

## **Supplemental Material**

### **Dissection of the factor requirements for spliceosome disassembly and the elucidation of its dissociation products using a purified splicing system**

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## **Inventory of Supplemental Material**

**Supplemental Figures:**      Figures S1-S7

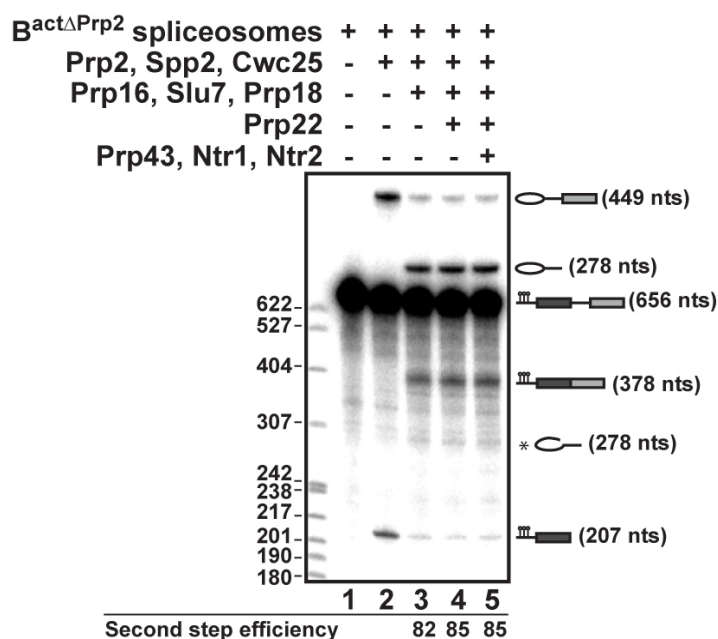
### **Supplemental Material and Methods:**

Spliceosome purification  
Cloning and expression strategy  
Northern blotting  
Immunoprecipitation  
Pulldown with biotinylated antisense RNA oligonucleotides  
Mass Spectrometry

### **Supplemental References**

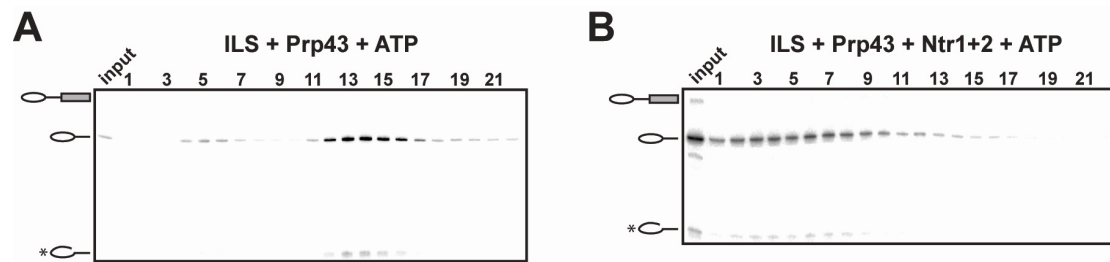
## Supplemental Material

## Supplemental Figure 1



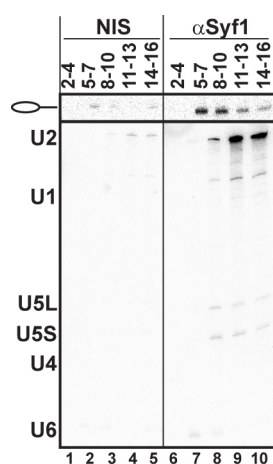
**Figure S1 (related to Figure 1). Spliceosomal factor requirements for step2 catalysis of the Actin7 substrate.** Kinetics of *in vitro* splicing catalyzed by affinity-purified spliceosomes assembled on the M3Act7 substrate. The B<sup>actΔPrp2</sup> complex (lane 1) was affinity-purified via a two step purification (see Methods). Fractions eluted from the amylose matrix were used for complementation assays with recombinant proteins at a 10-fold molar excess over the B<sup>actΔPrp2</sup> complex (10 fmol) as indicated above each lane (lanes 2-5). Reactions were incubated (45 min at 23°C; see Methods) in the presence of ATP. RNAs were extracted, separated by denaturing PAGE and visualized by autoradiography. Marker: ssDNA ladder. The position of pre-mRNA, splicing intermediates and products (and their lengths) are indicated on the right. Step 2 efficiency was calculated as follows: (mRNA+intron-lariat)/(mRNA+intron-lariat+5'exon+intron-3'exon) x100.

