The G-patch protein Spp2 couples the spliceosome-stimulated ATPase activity of the DEAH-box protein Prp2 to catalytic activation of the spliceosome

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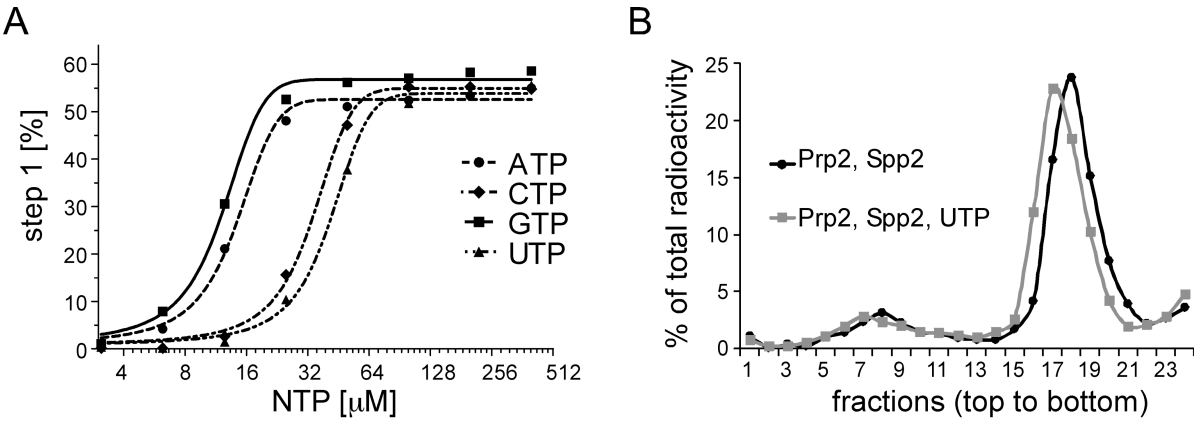

Figure S1 (Related to Fig. 2). (A) **All rNTPs allow efficient step 1 catalysis.** Splicing reconstitution *in vitro* was performed with affinity-purified $B^{act\ \Delta Prp2}$ spliceosomes in the presence of recombinant Prp2, Spp2 and Cwc25 and increasing concentrations of ATP, CTP, GTP and UTP for 1 h at 23 °C. The percent of catalyzed step 1 is plotted against the concentration of NTP (in logarithmic scale). Sigmoidal dose-response curves were fitted using GraphPad Prism software. (B) **Prp2 remodels the spliceosome in the presence of UTP as sole energy source.** Glycerol gradient sedimentation profiles of $B^{act\ \Delta prp2}$ spliceosomes (formed on ^{32}P-labeled wild-type actin pre-mRNA) incubated with Prp2 and Spp2 in the absence (black, B^{act}) or presence (grey, B*) of 1 mM UTP. Radioactivity contained in each fraction was determined by Cherenkov counting. The percent of total radioactivity present in each gradient fraction is plotted. 10-30% v/v glycerol gradients containing 75 mM KCl were loaded with 400 µl sample and centrifuged for 2 h at 60000 rpm in a TH660 rotor (Sorvall).

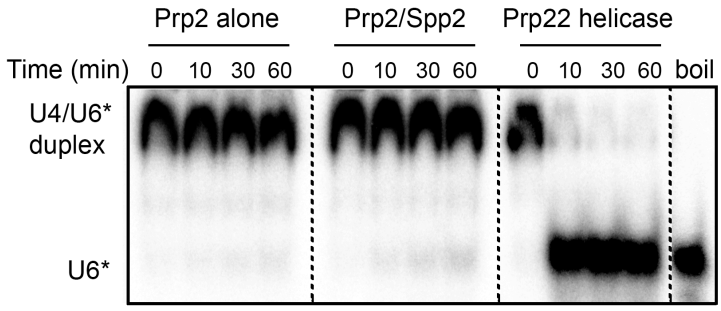

Figure S2 (Related to Fig. 5). Spp2 does not enable Prp2 to unwind dsRNA. RNA unwinding assays were performed with 0.25 nM U4/U6 snRNA duplex (U6 was ^{32}P-labeled) and 50 nM Prp2 or Prp22. When indicated 4 µM Spp2 was added to the reaction. Unwinding reactions were pre-incubated for 10 min at 35° C and initiated by the addition of 3 mM ATP. At the indicated time points, 5 µl aliquots of the unwinding reaction were withdrawn. Samples were separated by 8 % non-denaturing PAGE and visualized by autoradiography.

