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Activation of Prp28 ATPase by phosphorylated Npl3 at a critical step of spliceosome remodeling

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Splicing, a key step in the eukaryotic gene-expression pathway, converts precursor messenger RNA (pre-mRNA) into mRNA by excising introns and ligating exons. This task is accomplished by the spliceosome, a macromolecular machine that must undergo sequential conformational changes to establish its active site. Each of these major changes requires a dedicated DExD/H-box ATPase, but how these enzymes are activated remain obscure. Here we show that Prp28, a yeast DEAD-box ATPase, transiently interacts with the conserved 5′ splice-site (5′SS) GU dinucleotide and makes splicing-dependent contacts with the U1 snRNP protein U1C, and U4/U6.U5 tri-snRNP proteins, Prp8, Brr2, and Snu114. We further show that Prp28’s ATPase activity is potentiated by the phosphorylated Npl3, but not the unphosphorylated Npl3, thus suggesting a strategy for regulating DExD/H-box ATPases. We propose that Npl3 is a functional counterpart of the metazoan-specific Prp28 N-terminal region, which can be phosphorylated and serves as an anchor to human spliceosome.
Splicing is an essential step in the eukaryotic gene-expression pathway that converts pre-mRNA into mRNA by excising introns and ligating exons. This task, which demands single-nucleotide precision, is accomplished by the spliceosome, a macromolecular machine made of five small nuclear RNAs (snRNAs) and numerous proteins. Unique among ribonucleoprotein (RNP) machines, the spliceosome is assembled anew upon each intron and undergoes sequential conformational changes to establish its active site. Each of these major changes requires a dedicated DExD/H-box ATPase, but how these enzymes are rigorously regulated to trigger specific conformational changes remain obscure.

Spliceosome assembly follows a canonical pathway in which the pre-mRNA 5'SS is first recognized by U1 snRNP to form the early (E) complex, which then progresses through complexes A (containing U1 and U2 snRNPs), B (U1, U2, and U4/U6.U5 tri-snRNP), B' (U4 snRNP departed; 1st chemical step activation), B* (5'SS cleavage and lariat intron formation), C (2nd step activation), C* (3'SS cleavage), and P (product complex with ligated exons and lariat intron). Release of the spliced mRNA creates the intron lariat spliceosome (ILS; containing U2/5' and lariat intron), which is disassembled to recycle the snRNPs and degrade the intron. At almost every step of the way, one or two DExD/H-box ATPases are dedicated to driving this pathway forward. For example, Prp28 acts on pre-B complex to promote a U1/U6 switch at the 5’S’ such that the U6 snRNA’s invariant ACAGAGA box can bind to 5’S’, whereupon Brr2 unwinds the U4/U6 RNA duplex within the B complex to liberate U4 snRNP.

We have previously shown that yeast Prp28 (hereafter Prp28) can be made dispensable upon weakening U1 snRNP/5'S’ interaction by specific alterations of U1 snRNP components, including U1C protein and U1 snRNA, suggesting these components are Prp28’s molecular targets. Prp28, however, differs from its human counterpart (hPrp28) by lacking a metazoan-specific N-terminal region and by existing in free form rather than being a part of U5-snRNP. Both purified Prp28 and hPrp28 exhibit negligible ATPase activity, raising the hypothesis that a cofactor, a spliceosomal environment, or both are required to turn on their activities in the right place at the right time. The present study seeks to investigate how Prp28 comes in contact with the spliceosome and how its ATPase activity can be potentiated within the spliceosome environment.

Results and discussion

Prp28 contacts several key spliceosomal proteins during splicing. To explore the preceding hypothesis, we first examined Prp28’s presence in various splicing complexes enriched by low ATP concentrations. For example, Prp28 acts on pre-B complex to promote a U1/U6 switch at the 5’S’ such that the U6 snRNA’s invariant ACAGAGA box can bind to 5’S’, whereupon Brr2 unwinds the U4/U6 RNA duplex within the B complex to liberate U4 snRNP.

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**Fig. 1 Prp28 contacts key proteins at the heart of spliceosome.**

(a) Low ATP traps a transient interaction of Prp28 with the spliceosome. Splicing reactions (lanes 1–5) were done in V5-tagged Prp28 extracts at 0, 0.02, 0.05, 0.2, or 2 mM ATP and a portion was subjected to immunoprecipitation without antibody (PAS; lanes 6–10) or with anti-V5 antibody (lanes 11–15). Relative loadings are 1:10 for splicing reactions alone (lanes 1–5) vs. immunoprecipitated reactions (lanes 6–15). Positions of pre-mRNA, splicing intermediates, and mRNA are indicated to the left. The experiment was repeated three times with similar results. Schematic diagram showing a BPA-marked Prp28 cross-linked to protein X in a spliceosome assembled on MS2 stem-loop-tagged pre-mRNA, which can be pulled down by MS2-maltose-binding protein-(MS2-MBP)-conjugated agarose beads. Thunderbolt, 365-nm UV irradiation. The experiments were repeated three times with similar results. b) Identification of the X proteins as Prp8, Br2, Snu114, and U1C by using anti-Prp8, anti-Br2, anti-Snu114, or anti-V5 (U1C-V5) antibody, respectively. The experiments were repeated three times with similar results. c) Schematic summary of the cross-linking data. Splicing complexes accumulated at various ATP concentrations are shown to the left. The changing amount of Prp28 associated with the spliceosome is depicted to the right. Source data are provided as a Source Data file.
The amount of the pre-mRNA co-precipitated by anti-Prp28 antibody peaked at 0.05 mM ATP (Fig. 1a, lane 13). A reciprocal experiment using M52 loop-tagged pre-mRNA for pulling down splicing complexes yielded the same result (Supplementary Fig. 1a, b). Together, these data suggest that ATP hydrolysis is required for releasing Prp28 from splicing complexes (Fig. 1e and Supplementary Fig. 1c).