## Supplemental Figure 2



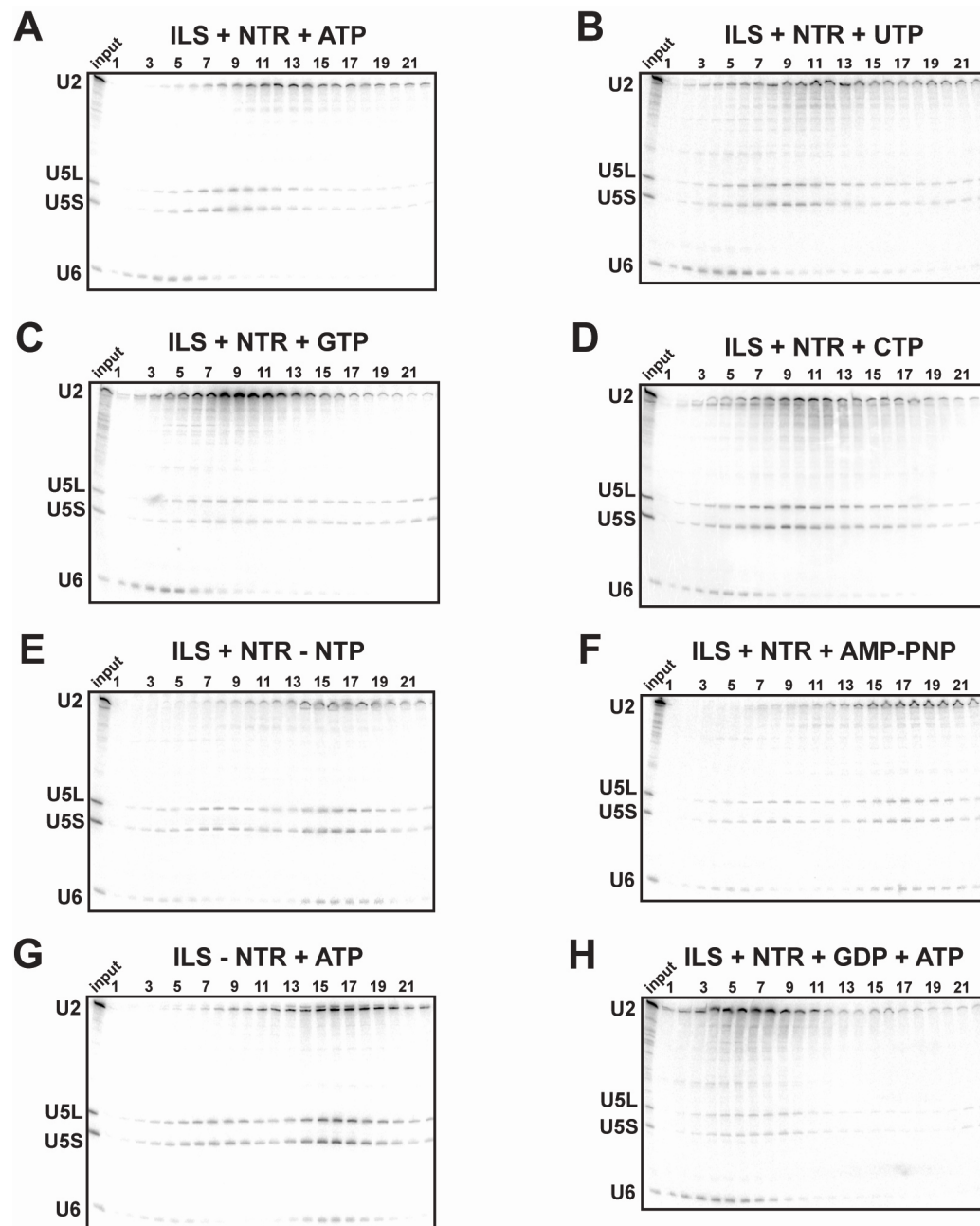
**Figure S2 (related to Figure 3). Dissociation of the intron-lariat spliceosome in solution requires Prp43, Ntr1 plus Ntr2 and ATP. A.** The ILS was incubated in solution with ATP and recombinant Prp43, or **B.** with ATP and recombinant Prp43 plus Ntr1 and Ntr2. The resulting complexes were separated on a 10 to 30% glycerol gradient. RNA from the gradient fractions shown was analyzed as in Figure S1. Input lanes indicate the samples before sedimentation.