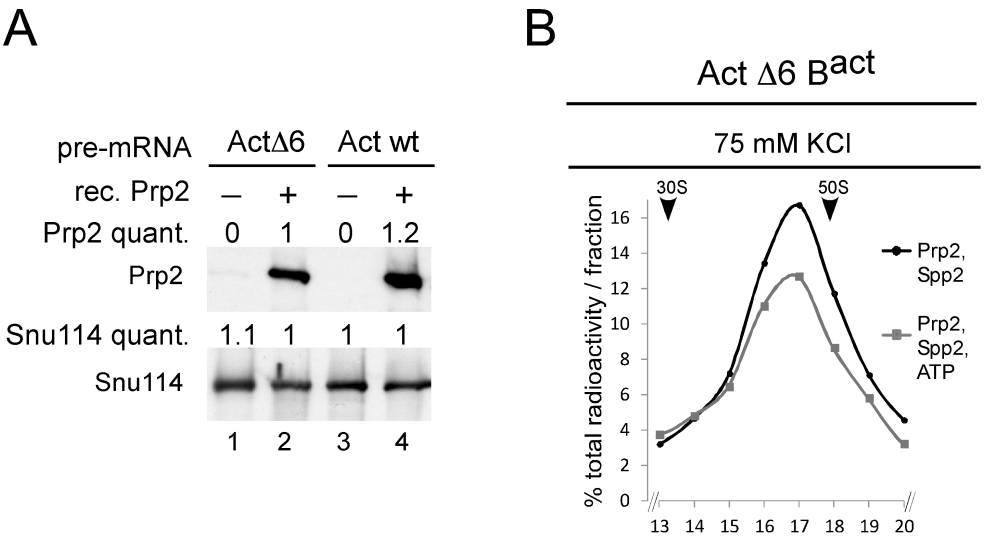

Figure S3 (Related to Fig. 4). Recombinant Prp2 binds to the ActΔ6 B^{act} spliceosomes in the absence of Spp2. (A). Western blot analysis of the presence of Prp2 (top panel) and Snu114 (bottom panel) in the ActΔ6 (lanes 1 and 2) and wild-type B^{act} spliceosomes (lanes 3 and 4). The purified spliceosomes (bound to affinity resin) were incubated with excess of Prp2, unbound protein was removed by washing, and spliceosomes were eluted and further purified on a glycerol gradient. Approximate quantification of the western blot signals (indicated above each blot) was performed using ImageJ. (B) **ActΔ6 B^{act} spliceosomes are not converted into catalytically activated B* complexes.** Profiles of analytical 10-30% (v/v) glycerol gradient centrifugations of the ActΔ6 B$^{act\ \Delta Prp2\ \Delta Spp2}$ spliceosomes incubated with Prp2 and Spp2 (black) or with Prp2, Spp2 and ATP (grey). No shift to the left of the spliceosome was observed, demonstrating that ActΔ6 B^{act} was not catalytically activated. Radioactivity contained in each fraction was determined as shown in Fig. 3A. Positions of migration markers – dissociated prokaryotic ribosomal subunits (30S and 50S), are indicated.

