Supplemental Material

Molecular dissection of step 2 catalysis of yeast pre-mRNA splicing investigated in a purified system

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Inventory of Supplemental Material

Supplemental Figure S1

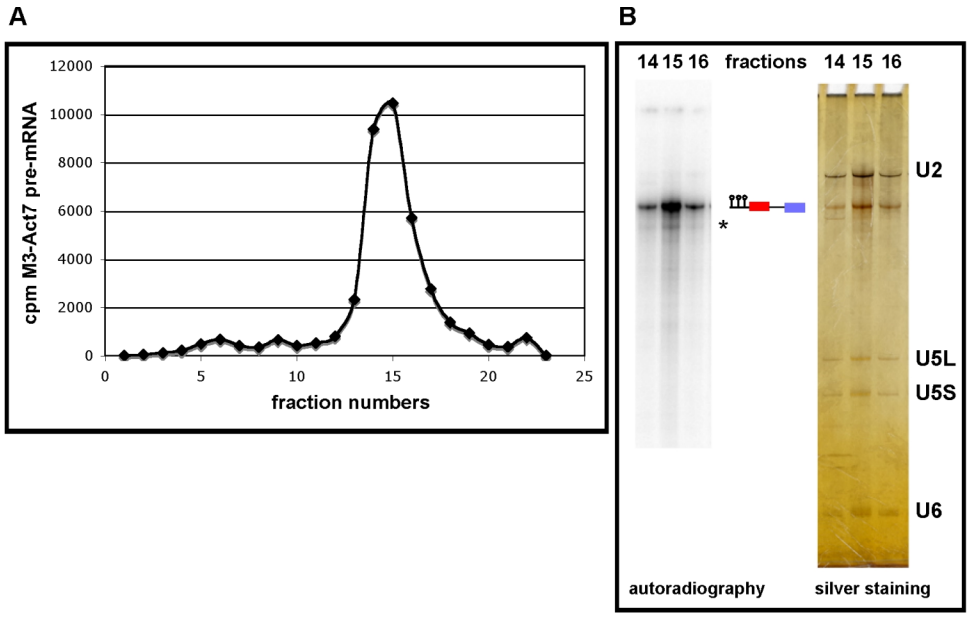

Figure S1 related to Figure 1. Characterization of purified B^{act} complex assembled on Act7 pre-mRNA. The B^{act} complex assembled on Act7 pre-mRNA was purified via three steps as described previously (Fabrizio et al., 2009), but with heat inactivated extract from the *prp2-1* strain (Warkocki et al., 2009). (A) Glycerol gradient profile of purified B^{act} complex assembled on Act7 with a specific activity of 300 cpm/fmol. The gradient was collected from the top and fractions analyzed by Cherenkov counting. (B) RNAs from fractions 14-16 were recovered, separated by denaturing PAGE and visualized by autoradiography and silver staining. The positions of the pre-mRNA and the snRNAs U2, U5 and U6 are indicated.