## Supplemental Figure 3



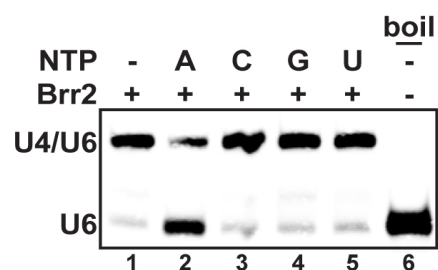
**Figure S3 (related to Figure 4). Syf1 (a NTC protein) associates with the U2 snRNA and the intron-lariat after dissociation of the ILS by the action of Prp43 plus Ntr1 and Ntr2.** The ILS was incubated with Prp43 plus Ntr1 and Ntr2 and separated on a 10 to 30% glycerol gradient. Every three gradient fractions from 2 to 16 were combined and immunoprecipitated with non-immunoserum (NIS) or with anti-Syf1 antibodies, respectively. RNA was analyzed as in Figure S1.

## Supplemental Figure 4



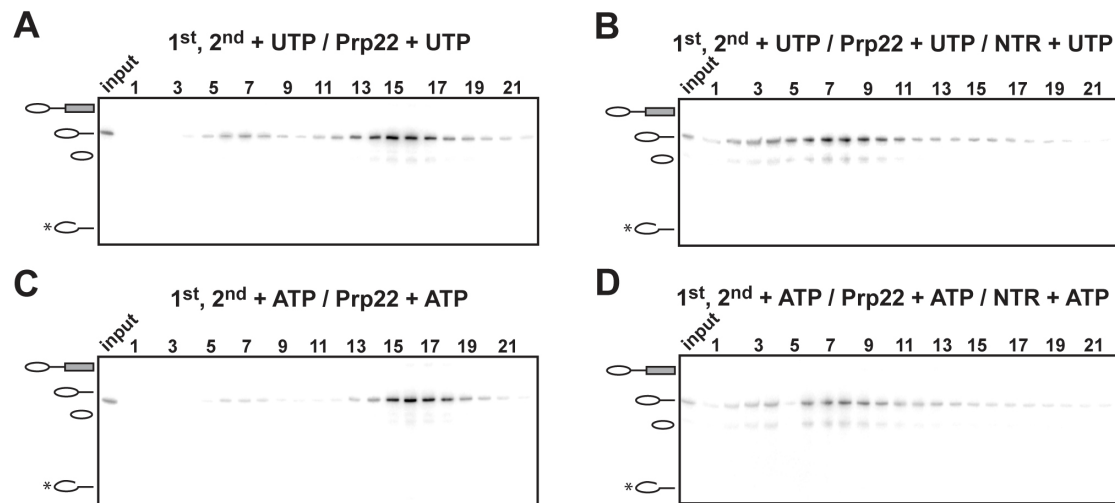
**Figure S4 (related to Figure 5). The ILS is dissociated by Prp43 plus Ntr1 and Ntr2 in the presence of each of the four rNTPs.** Post-catalytic spliceosomes bound to the matrix were reconstituted in the presence of ATP and recombinant Prp2, Spp2, Cwc25, Prp16, Slu7 and Prp18. Intron-lariat spliceosomes were eluted with Prp22 and ATP. Then, ATP was depleted by adding hexokinase and glucose. Prp43, Ntr1, Ntr2 were added along with the indicated rNTP (A-D), or without NTP (E), or with AMP-PNP (F). In further controls ATP was present, but without NTR proteins (G), or the NTR proteins were present along with a mixture of GDP and ATP (H). Each reaction was separated on a 10 to 30% glycerol gradient. RNA from the gradient fractions shown was recovered, separated by denaturing PAGE and visualized by Northern blot analysis. SnRNA identities are indicated on the left. Input lanes indicate the samples before sedimentation.