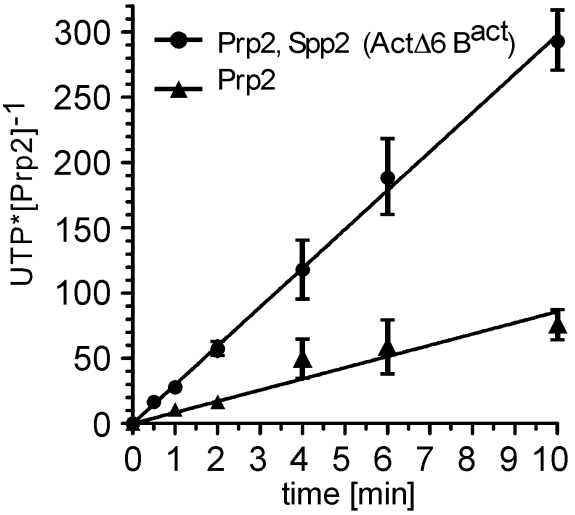

Figure S4 (Related to Fig. 4). ActΔ6 B^{act} spliceosomes stimulate Prp2's UTPase activity inefficiently. Prp2 UTPase activity analyzed in B$^{act\ \Delta Prp2\ \Delta Spp2}$ spliceosomes assembled on ActΔ6 pre-mRNA in the presence of Spp2. UTP hydrolysis by Prp2 (50 nM) plus Spp2 (150 nM), in the absence or presence of purified ActΔ6 spliceosomes was monitored as in Fig. 2. A 10 min time course is shown. Background hydrolysis by spliceosomes without added Prp2 was subtracted. Data points are mean values from two experiments (see Table S2).

Table S1. Calculation of UTP hydrolysis by $B^{act\ \Delta Prp2}$ spliceosomes in the absence and presence of Prp2

1	2	3	4	5	6
	min	fraction of UDP	UTP hydrolyzed [fmol]	UTP hydrolyzed per single spliceosome (background) or spliceosome and Prp2	UTP hydrolyzed per single Prp2 on the spliceosome
$B^{act\ \Delta Prp2}$ spliceosomes	0	0.0000	0	0	
	0.5	0.0039	4631	8	
	1	0.0065	7845	13	
	2	0.0118	14163	24	
	4	0.0204	24496	41	
	6	0.0328	39344	66	
	10	0.0611	73337	122	
$B^{act\ \Delta Prp2}$ spliceosomes + Prp2	0	0.0000	0	0	0
	0.5	0.0286	34265	57	51
	1	0.0581	69720	116	104
	2	0.1159	139095	232	208
	4	0.2062	247412	412	365
	6	0.2831	339720	566	496
	10	0.4141	496961	828	711

Table S2. Time course of UTP hydrolysis

column	1	2	3	4	5	6	7	8	9	10	11
		"Prp2"		"Prp2, Spp2"		"Prp2, Cwc25"		"Prp2, Spp2, Cwc25"		ActΔ6 B^{act} + Prp2, Spp2	
	exp #	1	2	1	2	1	2	1	2	1	2
min	0	0	0	0	0	0	0	0	0	0	0
	0.5	74	72	59	62	60	71	51	74	15	18
	1	102	110	94	97	113	118	77	79	28	28
	1.5	150		150		171		100			
	2	212	202	179	180	223	222	128	135	63	52
	4	403	398	312	368	389	399	212	250	140	96
	6	563	509	428	355	549	521	274	327	216	161
	10	773	749	517	576	715	722	367	492	314	272
		mean	sem	mean	sem	mean	sem	mean	sem	mean	sem
min	0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
	0.5	73	0.8	61	1.4	65	5.5	62	11.2	17	1.4
	1	106	3.8	96	1.4	115	2.8	78	1.2	28	0.1
	1.5	150		150		171		100			
	2	207	5.3	180	0.2	223	0.3	132	3.6	58	5.8
	4	401	2.6	340	28.1	394	4.9	231	18.8	118	21.9
	6	536	27.0	392	36.6	535	14.0	300	26.6	189	27.4
	10	761	11.8	546	29.6	719	3.6	429	62.4	293	21.0

sem = standard error of the mean

Number of UTP molecules hydrolyzed at the indicated time points by the $B^{act\ \Delta Prp2\ \Delta Spp2}$ spliceosome (wild-type columns: 1-9, or ActΔ6 columns: 10 and 11) after addition of the indicated recombinant proteins.