Supplemental Figure S2

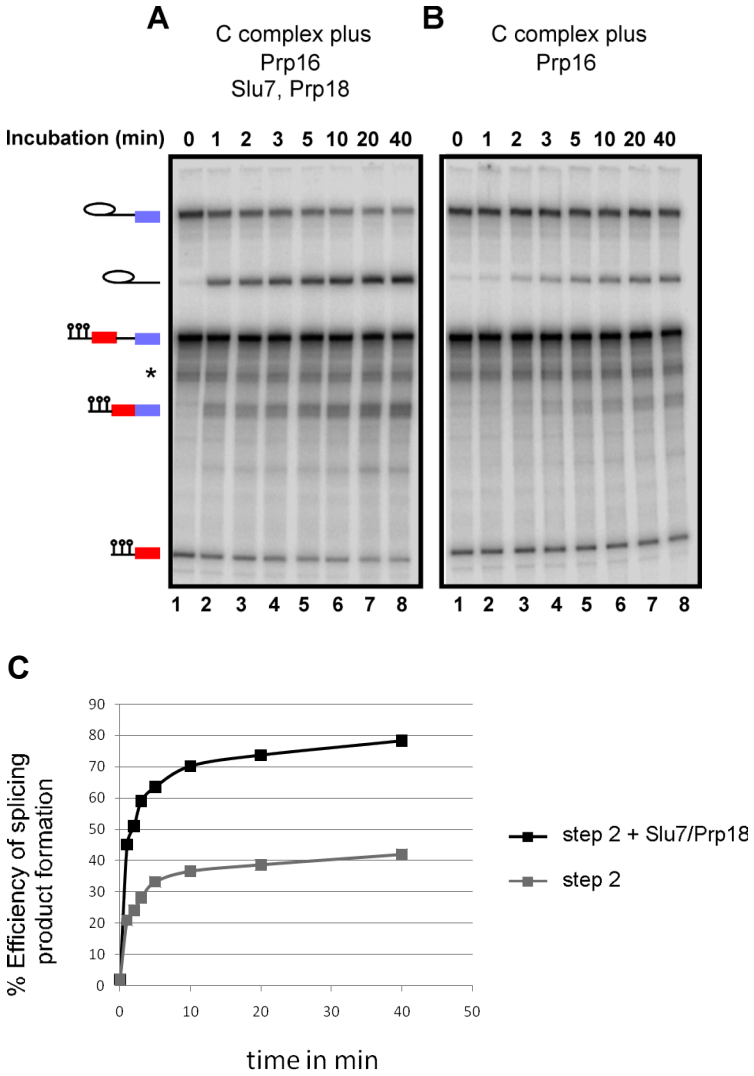

Figure S2 related to Figure 1. Time course of step 2 catalysis for Act7 substrates. B^{act} complexes assembled on Act7 were purified and eluted from the amylose matrix. Spliceosomes were then supplemented with Prp2, Spp2, Cwc25 and ATP at 4 °C and the reaction incubated for 40 min at 23 °C to obtain C complexes. Subsequently, Prp16, Slu7 and Prp18 (A) or only Prp16 (B) were added at 4 °C and the reactions incubated for another 40 min at 23 °C. Aliquots were taken at the time points indicated (lanes 1-8). RNAs were recovered, separated by denaturing PAGE and exposed to a Phosphorimager screen. Time points are indicated on top, symbols for pre-mRNA, splicing intermediates and products are indicated on the left: the 5' exon (with the three MS2 loops shown) in red, intron as a thin black line, 3' exon in blue. Asterisks: uncharacterised pre-mRNA bands. (C) The intensities of the bands of pre-mRNA, splicing intermediates and products in each lane were quantified and efficiencies for the second catalytic step were calculated as follow: mRNA+intron-lariat)/(mRNA+intron-lariat+5'exon+intron-3' exon) x100. Efficiencies were plotted against the indicated time points. Quantification was performed with a PhosphorImager using ImageQuant software (Molecular dynamics).

Supplemental Figure S3

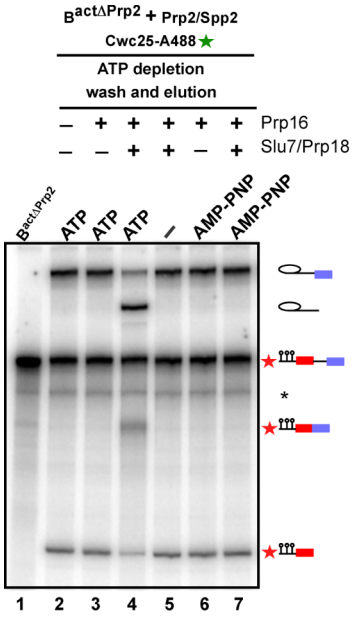

Figure S3 related to Figure 3A. Slu7 and Prp18 promote displacement of the step 1 factor Cwc25 from the spliceosome. (A) Affinity-purified B^{act} complexes assembled on Atto647N-maleimide-Actwt (red star) were complemented on the matrix with Prp2, Spp2, ATP and Cwc25-Alexa488 (green star). The reaction was incubated and then ATP was depleted. After extensive washing of the matrix with GK75 buffer, complexes were eluted and then supplemented with the proteins indicated, either in the absence (lane 5) or in the presence of ATP (lanes 2, 3 and 4) or ATP analogues (lanes 6 and 7) as indicated. After incubation, RNA was extracted, separated by denaturing PAGE and visualized by autoradiography. The position of the pre-mRNA, splicing intermediates and products are indicated on the right: the 5' exon (with the three MS2 loops shown) in red, intron as a thin black line, 3' exon in blue. Asterisks: uncharacterised pre-mRNA bands.

