFFDEVQSENDGQSSDVEEVDAKAIFVSSQNIADFISSKLLNAGIVTMAIHAGKPYQERLMNLEKFKREKNSILLATEVLSRGLNVPEVS<br>LVI IYNAVKTFAQYVHTTGRTARGSRSGTAITLLHDELHSGAYILSKAMRDEEIKALDPLQAKELQEMSAK FESGMKKGKFRLSKGFGGKGLNENIKSKREEAQNKDLELKKNDKRSDDL<br>EKKISNPREGHDSVSESSALIPRLNYELFKESTDGSIFYAKVYINDLPQIVRWEATKNTTLFLIKHETGASITNKGKGYFPEGKEPKNENDEPKLYLLIEGQDEKDIQLSIELLEQKVK<br>EGVVKAASLSLKSTKY |
| Prp5 <sup>VK/CC</sup> GAG   | METIDSKQNINRESLLEERRKKLAKWKQKKAQFDAQKEHQTSRNDIVTNSLEGGQTTEKFTERQERVKEELRKRKNEFRKSDPEVSVKPSKSKKSKRSKVKKKISDFDSDDDDSEIGVSF<br>RSKEHIQKAPEDHNEKDPDEFMTSLKEEKMSNSKGMYDRGDIILDVEDQLFELGGTDEDEDVNTDNSNIAKIAKLAKKRVKQIYYSPEELEPFQKNFYIESETVSSMSEMEVEE LRL<br>SLDNIKIKGTGPKPVTKWSQLGLSTD TMLITEKLHFGSLTPIQSALPAIMSGRDVIGISKTGSGKTSYLLPRLRQVKAQRPLSKHETGPMGLILAPTRELALQIHEEVTKFTTEAD<br>TSIRSVVVTGGSEMKKQITDLKRGTEIVVATPGRFIDILTLNDGKLLSTKRITFVVMDEADRLFDLGFEPQITQIMKTVRPDKQTVLFSATFPNKLRSFAVRVLHSPISITINSKGMVN<br>ENVKQKFRIVHSEDEKFDNLVQLIHERSEFFDEVQSENDGQSSDVEEVDAKAIFVSSQNIADFISSKLLNAGIVTMAIHAGKPYQERLMNLEKFKREKNSILLATEVLSRGLNVPEVS<br>LVI IYNAVKTFAQYVHTTGRTARGSRSGTAITLLHDELHSGAYILSKAMRDEEIKALDPLQAKELQEMSAK FESGMKKGKFRLSKGFGGKGLNENIKSKREEAQNKDLELKKNDKRSDDL<br>EKKISNPREGHDSVSESSALIPRLNYELFKESTDGSIFYAKVYINDLPQIVRWEATKNTTLFLIKHETGASITNKGKGYFPEGKEPKNENDEPKLYLLIEGQDEKDIQLSIELLEQKVK<br>EGVVKAASLSLKSTKY |
| Prp5 <sup>VK/CC</sup> GAR   | METIDSKQNINRESLLEERRKKLAKWKQKKAQFDAQKEHQTSRNDIVTNSLEGGQTTEKFTERQERVKEELRKRKNEFRKSDPEVSVKPSKSKKSKRSKVKKKISDFDSDDDDSEIGVSF<br>RSKEHIQKAPEDHNEKDPDEFMTSLKEEKMSNSKGMYDRGDIILDVEDQLFELGGTDEDEDVNTDNSNIAKIAKLAKKRVKQIYYSPEELEPFQKNFYIESETVSSMSEMEVEE LRL<br>SLDNIKIKGTGPKPVTKWSQLGLSTD TMLITEKLHFGSLTPIQSALPAIMSGRDVIGISKTGSGKTSYLLPRLRQVKAQRPLSKHETGPMGLILAPTRELALQIHEEVTKFTTEAD   |

|                            |   |
|----------------------------|---|
|                            | TSIRSVVVTGGSEMKKQITDLKRGTEIVVATPGRFIDILTLNDGKLLSTKRITFVVMDEADRLFDLGFEPQITQIMKTVRPDKQTVLFGARFPNKLRSFAVRVLHSPISITINSGMVN<br>ENVKQKFRIVHSEDEKFDNLVQLIHERSEFFDEVQSENDGQSSDVEEVDAKAIIFVSSQNIADFISSKLLNAGIVTMAIHAGKPYQERLMNLEKFKREKNSILLATEVLSRGLNVPEVS<br>LVIYNAVKTFAQYVHTTGRRTARGSRSGTAITLLHDELGSAYILSKAMRDEEIKALDPLQAKELQEMSA CFESGMKKGKFRLSKGFGGKLENIKSKREEAQNKDLELKKNDKRSDDL<br>EKKISNPREGHDSVSESSALIPRLNYELFKES'TDGSIFYAKVYINDLPQIVRWEATKNTTLFIFKHETGASITNKGFYPEGKEPKNENDEPKLYLLIEGQDEKDIQLSIELLEQKVK<br>EGVVKAASLSLKSTKY                     |