## Supplemental Figure 5



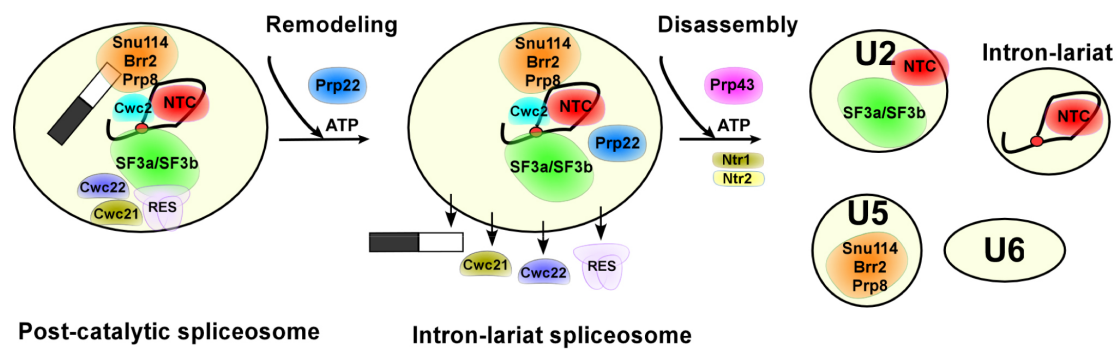
**Figure S5 (related to Figure 5). Brr2 is an ATP-specific RNA-helicase.** U4/U6 RNA duplex (U6 labeled) was incubated with recombinant Brr2 (produced in *S. cerevisiae*) in the presence of each individual NTP (lane 2 to 5) or in the absence of NTP (lane 1), analyzed by native 5% PAGE and visualized by autoradiography. The RNA duplex U4/U6 was boiled 5 min at 95°C (lane 6).

## Supplemental Figure 6



**Figure S6 (related to Figure 5). Both catalytic steps and the disassembly of the spliceosome can occur in the presence of solely UTP. A.**  $B^{act\Delta prp2}$  spliceosomes assembled on Actin wild type pre-mRNA were incubated with recombinant Prp2, Spp2, Cwc25 (1<sup>st</sup>) and Prp16, Slu7, Prp18 (2<sup>nd</sup>) and with UTP to obtain post-catalytic spliceosomes bound to the amylose matrix which were further incubated with Prp22 plus UTP, leading to the release of the ILS from the matrix. The ILS was separated on a 10 to 30% glycerol gradient. RNA from the gradient fractions shown was recovered, separated by denaturing PAGE and visualized by autoradiography. **B.** The ILS was incubated in solution with recombinant Prp43, Ntr1 and Ntr2 and with UTP and separated on a 10 to 30% glycerol gradient. RNA was analyzed as in panel A. **C.** and **D.** The experiments were performed in the same way as described in panel A. and B. respectively, except they were performed entirely in the presence of ATP. Input lanes indicate the samples before sedimentation.