Supplemental Figure S4

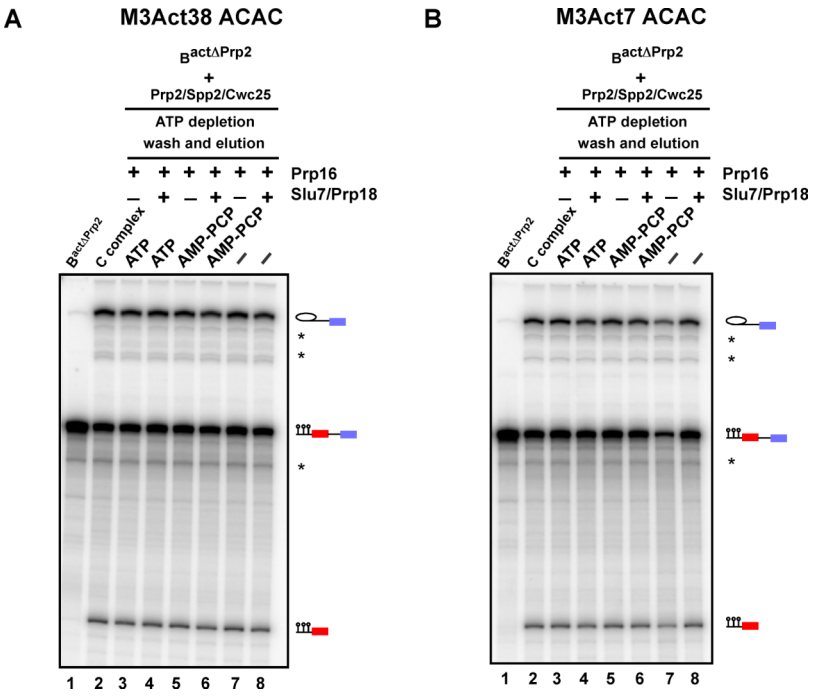

Figure S4 related to Figure 3B and D. Addition of Prp16 plus ATP, Slu7 and Prp18 does not lead to step 2 products with pre-mRNAs mutated at the 3' splice site. Act38 ACAC (A) and Act7 ACAC (B) pre-mRNAs were extracted from spliceosomal complexes obtained as described above each lane and in the main text, separated by denaturing PAGE and visualized by autoradiography. The positions of the pre-mRNAs and the splicing intermediates are indicated on the right. Asterisks: uncharacterised pre-mRNA bands.

Supplemental Figure S5

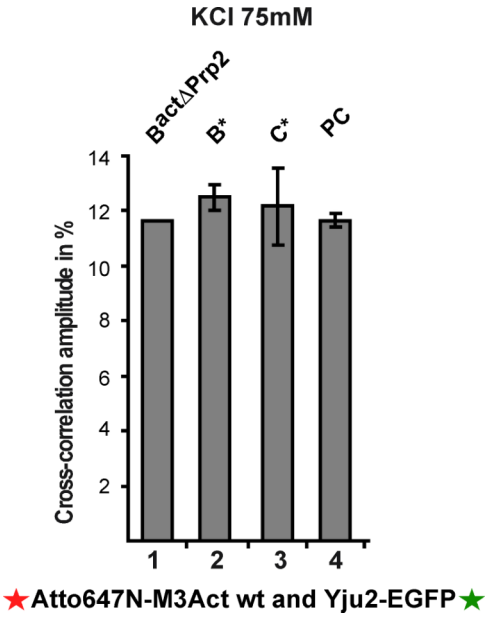

Figure S5 related to Figure 3. Yju2-EGFP remains bound to the spliceosome upon catalytic activation and step 2 catalysis. Affinity-purified $B^{actΔPrp2}$ complexes assembled on Atto-Actwt (red star) and carrying Yju2-EGFP (Ohrt et al., 2012) (column 1), were complemented with Prp2, Spp2 and ATP (column 2, B*), and additionally with Cwc25 and Prp16 (column 3, C*) and Slu7/Prp18 (column 4, PC, post-catalytic spliceosome). After the standard incubation at 75 mM KCl, samples were subjected to dcFCCS measurements. The different percentages of cross-correlation between different fluorescently labeled samples are due to the inherent variability of the experimental set up (see also (Ohrt et al., 2012).