| Prp5 <sup>core</sup>       | GLNDIFEAQKIEWHEHMSPEELEPFQKNFYIESETVSSMSEMEVEELRRLSLDNIKIKGTGVPKPVTKWSQLGLSTDTCCLITEKLHFGSLTPIQSQUALPAIMSGRDVIGISKTGSGKTI<br>SYLLPLLRQVKAQRPLSKHETGPMGLILAPTRELALQIHEEVTKFTEADTSIRSVVVTGGSEMKKQITDLKRGTEIVVATPGRFIDILTLNDGKLLSTKRITFVVMDEADRLFDLGFEP<br>QITQIMKTVRPDKQTVLFSATFPNKLRSFAVRVLHSPISITINSGMVNENVKQKFRIVHSEDEKFDNLVQLIHERSEFFDEVQSENDGQSSDVEEVDAKAIIFVSSQNIADFISSKLL<br>NAGIVTMAIHAGKPYQERLMNLEKFKREKNSILLATEVLSRGLNVPEVSLVIYNAVKTFAQYVHTTGRRTARGSRSGTAITLLHDELGSAYILSKAMRDEEIKALDPLQAKELQEMSA<br>CFESGMKKGKFRLSKGFGGKLENIKSKREEAQN |
| Prp5 <sup>core2</sup>      | GLNDIFEAQKIEWHEHMSPEELEPFQKNFYIESETVSSMSEMEVEELRRLSLDNIKIKGTGVPKPVTKWSQLGLSTDTCCLITEKLHFGSLTPIQSQUALPAIMSGRDVIGISKTGSGKTI<br>SYLLPLLRQVKAQRPLSKHETGPMGLILAPTRELALQIHEEVTKFTEADTSIRSVVVTGGSEMKKQITDLKRGTEIVVATPGRFIDILTLNDGKLLSTKRITFVVMDEADRLFDLGFEP<br>QITQIMKTVRPDKQTVLFSATFPNKLRSFAVRVLHSPISITINSGMVNENVKQKFRIVHSEDEKFDNLVQLIHERSEFFDEVQSENDGQSSDVEEVDAKAIIFVSSQNIADFISSKLL<br>NAGIVTMAIHAGKPYQERLMNLEKFKREKNSILLATEVLSRGLNVPEVSLVIYNAVKTFAQYVHTTGRRTARGSRSGTAITLLHDELGSAYILSKAMRDEEIKALDPLQAKELQEMSA<br>KFESGMKKGKFRLSKGFGGKLENIKSKREEAQN |
| Prp5 <sup>core</sup> E235A | GLNDIFEAQKIEWHEHMSPEELEPFQKNFYIESETVSSMSEMEVEELRRLSLDNIKIKGTGVPKPVTKWSQLGLSTDTCCLITEKLHFGSLTPIQSQUALPAIMSGRDVIGISKTGSGKTI<br>SYLLPLLRQVKAQRPLSKHETGPMGLILAPTRELALQIHEEVTKFTEADTSIRSVVVTGGSEMKKQITDLKRGTEIVVATPGRFIDILTLNDGKLLSTKRITFVVMDEADRLFDLGFEP<br>QITQIMKTVRPDKQTVLFSATFPNKLRSFAVRVLHSPISITINSGMVNENVKQKFRIVHSEDEKFDNLVQLIHERSEFFDEVQSENDGQSSDVEEVDAKAIIFVSSQNIADFISSKLL<br>NAGIVTMAIHAGKPYQERLMNLEKFKREKNSILLATEVLSRGLNVPEVSLVIYNAVKTFAQYVHTTGRRTARGSRSGTAITLLHDELGSAYILSKAMRDEEIKALDPLQAKELQEMSA<br>CFESGMKKGKFRLSKGFGGKLENIKSKREEAQN |
| Prp5 <sup>core</sup> TAG   | GLNDIFEAQKIEWHEHMSPEELEPFQKNFYIESETVSSMSEMEVEELRRLSLDNIKIKGTGVPKPVTKWSQLGLSTDTCCLITEKLHFGSLTPIQSQUALPAIMSGRDVIGISKTGSGKTI<br>SYLLPLLRQVKAQRPLSKHETGPMGLILAPTRELALQIHEEVTKFTEADTSIRSVVVTGGSEMKKQITDLKRGTEIVVATPGRFIDILTLNDGKLLSTKRITFVVMDEADRLFDLGFEP<br>QITQIMKTVRPDKQTVLFSATFPNKLRSFAVRVLHSPISITINSGMVNENVKQKFRIVHSEDEKFDNLVQLIHERSEFFDEVQSENDGQSSDVEEVDAKAIIFVSSQNIADFISSKLL<br>NAGIVTMAIHAGKPYQERLMNLEKFKREKNSILLATEVLSRGLNVPEVSLVIYNAVKTFAQYVHTTGRRTARGSRSGTAITLLHDELGSAYILSKAMRDEEIKALDPLQAKELQEMSA<br>CFESGMKKGKFRLSKGFGGKLENIKSKREEAQN |