## Supplemental Figure 7



**Figure S7. Summary of the action of Prp22 and Prp43 during disassembly of the spliceosome.** The addition of Prp22 and ATP to post-catalytic spliceosomes triggers the separation of mRNA and the intron-lariat spliceosome (ILS) as well as Cwc21, Cwc22 and the RES-complex. The addition of Prp43, ATP, Ntr1 and Ntr2 to the purified ILS promotes its disassembly into distinct components: the intron-lariat, U6 snRNA, a 20-25S U2 snRNP and an 18S U5 snRNP. The complete set of the U2 proteins and the U5 proteins Brr2, Snu114 and Prp8 are associated with U2 and U5 snRNAs, respectively. The NTC-complex associates primarily with both the released U2 snRNP and intron-lariat RNA. Prp43 activity alone is sufficient to dissociate the ILS.



## Supplemental Material and Methods

### Spliceosome purification

Yeast  $B^{\text{act}\Delta\text{Prp2}}$  spliceosomal complexes were purified essentially as described in (Warkocki et al. 2009) by using heat inactivated extracts from the yeast strain *prp2-1* (Yean and Lin 1991). Before splicing, M3Act7 pre-mRNA wild-type was incubated with a 30-fold molar excess of purified MS2-MBP fusion protein at 4°C for 30 min in 20 mM HEPES-KOH (pH 7.9). Typically, a 36 ml splicing reaction containing 1.8 nM of  $^{32}\text{P}$ -labeled M3Act pre-mRNA (specific activity 170–500 cpm/fmol) was performed in 62.5 mM  $\text{KPO}_4$  (pH 7.4), 3% PEG 8000, 2.5 mM  $\text{MgCl}_2$ , 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).  $B^{\text{act}\Delta\text{Prp2}}$  complexes were assembled by incubating at 23°C for 45 min. Six ml aliquots of the splicing reaction were loaded onto 36-ml linear 10–30% (v/v) glycerol gradients containing GK150 buffer (20 mM HEPES-KOH pH 7.9, 1.5 mM  $\text{MgCl}_2$ , 150 mM KCl). Gradients were centrifuged for 14 h at 23,000 rpm in a SureSpin 630 rotor (Thermo Scientific) and harvested manually in 1.5 ml 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 200–450  $\mu\text{l}$  of amylose matrix equilibrated with GK150 buffer. The matrix was washed two times with 10 ml GK150 buffer and two times with GK75 buffer (containing 75 mM instead of 150 mM KCl) supplemented with 5% glycerol, 0.01% NP40, 0.5 mM DTT and 0.5 mM PMSF.  $B^{\text{act}\Delta\text{Prp2}}$  spliceosomes bound to the amylose matrix were then transferred to a new tube for reconstitution and disassembly assays.

### Cloning and expression strategy

Prp2, Spp2, Cwc25, Prp16, Slu7, Prp18 and Prp22 were obtained as previously described (Warkocki et al. 2009). Prp43 was expressed from pET-m11 in *E. coli* Rosetta II (DE3) and Cwc23 from pGEX-6P1 *E. coli* Rosetta II (DE3). Both recombinant proteins were further purified as described (Warkocki et al. 2009). Ntr1 and Ntr2 were produced as follows: the genes encoding Ntr1 and Ntr2 were amplified from genomic DNA of *Saccharomyces cerevisiae* S288c using the oligonucleotides: Ntr1\_His\_SG\_for(5'AATGCATCACCATCACCATCACGAGGATTCGGACTCCAACACAGATAAAAAG3'), Ntr2\_SG\_for(5'AATGGCGATCAAGAAAAGAAATAAAATTAGGTTACCAAGT) Ntr1\_SG\_rev(5'TCCCGAGGTCAAGGGCCCCATAAATAAATTGGC3') Ntr2\_SG\_rev(5'TCCCGTTACCAATGAGCTTATTTATTAGGCTTGC3') and inserted into pENTRY-IBA10 vector (IBA GmbH, Germany). Using the StarGate system (IBA, Germany) DNA was transferred into two distinct pASG-IBA3 vectors. The sequence of Ntr1 and Ntr2 contained a C-terminal Strep-tag, and Ntr1 additionally contained N-terminal His-tag. They were transformed into *E. coli* Rosetta II (DE3) cells and were grown at 37°C in 2YT medium supplemented with ampicillin at 50  $\mu\text{g/ml}$  and chloramphenicol at 34  $\mu\text{g/ml}$ , until an OD600 of 0.9 was reached. The expression of His<sub>6</sub>-Ntr1-Strep and Ntr2-Strep was induced by addition of 200  $\mu\text{g}$