Supplemental Figure S6

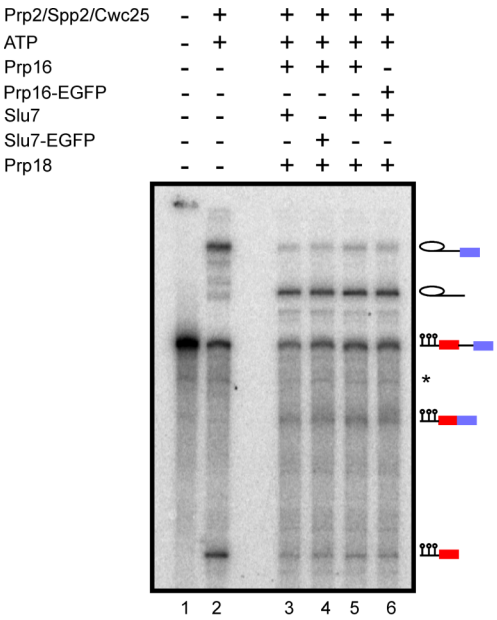

Figure S6 related to Figures 4 and 5. Prp16-EGFP and Slu7-EGFP are functional. B^{act} complexes assembled on Actwt (lane 1) were affinity-purified, eluted from the amylose matrix and complemented with Prp2, Spp2, ATP and Cwc25 (lane 2). Complexes were additionally complemented with Prp16, Slu7 and Prp18 (lanes 3 and 5) or fluorescently-labelled Slu7-EGFP (lane 4) or Prp16-EGFP (lane 6). RNA was analysed on an 8% polyacrylamide–urea gel and visualised by autoradiography. The positions of the pre-mRNAs and the splicing intermediates and products are indicated on the right. Asterisks: uncharacterised pre-mRNA bands.

Supplemental Figure S7

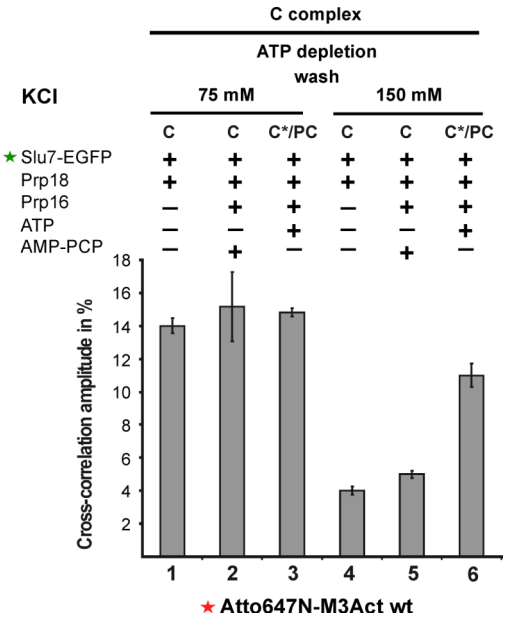

Figure S7 related to Figure 4. Stable binding of Slu7 after the ATP-dependent action of Prp16. The C complex was assembled on Atto-Actwt (red star) and retained on the amylose matrix. ATP was depleted and the matrix was washed with GK75 buffer. Half of the reaction was supplemented with KCl to a final concentration of 150 mM. Both reactions were then complemented with Slu7-EGFP (green star), Prp18 and Prp16 either in the presence of AMP-PCP (columns 2 and 5) or ATP (columns 3 and 6). C*/PC indicates that Slu7/Prp18 binds to the C* complex which is transformed into the post-catalytic complex (PC) upon their binding. Reactions were washed with GK75 or GK150 buffer, as indicated and spliceosomes eluted from the amylose matrices with maltose and analyzed by dcFCCS as above.