| Prp5 <sup>core</sup> GAG   | GLNDIFEAQKIEWHEHMSPEELEPFQKNFYIESETVSSMSEMEVEELRRLSLDNIKIKGTGVPKPVTKWSQLGLSTDTCCLITEKLHFGSLTPIQSQUALPAIMSGRDVIGISKTGSGKTI<br>SYLLPLLRQVKAQRPLSKHETGPMGLILAPTRELALQIHEEVTKFTEADTSIRSVVVTGGSEMKKQITDLKRGTEIVVATPGRFIDILTLNDGKLLSTKRITFVVMDEADRLFDLGFEP<br>QITQIMKTVRPDKQTVLFSATFPNKLRSFAVRVLHSPISITINSGMVNENVKQKFRIVHSEDEKFDNLVQLIHERSEFFDEVQSENDGQSSDVEEVDAKAIIFVSSQNIADFISSKLL<br>NAGIVTMAIHAGKPYQERLMNLEKFKREKNSILLATEVLSRGLNVPEVSLVIYNAVKTFAQYVHTTGRRTARGSRSGTAITLLHDELGSAYILSKAMRDEEIKALDPLQAKELQEMSA<br>CFESGMKKGKFRLSKGFGGKLENIKSKREEAQN |
| Prp5 <sup>core</sup> GAR   | GLNDIFEAQKIEWHEHMSPEELEPFQKNFYIESETVSSMSEMEVEELRRLSLDNIKIKGTGVPKPVTKWSQLGLSTDTCCLITEKLHFGSLTPIQSQUALPAIMSGRDVIGISKTGSGKTI<br>SYLLPLLRQVKAQRPLSKHETGPMGLILAPTRELALQIHEEVTKFTEADTSIRSVVVTGGSEMKKQITDLKRGTEIVVATPGRFIDILTLNDGKLLSTKRITFVVMDEADRLFDLGFEP<br>QITQIMKTVRPDKQTVLFSATFPNKLRSFAVRVLHSPISITINSGMVNENVKQKFRIVHSEDEKFDNLVQLIHERSEFFDEVQSENDGQSSDVEEVDAKAIIFVSSQNIADFISSKLL<br>NAGIVTMAIHAGKPYQERLMNLEKFKREKNSILLATEVLSRGLNVPEVSLVIYNAVKTFAQYVHTTGRRTARGSRSGTAITLLHDELGSAYILSKAMRDEEIKALDPLQAKELQEMSA<br>CFESGMKKGKFRLSKGFGGKLENIKSKREEAQN |

**Supplemental Table S4. Results from fitting of histograms of  $E_{\text{FRET}}$  Distributions**

| <u>Related Figure</u> | <u>Experimental Description</u>               | <u># of Gaussian Terms in Fit</u> | <u>Peak 1</u> | <u>Peak 2</u> |
|-----------------------|---|-----------------------------------|---------------|---------------|
| 2C                    | Prp5 <sup>core</sup>                          | 1                                 | 0.886 ± 0.001 | N/A           |
| 2E                    | Prp5 <sup>core</sup> + polyA RNA + ATP        | 2                                 | 0.895 ± 0.003 | 0.52 ± 0.11   |
| 3G                    | Prp5 <sup>core</sup> -E235A                   | 2                                 | 0.799 ± 0.002 | 0.53 ± 0.03   |
| 3I                    | Prp5 <sup>core</sup> -E235A + polyA RNA + ATP | 2                                 | 0.775 ± 0.002 | 0.523 ± 0.057 |
| 4A                    | Prp5 <sup>core</sup> -GAG + polyA RNA + ATP   | 2                                 | 0.895 ± 0.006 | 0.51 ± 0.08   |
| 4B                    | Prp5 <sup>core</sup> -TAG + polyA RNA + ATP   | 1                                 | 0.869 ± 0.002 | N/A           |
| 4C                    | Prp5 <sup>core</sup> -GAR + polyA RNA + ATP   | 1                                 | 0.882 ± 0.003 | N/A           |
| Sup. Fig. S2B         | Prp5 <sup>core</sup> + ATP                    | 1                                 | 0.882 ± 0.001 | N/A           |