anhydrotetracycline per liter cell culture and the cells were then incubated at 16°C for 16 h. Cells were harvested by centrifugation and resuspended in buffer S [50 mM Tris (pH 7.5), 400 mM NaCl, 2 mM MgCl<sub>2</sub>]. Cells expressing Ntr2-Strep were lysed using a Microfluidizer (Microfluidics, USA) and then subjected to ultracentrifugation in order to separate the soluble and insoluble fractions. The Strep-tagged Ntr2 was isolated from the soluble fraction using a StrepTactin column (IBA GmbH, Germany) and eluted with Buffer S supplemented with 2.5 mM desthiobiotin. After a desalting step in buffer A [10 mM Tris (pH 7.5), 50 mM NaCl, 2 mM MgCl<sub>2</sub>] using a HiPrep Desalting Column (GE Healthcare, Germany), the sample was bound to a Source 30 Q anion exchange column (GE Healthcare, Germany) and eluted by a 200 ml gradient of 0-40% buffer B [100 mM Tris (pH 7.5), 600 mM NaCl, 2 mM MgCl<sub>2</sub>]. After the addition of purified Ntr2-Strep, cells expressing His<sub>6</sub>-Ntr1-Strep were lysed using a Microfluidizer (Microfluidics, USA), and subsequently ultracentrifuged. The complex of His<sub>6</sub>-Ntr1-Strep and Ntr2-Strep was isolated from the soluble fraction using a StrepTactin column (IBA GmbH, Germany) and eluted with Buffer S supplemented with 2.5 mM desthiobiotin. The eluates were applied to a HisTrap column (GE Healthcare, Germany) and eluted using Buffer S supplemented with 250 mM Imidazole.

### **Northern blotting.**

For Northern blots, RNA was separated by electrophoresis on an 8% polyacrylamide gel containing 8 M urea, transferred to nylon membrane (Pall Life Sciences) and fixed by UV irradiation. <sup>32</sup>P-labelled probes for U1, U2, U4, U5 and U6 snRNAs were generated from the corresponding yeast gene by the random priming method using a Prime-It kit (Stratagene). Blots were hybridized overnight at 42°C in buffer containing 25 mM NaPO<sub>4</sub> (pH 6.5), 6xSSC solution, 5x Dehnardt's solution, 0.5% SDS, 50% formamide and 0.1 mg/ml Salmon sperm DNA. Blots were washed twice at room temperature with 2xSSC solution containing 0.5% SDS, twice with 2xSSC solution plus 0.1% SDS and once with the latter solution at 50°C. Membranes were dried and bands were visualized using a phosphorimager.