Table S3. Protein composition of ActΔ6 B$^{act\ \Delta Prp2\ \Delta Spp2}$ spliceosomes reconstituted *in vitro* in the presence of Prp2, Spp2, without or with ATP.

Yeast Protein name	Systematic Gene name	MW [kDa]	Human Homologue	Number of peptides sequenced	
			Column	**1**	**2**
			Pre-mRNA	ActΔ6	
			incubation with ATP		**+**
			KCl concentration in the glycerol gradient [mM]	75	
			fmoles analysed	100	100
			Device used for analysis	Orbitrap	
Sm proteins					
B	YER029C	22.4	B	42	41
D1	YGR074W	16.3	D1	27	25
D2	YLR275W	12.8	D2	31	33
D3	YLR147C	11.2	D3	23	25
E	YOR159C	10.4	E	7	12
F	YPR182W	9.6	F	3	3
G	YFL017W-A	8.5	G	9	8
U2 snRNP proteins					
Rse1	YML049C	153.8	SF3b130	283	305
Hsh155	YMR288W	110	Sf3b155	208	166
Prp9	YDL030W	63	SF3a60	131	115
Cus1	YMR240C	50.2	SF3b145	120	86
Prp21	YJL203W	33	SF3a120	53	46
Prp11	YDL043C	29.9	SF3a66	31	36
Lea1	YPL213W	27.2	U2-A′	50	55
Hsh49	YOR319W	24.5	SF3b49	12	14
Msl1	YIR009W	12.8	U2-B″	22	19
Rds3	YPR094W	12.3	SF3b14b	14	15
Ysf3	YNL138W-A	10	SF3b10	4	7
U5 snRNP proteins					
Prp8	YHR165C	279.5	220K	470	467
Brr2	YER172C	246.2	200K	510	493
Snu114	YKL173W	114	116K	245	210
RES complex proteins					
Bud13	YGL174W	30.5	MGC13125	53	65
Pml1	YLR016C	23.6	SNIP1 ?	6	10
Ist3/Snu17	YIR005W	17	CGI-79 ?	17	13
NTC/Prp19 complex proteins					
Syf1/ Ntc90	YDR416W	100	hSyf1/XAB2	172	168
Clf1/ Ntc77	YLR117C	82.4	CRNKL1	118	125
Cef1/ Ntc85	YMR213W	68	CDC5L	108	118
Prp19	YLL036C	56.6	hPRP19	294	293
Isy1/ Ntc30	YJR050W	28	KIAA1600	28	31
Syf2/ Ntc31	YGR129W	25	GCIP p29	24	49
Snt309/Ntc25	YPR101W	21	SPF27	10	8
Ntc20	YBR188C	16	-------	15	16
"NTC related proteins"					
Prp46	YPL151C	51	PRL1	76	85
Prp45	YAL032C	42.5	SKIP1	73	84
Ecm2	YBR065C	41	RBM22 ?	52	45
Cwc2	YDL209C	38.4	RBM22	53	49
Bud31/Cwc14	YCR063W	18.4	G10	13	14
Cwc15	YDR163W	20	AD-002	37	40
Known splicing proteins					
Yju2/Cwc16	YKL095W	32	CCDC130	24	28
Cwc21	YDR482C	15.7	Srm300	22	15
Cwc22	YGR278W	67.3	KIAA1604	139	68
Cwc24	YLR323C	28	RNF113A	21	23
Cwc27	YPL064C	35	NY-CO-10	44	29
Prp17/Cdc40	YDR364C	52	hPRP17	38	44
Added recombinant proteins					
Spp2	YOR148C	20.6	GPKOW/T54	9	2
Prp2	YNR011C	100	DDX16/DHX16	69	21

Proteins were identified by LC-MSMS after separation by PAGE. The table contains information about the concentration (in fmoles) of each complex analyzed by mass spectrometry. The table shows the *S. cerevisiae* protein, systematic gene name and the calculated molecular weight of the (*S. cerevisiae*) protein in kDa. The fourth column contains the name of the human protein to aid

comparison with previous studies of human spliceosomal complexes. Proteins are grouped in organisational and/or functional subgroups. Note that Cwc24, Cwc27 and the RES complex proteins are equally represented in the absence or presence of ATP, while they are displaced from a catalytically activated Act-wt B* complex (Warkocki et al. 2009).