| Sup. Fig. S2D         | Prp5 <sup>core</sup> + polyA RNA              | 1                                 | 0.896 ± 0.001 | N/A           |
| Sup. Fig. S3C         | Prp5 <sup>core</sup> + U2 RNA                 | 1                                 | 0.88 ± 0.01   | N/A           |
| Sup. Fig. S3E         | Prp5 <sup>core</sup> + U2 RNA + ATP           | 2                                 | 0.890 ± 0.001 | 0.77 ± 0.04   |
| Sup. Fig. S4E         | Prp5 <sup>core2</sup>                         | 1                                 | 0.371 ± 0.002 | N/A           |
| Sup. Fig. S4G         | Prp5 <sup>core2</sup> + U2 RNA + ATP          | 2                                 | 0.354 ± 0.002 | 0.68 ± 0.06   |
| Sup. Fig. S5B         | Prp5 <sup>core</sup> + polyA RNA + ADP        | 1                                 | 0.894 ± 0.001 | N/A           |
| Sup. Fig. S5D         | Prp5 <sup>core</sup> + U2 RNA + ADPNP         | 2                                 | 0.883 ± 0.001 | 0.72 ± 0.03   |
| Sup. Fig. S8A         | Prp5 <sup>core</sup> -GAG                     | 1                                 | 0.882 ± 0.003 | N/A           |
| Sup. Fig. S8B         | Prp5 <sup>core</sup> -TAG                     | 1                                 | 0.896 ± 0.001 | N/A           |
| Sup. Fig. S8C         | Prp5 <sup>core</sup> -GAR                     | 1                                 | 0.897 ± 0.002 | N/A           |
| Sup. Fig. S9B         | Prp5 <sup>core</sup> -E235A + U2 RNA + ATP    | 2                                 | 0.809 ± 0.003 | 0.54 ± 0.02   |
| Sup. Fig. S9D         | Prp5 <sup>core</sup> -GAG + U2 RNA + ATP      | 1                                 | 0.893 ± 0.002 | N/A           |
| Sup. Fig. S9F         | Prp5 <sup>core</sup> -TAG + U2 RNA + ATP      | 1                                 | 0.898 ± 0.001 | N/A           |
| Sup. Fig. S9H         | Prp5 <sup>core</sup> -GAR + U2 RNA + ATP      | 1                                 | 0.904 ± 0.002 | N/A           |

N/A = not applicable

**Supplemental Table S5. Results from maximum likelihood fitting of histograms of lifetime distributions**

| <u>Related Figure</u> | <u>Experimental Description</u>         | <u>RNA</u>          | <u><math>\tau_{open}</math> (s)</u> | <u><math>\tau_{closed}</math> (s)</u> | <u><math>K_{close}</math></u> |