### **Immunoprecipitation**

Protein A-Sepharose beads (GE Healthcare) and protein G-Sepharose beads (GE Healthcare) were prepared by washing 1 ml of the slurry 3 times with 1 ml of NET-150 buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% NP-40]. Beads were resuspended in 50 % NET-150 buffer and 60 µl was used per assay. 10 µl of antibody against Snul14, Prp19, Syf1, Cwc2, GFP (Cus1) or NIS (Non Immuno Serum) were added and incubated 1h at 4°C with end-over-end rotation. Beads were washed 3 times with 500 µl of NET-150 buffer and once with 500 µl of NET-75 buffer [50 mM Tris-HCl (pH 7.4), 75 mM NaCl, 0.05% NP-40], then 500 µl of NET-75 buffer together with typically, 200 to 300 µl of pooled fractions (2-4, 5-7, 8-10, 11-13 or 14-16) from a gradient of Prp43-dissociated ILS, were added to the beads and then incubated 2h at 4°C with end-over-end rotation. The beads were washed 3 times with 500 µl of NET-75, and subjected to proteinase K digestion. The RNAs were extracted by PCI, precipitated by ethanol, and then separated by 8% denaturing PAGE, followed by autoradiography and Northern Blot analysis.

### Pulldown with biotinylated antisense RNA oligonucleotides.

Streptavidin-coated Dynabeads (Dynal m270, Invitrogen) were prepared by washing 40 µl of the slurry 3 times with 500 µl of B&W buffer [10 mM Tris-HCl (pH7.4), 500 mM NaCl, 0.025% NP-40, 0.1 mM EDTA]. Beads were incubated end-over-end at room temperature for 15 min with 500 µl of B&W buffer containing 600 pmol biotinylated oligonucleotide complementary to U6 (+92/+109) U6BioTEG 5'-AAAACGAAAUAAAUCUCUUUG, or the intron-lariat (+13/+33) ILBioTEG 5'-GUUAAAUGGGAUGGUGCAAGC) or for the control beads without oligo, in the presence of 0.12 mg tRNA and 0.64 mg acetylated BSA. Beads were washed 3 times with 500 µl of B&W buffer, then 3 times with 500 µl of NET-75 buffer. Glycerol gradient fractions 2 to 6 for U6 and IL were added to the beads together with 500 µl of NET-75 buffer, and then incubated 2h at 4°C with end-over-end rotation. Beads were washed 3 times with 500 µl of NET-75 buffer, proteinase K digested, heated at 95°C 5 min and the liquid phase was collected. RNAs were recovered and analysed as described above.

### Mass Spectrometry

For mass spectrometric identification of proteins associated with the various gradient-fractionated spliceosomal complexes, the following fractions were pooled in low binding 1.5 ml reaction tubes (Eppendorf): the peak fractions of the B<sup>actΔPrp2</sup>, post-catalytic spliceosome and ILS; every two fractions of the gradient containing the ILS disassembled by Prp43 and, additionally, the five top gradient fractions of the post-catalytic spliceosome and ILS. 300 µl of the pooled fractions were supplemented with 40 µg glycoblue, 30 µl of 3M NaOAc (pH 5.2) and 1 ml of ethanol. After thorough mixing the solution was stored over night at -80 °C and then centrifuged for 30 min at 13,000 rpm, at 4°C in a microfuge. Subsequently, the pellet was washed with 70% ethanol, dried in a vacuum dryer and resuspended in 1x SDS PAGE loading buffer from Invitrogen and heated 10 min at 70°C. Proteins recovered from purified spliceosomal complexes (~80-120 fmol) or from the Prp43-disassembled ILS (same volume), were separated by SDS-PAGE on 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 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.

### References

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- Warkocki, Z., Odenwälder, P., Schmitzová, J., Platzmann, F., Stark, H., Urlaub, H., Ficner, R., Fabrizio, P., and Lührmann, R. 2009. Reconstitution of both steps of *Saccharomyces cerevisiae* splicing with purified spliceosomal components. *Nat Struct Mol Biol* **16**: 1237-1243.

Yean, S.L. and Lin, R.J. 1991. U4 small nuclear RNA dissociates from a yeast spliceosome and does not participate in the subsequent splicing reaction. *Mol Cell Biol* **11**: 5571-5577.