Supplemental methods

Purification of spliceosomes

Preparative purifications of the $B^{act\ \Delta Prp2}$ or $B^{act\ \Delta Prp2\ \Delta Spp2}$ spliceosome were performed as described previously (Warkocki et al. 2009). Briefly, *in vitro* splicing reactions with 1.7-2 nM pre-mRNA (containing trace ^{32}P-labeled pre-mRNA, ca. 20-100 cpm/fmole) and 40% v/v heat-treated (35^{o}C, 30 min) yeast whole-cell extract from *prp2*-1 cells [strain 3.2.AID; alpha, *prp2-1*, *ade2*, *his3*, *lys2-801*, *ura3*, carrying a G360D mutation in Prp2; (Yean and Lin 1991)] were incubated for 30-40 min at 23^{o}C. The reactions were layered on 10-30% v/v glycerol gradients (20 mM Hepes-KOH pH 7.9, 150 mM KCl, 1.5 mM $MgCl_2$, 0.01% NP40; up to 6 ml of splicing reaction were layered onto one gradient, total volume was 36 ml) and centrifuged in a SureSpin rotor (Kendro) at 23000 rpm at 4^{o}C for 14h. Gradients were fractionated manually into 24 fractions from top to bottom. Spliceosomes in fractions 15-20 were pooled and affinity selected on 0.6 ml amylose resin (NEB) in 10 ml columns (BioRad). Beads were washed with 20 ml GK150 or GK75 buffer (150 or 75 mM KCl, 20 mM Hepes-KOH pH 7.9, 1.5 mM $MgCl_2$, 5% v/v glycerol, 0.01% NP40) as indicated. Spliceosomes were eluted with 0.6 ml of GK75 containing 12 mM maltose. The amounts and molarity of the eluted spliceosomes was estimated based on the specific activity of the pre-mRNA and the cpms determined by Cherenkov counting.

Reconstitution of Prp2 activity *in vitro*

Affinity purified $B^{act\ \Delta Prp2\ \Delta Spp2}$ spliceosomes (Fig. 3A), were incubated in the presence of 0.12 U/µl RNAsin (Promega), Prp2 with or without Spp2 (both 40 nM), and 1 mM ATP for 30 min at 23 ^{o}C. 400 µl of the sample were loaded on a 10-30% v/v glycerol gradient in GK75 buffer and centrifuged for 2 h at 60000 rpm at 4 ^{o}C in a TH660 rotor (Thermo Scientific). Gradients were fractionated manually into 24 fractions from top to bottom. Radioactivity in each fraction was measured by Cherenkov counting and plotted as percent of total radioactivity present in the gradient (including pelleted material).

Reconstitution of step 1 catalysis *in vitro*

The reconstitution reactions in Figs. S1 and 3 were assembled on ice and performed as described in (Warkocki et al. 2009). Briefly, purified spliceosomes (ca. 1 nM) were incubated in the presence of 0.12 U/µl RNAsin, with Cwc25, Prp2, Spp2 (each 40 nM) and ATP (1 mM) as indicated (or with increasing concentrations of ATP, CTP, GTP and UTP, Fig. S1A) for 1 h at 23 ^{o}C. All reactions were stopped by addition of EDTA, SDS and proteinase K (10 mM, 0.2%, 0.2 mg/ml) and processed as described above for splicing *in vitro*. In Fig. 3B the spliceosomes were collected from peak fractions of glycerol gradients.