Supplemental Table 1

Yeast Protein name	MW [kDa]	Human Homologue Name	Number of peptides sequenced				
Column			**1**	**2**	**3**	**4**	**5**
Actwt-B^{act} spliceosomes supplemented with			**-**	Prp2 Spp2	Prp2 Spp2 Cwc25	Prp2 Spp2 Cwc25 Prp16	Prp2 Spp2 Cwc25 Prp16 Slu7 Prp18
Name of the obtained complex			**Bact**	**B***	**C**	**C***	**PC**
fmoles analysed with Orbitrap			80				
KCl concentration in the gradient (mM)			75				
Sm proteins							
B	22.4	B	25	20	13	19	12
D1	16.3	D1	11	16	17	22	17
D2	12.8	D2	34	25	25	20	16
D3	11.2	D3	21	10	16	10	9
E	10.4	E	7	7	12	9	7
F	9.6	F	6	6	7	4	4
G	8.5	G	4	2	5	6	5
U2 snRNP proteins							
Rse1	153.8	SF3b130	208	180	160	270	203
Hsh155	110	Sf3b155	194	167	106	133	100
Prp9	63	SF3a60	101	92	68	123	76
Cus1	50.2	SF3b145	71	74	46	58	43
Prp21	33	SF3a120	50	48	40	39	24
Prp11	29.9	SF3a66	32	31	23	36	18
Lea1	27.2	U2-A'	46	32	26	38	36
Hsh49	24.5	SF3b49	9	7	22	22	17
Msl1	12.8	U2-B''	9	8	11	9	9
Rds3	12.3	SF3b14b	4	5	6	10	8
Ysf3	10	SF3b10		2	4	5	4
U5 snRNP proteins							
Prp8	279.5	220K	489	578	319	436	408
Brr2	246.2	200K	449	326	211	420	362
Snu114	114	116K	210	234	127	212	145
RES complex proteins							
Bud13	30.5	MGC13125	25	21	15	13	11
Pml1	23.6	SNIP1	25	13	7	4	6
Ist3|Snu17	17	CGI-79	10	8	5	5	4
NTC/Prp19 complex proteins							
Syf1| Ntc90	100	hSyf1|XAB2	239	229	99	174	142
Clf1| Ntc77	82.4	CRNKL1	121	119	52	98	74
Cef1| Ntc85	68	CDC5L	112	95	55	112	92
Prp19	56.6	hPRP19	245	247	140	227	203
Isy1| Ntc30	28	KIAA1600	30	28	16	27	23
Syf2| Ntc31	25	GCIP p29	23	34	27	30	32
Snt309|Ntc25	21	SPF27	6	5	15	21	21
Ntc20	16	-------	10	10	8	10	12
NTC related proteins							
Prp46	51	PRL1	68	65	40	87	67
Prp45	42.5	SKIP1	70	65	47	75	60
Ecm2	41	RBM22	61	61	30	25	18
Cwc2	38.4	RBM22	56	51	33	33	35
Bud31|Cwc14	18.4	G10	10	15	10	13	8
Cwc15	20	AD002|HSPC148	36	33	33	37	26
Yju2| Cwc16	32	CCDC130	24	36	18	26	20
Cwc21	15.7	Srm300	18	19	14	12	8
Cwc22	67.3	KIAA1604	105	93	40	52	44

Column			1	2	3	4	5
Name of the obtained complex			**Bact**	**B***	**C**	**C***	**PC**
NTC related proteins continue							
Cwc24	28	RNF113A	29	6			
Cwc27	35	NY-CO-10	38	11	4		
Cwc23	33.2	DNAJ A1					
Recombinant step1 proteins							
Cwc25	20.4	CCDC49			27	22	5
Spp2	20.6	GPKOW|T54					
Prp2	100	DDX16|DHX16		84	32	49	37
Step2 proteins							
Prp17|Cdc40	52	hPRP17	31	25	28	40	25
Recombinant step2 proteins							
Prp16	121.6	hPRP16				240	167
Slu7	44.6	hSLU7					24
Prp18	28.4	hPRP18					15
Disassembly proteins							
Prp22	130	hPRP22					
Prp43	87.6	hPRP43					
Ntr1| Spp382	83	TFIP11					
Ntr2	36.6	-------					