|-----------------------|---|---------------------|-------------------------------------|---------------------------------------|-------------------------------|
| 2D/E                  | Prp5 <sup>core</sup> + RNA + ATP        | 310 $\mu$ M Poly(A) | 3.97 $\pm$ 0.31                     | 0.46 $\pm$ 0.04                       | 0.12 $\pm$ 0.01               |
| 3H/I                  | Prp5 <sup>core</sup> -E235A + RNA + ATP | 310 $\mu$ M Poly(A) | 1.09 $\pm$ 0.05                     | 0.49 $\pm$ 0.02                       | 0.45 $\pm$ 0.03               |
| 4A                    | Prp5 <sup>core</sup> -GAG + RNA + ATP   | 310 $\mu$ M Poly(A) | 5.43 $\pm$ 0.45                     | 0.34 $\pm$ 0.03                       | 0.063 $\pm$ 0.007             |
| 4B                    | Prp5 <sup>core</sup> -TAG + RNA + ATP   | 310 $\mu$ M Poly(A) | 13.58 $\pm$ 1.28                    | 0.33 $\pm$ 0.04                       | 0.024 $\pm$ 0.004             |
| 4C                    | Prp5 <sup>core</sup> -GAR + RNA + ATP   | 310 $\mu$ M Poly(A) | 6.12 $\pm$ 0.46                     | 0.26 $\pm$ 0.02                       | 0.042 $\pm$ 0.004             |
| Sup. Fig. S3D/E       | Prp5 <sup>core</sup> + RNA + ATP        | 1 $\mu$ M U2        | 8.30 $\pm$ 0.84                     | 0.71 $\pm$ 0.12                       | 0.09 $\pm$ 0.02               |
| Sup. Fig. S5A/B       | Prp5 <sup>core</sup> + RNA + ADPNP      | 1 $\mu$ M U2        | 4.75 $\pm$ 0.49                     | 1.60 $\pm$ 0.21                       | 0.34 $\pm$ 0.06               |
| Sup. Fig. S9 A/B      | Prp5 <sup>core</sup> -E235A + RNA + ATP | 1 $\mu$ M U2        | 2.17 $\pm$ 0.52                     | 0.71 $\pm$ 0.06                       | 0.33 $\pm$ 0.08               |
| Sup. Fig. S9 C/D      | Prp5 <sup>core</sup> -GAG + RNA + ATP   | 1 $\mu$ M U2        | 6.13 $\pm$ 1.40                     | 0.51 $\pm$ 0.11                       | 0.08 $\pm$ 0.03               |
| Sup. Fig. S9 E/F      | Prp5 <sup>core</sup> -TAG + RNA + ATP   | 1 $\mu$ M U2        | 9.50 $\pm$ 2.11                     | 0.61 $\pm$ 0.30                       | 0.06 $\pm$ 0.03               |
| Sup. Fig. S9 G/H      | Prp5 <sup>core</sup> -GAR + RNA + ATP   | 1 $\mu$ M U2        | 13.90 $\pm$ 2.15                    | 0.36 $\pm$ 0.11                       | 0.03 $\pm$ 0.01               |