Splicing substrates

All pre-mRNAs were generated by run-off transcription *in vitro* with T7 RNA polymerase on PCR-amplified DNA templates of yeast actin pre-mRNA tagged with three MS2 stem-loops at the 5' end as described in (Fabrizio et al. 2009). The tagged transcripts contain 687 (wild-type) and 480 (ActΔ6) nucleotides. Radioactively body-labeled pre-mRNAs were

produced in the presence of α^{32}P UTP at concentrations that on average allowed incorporation of 1-1.2 ^{32}P uridine residues per transcript. Transcripts were PAGE purified. The concentration and purity of unlabeled transcripts were estimated by measuring absorbance at 260 nm.

Production of site-specific labeled pre-mRNA and DNA splint-directed RNA ligation

Site-specific labeled pre-mRNA was produced by ligation of RNA fragments prepared previously by site-specific cleavage of the pre-mRNA with DNA enzymes essentially as described by (Cameron and Uhlenbeck 1977; Silverman and Baum 2009). RNA fragments were ligated by DNA splint directed RNA ligation essentially as described previously (Moore and Sharp 1992).

Preparation of yeast whole-cell extract

The yeast cells *prp2*-1 (MATalpha, prp2-1, ade2, his3, lys2-801, ura3; kindly provided by Dr. Ren-Jang Lin), were grown to OD_{600} = 5-6. The cells were harvested and washed with ice-cold water. Each 50 g of washed and pelleted cells were suspended in 45 ml AGK buffer (20 mM HEPES-KOH pH 7.9, 400 mM KCl, 1.5 mM $MgCl_2$, 8% (v/v) glycerol) containing 1 mM DTT and protease inhibitors (Roche) and drops were frozen in liquid nitrogen. Frozen yeast-cell beads were ground and the resulting powder was thawed in a water bath at room temperature. The crude lysate was centrifuged twice at 4 °C in a SS34 rotor (Sorvall) at 17000 rpm for 30 min. The supernatant was then centrifuged for 1 h in a T647.5 rotor (Thermo Scientific) at 42000 rpm. The resulting cleared-lysate (middle phase, ca. 70% of total volume) was dialyzed twice in dialysis membranes (Spectrum, membrane cut-off 6-8 kDa) against 5 L of buffer D (20 mM HEPES-KOH pH 7.9, 50 mM KCl, 0.2 mM EDTA, 20% (v/v) glycerol, 0.5 mM DTT, 0.5 mM PMSF, 2 mM benzamidine) for 1 h 45 min-2 h or subjected to cross-flow dialysis (Sartorius Stedim Biotech). The dialysed extract was centrifuged in a SS34 rotor (Sorvall) for 10 min at 4°C, divided into aliquots, frozen in liquid nitrogen, and stored at – 80°C.

Calculation of UTP hydrolysis

We calculated the fraction of UDP and UTP in a single aliquot on the basis of ImageQuant 5.1-reported intensities according to UDP=[UDP]/([UTP]+[UDP]) (1). The amount of hydrolyzed UTP (formed UDP) in fmol was calculated by multiplying (1) by the total amount of UTP in the test tube and 1000 (1)·(12 µl·100 pmol/µl)·(1000) (2). To obtain the number of UTP molecules hydrolyzed per Prp2 and the spliceosome (2) was divided by the amount of Prp2-bound spliceosomes in the test tube. To obtain the number of UTP molecules hydrolyzed exclusively by Prp2 in the presence of the spliceosome, we first subtracted from (2) the spliceosome-dependent background UTP hydrolysis estimated on the basis of linear regression from the sample lacking Prp2. Subsequently, the obtained value was divided by the amount of Prp2 in the test tube.
UDP=[UDP]/([UTP]+[UDP]) = (1)
number of UTP molecules hydrolyzed in a test tube

(1) x (12 µl x100 pmol/µl) x (1000) = (2)
number of UTP hydrolyzed per Prp2 and the spliceosome

{(1)sample – (1)background by linear regression x time)/(12 x [Prp2]} = (3)
number of UTP hydrolyzed exclusively by Prp2 in the presence of the spliceosome