Table S1 related to Figure 2. Proteins identified by mass spectrometry in B^{act} complexes, supplemented stepwise with the recombinant proteins indicated. Proteins from peak fractions as described in Figure 2A were separated by SDS-PAGE, stained with Coomassie and the gel was cut into 23 slices. Proteins were digested with trypsin and resulting peptides were eluted and analyzed by LC-MS/MS. The absolute number of sequenced peptides is shown for each identified protein. B^{act} and B^{act} complexes supplemented with Prp2, Spp2 and ATP (i.e. B*), which were purified under the same conditions are shown for comparison. The post-catalytic complex is named PC.

Supplemental Methods

Spliceosome Purification

Yeast spliceosomal complexes were purified essentially as described in (Fabrizio et al., 2009) except that heat inactivated extracts from the yeast strain *prp2-1* (Yean and Lin, 1991) were used in the assembly reactions. Before splicing, wildtype Actin pre-mRNA or other constructs were incubated with a 35-fold molar excess of purified MS2-MBP fusion protein at 4°C for 30 min in 20 mM HEPES-KOH (pH 7.9). Depending on the required amount of spliceosomal complexes, a 6–72 ml splicing reaction containing 1.8 nM of ^{32}P-labeled M3Act pre-mRNA (specific activity 20–300 cpm/fmol), 60 mM KPO4 (pH 7.4), 3% PEG 8000, 2.5 mM $MgCl_2$, 2.0 mM ATP, 2.0 mM spermidine, and 40% yeast extract in buffer D (20 mM HEPES-KOH, pH 7.9, 50 mM KCl, 0.2 mM EDTA pH 8.0, 20% (v/v) glycerol, 0.5 mM DTT, and 0.5 mM PMSF) was performed. B^{act} complexes were assembled by incubating at 23°C for 50 min. Six ml aliquots of the splicing reaction were loaded onto 36-ml linear 10-30% (w/w) glycerol gradients containing GK150 or in some experiments GK75 buffer (20 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 150 or 75 mM KCl). Gradients were centrifuged for 14 h at 23,000 rpm in a SureSpin 630 rotor (Thermo Scientific) and harvested manually in 1500 µl fractions from the top. Fractions were analyzed by Cherenkov counting in a scintillation counter and peak fractions containing complexes were pooled and loaded onto columns containing 250–300 µl of amylose matrix equilibrated with GK75 or GK150 buffer. The matrix was washed two times with 10 ml GK150 buffer and two times with GK75 buffer supplemented with 5% glycerol and 0.01% NP40, and spliceosomal complexes were eluted dropwise with 750 µl elution buffer (GK75 buffer containing 12-20 mM maltose, 5% glycerol and 0.01% NP40). The elution fractions were either used directly for reconstitution assays or for dcFCCS analysis or were loaded onto a 3.8-ml linear 10–30% (w/w) glycerol gradient containing GK75 buffer. Gradients were centrifuged for 107 min at 490,000 g in a Sorvall TH660 rotor, and 175-µl fractions were harvested from the top of the gradient and analyzed by Cherenkov counting. Peak fractions were then used for mass spectrometry analysis or used in reconstitution assays.

Mass Spectrometry

For mass spectrometric analyses of proteins associated with spliceosomal complexes, the peak fractions of the respective second glycerol gradient were combined in low-protein binding 1.5 ml reaction tubes (Eppendorf). 350 µl of the combined fractions were supplemented with 40 µg glycoblue, 35 µl of 3 M NaOAc (pH 5.2) and 1100 µl of ethanol. After thorough mixing the solution was stored overnight at -20 °C and then centrifuged for 30 min at 13,000 rpm and 4°C in a table top centrifuge. Subsequently, the pellet was washed with 70 % ethanol, dried in a vacuum dryer and resuspended in 1x SDS PAGE loading buffer from Invitrogen. Proteins recovered from purified spliceosomal complexes (~400 fmol), were separated by SDS-PAGE using a 4-12% Bis-Tris-HCL (pH 7.0) NUPAGE polyacrylamide gel (Invitrogen) and stained with Coomassie. Entire lanes were cut into 23 slices and proteins were digested in-gel with trypsin and extracted as previously described (Shevchenko et al., 1996). Resulting peptides were analyzed in an LC-coupled ESI Q-ToF (Q-ToF Ultima, Waters) and/or OrbitrapXL (ThermoFisher Scientific) mass spectrometer

under standard conditions. Proteins were identified by searching fragment spectra against the NCBI nonredundant (nr) database using Mascot as search engine.