Protein purification

Proteins were full-length Prp2 and Cwc25, and a 35aa N-terminal truncation of Spp2. All proteins were fused to a hexahistidine tag (at the C terminus – Prp2 and Spp2, pET21a, or at N terminus – Cwc25, pETM11) for affinity purification. Protein purification was performed at 4°C. The Rosetta II *E. coli* pellets were suspended by vortexing in 5 ml of lysis buffer per 1 g cells and lysed with a fluidiser system at 80 psi 6 times (Microfluidics) in 50 mM HEPES-NaOH pH 7.5, 600 mM NaCl, 2 mM β-mercaptoethanol (β-ME), 20 mM imidazole and 10% (v/v) glycerol containing EDTA-free protease inhibitors (Roche). Insoluble material was removed by centrifugation for 40 min at 10,000 rpm in a SS-34 rotor (Sorvall). The supernatant was applied to HisTrap HP FF crude or GSTrap HP columns (GE Healthcare), pre-equilibrated with lysis buffer, using the Äkta Prime system (GE Healthcare). Contaminant proteins were removed by washing with 10 column volumes (CV) of lysis buffer followed by two CV of washing buffer (20 mM HEPES-NaOH pH 7.5, 2 M LiCl, 5% v/v glycerol) and further by two CV of lysis buffer, three CV of 5% of elution buffer (50 mM HEPES-NaOH pH 7.5, 600 mM NaCl, 2 mM β-ME, 250 mM imidazol and 10% (v/v) glycerol) followed by two CV of 10%, 15% and 20% of elution buffer. Bound proteins were eluted with a twenty CV gradient of 20-100% elution buffer. Elution of the proteins was monitored by SDS PAGE analysis of the obtained fractions. The elution buffers were exchanged by dialysis or with a desalting column 26/10 (GE Healthcare) against lysis buffer containing 20 mM imidazole and without reduced glutathione, respectively. The tags were cleaved with TEV protease. Proteins were then applied again to affinity columns, cleaved off tags, proteases, and purified proteins without tags were collected in the flow-through. Proteins were concentrated using Centricon concentrators (Millipore) and further purified by size exclusion chromatography (GE Healthcare) using buffers containing 20 mM HEPES-NaOH pH 7.5 and salt concentrations ranging from 100-300 mM NaCl (depending on the protein), 2 mM DTT and 5 % (v/v) glycerol. The purified proteins were analysed by 12.5% SDS PAGE. Fractions with 95 % or higher purity (estimated by Coomassie Blue staining) were aliquoted, quick frozen in liquid nitrogen and stored at – 80°C. The concentration of the proteins was determined by Bradford assay, and absorbance at 280 nm and calculation on the basis of extinction co-efficient.

Western blotting

Proteins were obtained from affinity-purified spliceosomes. Probing was performed with rabbit polyclonal antibodies against Prp2, GST-Spp2 (kind gifts from Ren-Jang Lin), Snu114 (Fabrizio et al. 1997), Cwc2 (Fourmann et al. 2013) and Prp19 (kind gift from Kum-Loong Boon). Secondary goat anti-rabbit antibodies (Jackson Immunoresearch, USA) were used at 1-to-50000 dilution. The blots were developed using an ECL kit (GE Healthcare) and exposed to film (Kodak).

RNA helicase assays

RNA unwinding assays were performed with 0.25 nM U4/U6 snRNA duplex and 50 nM Prp2 or Prp22 (produced in *E. coli* and purified) in buffer containing 20 mM HEPES-KOH pH 7.9, 50 mM KCl, 3 mM $MgCl_2$, 5 % (v/v) glycerol, 1 mM DTT, 100 ng/µl BSA. When indicated 4 µM Spp2 was present in the reaction. Unwinding reactions were pre-incubated for 10 min at 35° C and initiated by the addition of 3 mM ATP. At the indicated time points, 5 µl aliquots of the unwinding reaction were withdrawn and stopped with a buffer

containing 1 % SDS, 25 mM EDTA and 8 % glycerol. Samples were separated by 8 % non-denaturing PAGE, carried out in 0.5 X TBE at 4 °C, and visualized by autoradiography.

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