Dual-color FCCS set-up

The dcFCCS set-up (MicroTime 200, PicoQuant GmbH, Berlin, Germany) is based on an inverse epi-fluorescence microscope (IX-71, Olympus Europa, Hamburg, Germany). The system was equipped with two identical pulsed 470-nm diode lasers (LDH-P-C-470 B, PicoQuant GmbH, Berlin, Germany) and two identical pulsed 635-nm diode lasers (LDH-P-635, PicoQuant GmbH, Berlin, Germany) with linear polarisation and a pulse duration of 50 ps (FWHM). The lasers were pulsed alternately with an overall repetition rate of 40 MHz (PIE mode) corresponding to a delay between pulses of 25 ns (PDL 828 "Sepia II", PicoQuant GmbH, Berlin, Germany). The light was focused by the microscope's objective (UPLSAPO 60x W, 1.2 N.A., Olympus Europa, Hamburg, Germany). Fluorescence was collected by the same objective (epi-fluorescence mode), passed through the dichroic mirror, and focused by a tube lens through a single circular aperture (diameter 150 μm). After the pinhole, the light was re-collimated, split by a polarising beam-splitter cube (Ealing Catalogue, St. Asaph, UK) and two dichroic mirrors (640 dcxr, Chroma Technology, Rockingham, VT, USA), and focused onto two single-photon avalanche diodes for the red (two SPCM-AQR-13, Perkin Elmer, Wellesley, MA, USA), and two for the green emission (PDM 50-C, Micro Photon Devices, Milan, Italy), respectively. This kind of excitation/emission scheme yielded maximum information on fluorescence anisotropy and spectrum, although we subsequently used only the spectral information. Emission bandpass filters HC692/40 and HC520/35 (Semrock, USA), positioned directly in front of each detector, were used for blocking scattered light. Time-correlated single-photon counting electronics (HydraHarp 400, PicoQuant GmbH, Berlin, Germany) independently recorded the detected photons of all detectors with an absolute temporal resolution of 4 ps on a common time frame. To avoid artifacts due to evaporation-induced convection or unspecific binding to the cover slide during our dcFCCS measurements, cover slides were coated with Sigmacote (Sigma-Aldrich) according to the manufacturer's instructions, and spliceosomal samples were hermetically sealed between two coated cover glasses using a 1 mm silicone spacer (Grace Bio-Labs). Measurement was then performed for 10 min at room temperature.

Expression and Purification of Prp16-EGFP and Slu7-EGFP

Full-length yeast *PRP16* and *SLU7* genes were cloned into pYES2/CT plasmid containing an N-terminal His_6-tag in frame with an N-terminal EGFP-tag and transformed into the INVSc1 yeast strain (Invitrogen). Cells were grown to an $O.D_{600}$ of 4.0 in YP medium supplemented with 2% galactose. Whole cell extract was prepared as described in (Gottschalk et al., 1999). His_6-EGFP-Prp16 and His_6-EGFP-Slu7 were captured on Ni^{2+}-NTA resin (GE Healthcare), eluted with the buffer containing 200 mM imidazole, and purified by size exclusion chromatography S200 Sephadex 16/600 (GE Healthcare) equilibrated with 20 mM HEPES pH 7.5, 200 mM NaCl, 20% (w/v) glycerol, and 2 mM DTT. Pooled fractions were dialyzed against 40 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20% (w/v) glycerol, and 1 mM DTT. Recombinant Prp2, Spp2, Cwc25, Prp16, Slu7, Prp18 and Prp22 were expressed in *E. coli* and purified essentially as described previously (Warkocki et al., 2009). Cwc25-Alexa was expressed, labeled and purified as described in (Ohrt et al., 2012).

References

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