To understand Prp28’s action within the protein-rich RNP environment of the spliceosome, we adapted a p-benzoylphenylalanine (BPA; a photoactivatable unnatural amino acid) based cross-linking method for detecting potentially transient protein-protein interactions. In this approach, BPA is site-specifically incorporated in vivo into Prp28 using an orthogonal pair of aminoacyl tRNA synthetase and suppressor tRNA (UAG). There were several considerations for choosing which amino-acid residues for BPA replacement. We first selected hydrophilic amino-acid residues that are not strictly conserved among DExD/H-box proteins, arguing that they are more likely to situate on Prp28’s surface and that their BPA replacements are less likely to significantly impact on Prp28’s function. We then used the structural information of Vasa, another DExD/H-box protein, to computationally model Prp28 structure and to guide our final selections. Among the 42 UAG (stop codon)-containing Prp28 alleles tested, 36 supported cell growth in BPA-containing media (Supplementary Data 4). We next prepared active splicing extracts (Supplementary Fig. 1d) from these engineered strains for performing BPA-mediated protein-protein cross-linking (Fig. 1b). Among the 12 extracts that yielded detectable Prp28-cross-linked products, we found that most of those BPA-replaced residues are located on the surface of RecA1 domain or in the N-terminal region of Prp28 that is not resolved in the crystal structure (Supplementary Fig. 2). Data from the Prp28-K27BPA, -K41BPA, -K82BPA, and -K136BPA experiments are shown in Fig. 1c. These cross-linked species are splicing-dependent because their appearances depend on the presence of pre-mRNA, intron, functional 5′ SS and branch site, and UV irradiation (Fig. 1c and Supplementary Fig. 3). The addition of RNase A after UV irradiation did not abolish the cross-linking signals, suggesting that Prp28 makes direct contacts with targeted proteins (Supplementary Fig. 3). We then scaled up the Prp28-K136BPA reaction for mass-spectrometry analysis (Supplementary Fig. 4), which led to the identification of Prp8, a very large splicing factor in the spliceosome (Supplementary Fig. 4 and Supplementary Data 5). Immunoblotting using anti-Prp8 and anti-Prp28 antibodies confirmed this finding (Supplementary Figs. 1e, f and 4c). On the basis of a combination of cross-linked species’ molecular sizes, Prp8’s location in published U4/U6.U5 tri-snRNP structures and Prp28’s known genetic interactions, we systematically interrogated other cross-linked proteins using a panel of antibodies. This effort identified two additional U5-snRNP proteins, Br2 and Snu114, as well as U1C (Fig. 1d and Supplementary Fig. 3). There are, however, several other cross-linked species (Supplementary Fig. 1g) that remain to be identified.

To gain insight into Prp28’s interactions with these four proteins during spliceosomal assembly, we performed cross-linking experiments by varying ATP concentrations ranging from 0.02 to 2 mM (Fig. 1c–e), which yielded several key observations. First, Prp28 indeed contacts U1C as predicted, but only at ATP concentrations below 2 mM ATP, consistent with U1 snRNP’s departure prior to the occurrence of splicing chemistry at 2 mM ATP. The observed Prp28/U1C interaction at 0.02 mM ATP may correspond to Prp28’s ATP-independent role in stabilizing early splicing complexes. Second, Prp28 can contact Prp8, Br2, and Snu114 (e.g., K136BPA), suggesting an intimate functional relationship with U5-snRNP, reminiscent of hPrp28’s role in facilitating U4/U6.U5 tri-snRNP integration into the spliceosome. Third, Prp28’s contacts with Br2 suggest that the two DExD/H-box ATPases physically communicate with each other for sequential removal of U1 snRNP and U4 snRNP. Two pre-B structures are now available. In the yeast structure, Prp28 cannot be precisely positioned. Our cross-linking data, though, appears to fit well with both the yeast and human pre-B structures with respect to the locations of Prp8 and Snu114. In the human Pre-B structure, the main body of hPrp28 appears to be distant from the location of Br2 (Supplementary Fig. 2c and Supplementary Software 1). Yet, the N-terminal domain of hPrp28, which is not conserved in yPrp28 (see below), threads through Prp8 and Snu14 moieties to reach Br2 (Supplementary Fig. 2c). In both human and yeast cases, Br2 is observed or predicted, respectively, to undergo a rotation and translocation in the pre-B-to-B transition. In this light, we note that in the published yeast Pre-B structure, one of the speculated locations of Prp28 is close to Br2, while the other location is not; whereas the human Br2, upon the predicted dramatic translocation, would also be physically close to the Prp28 main body. It is therefore tempting to speculate that this translocation may then place Br2 in the vicinity of Prp28 main body that is made up of the two RecA-like (i.e., the enzymatic) domains. At the moment, we cannot rule out that the cross-linking between Prp28 and Br2 can occur without translocation, because the contact can be through the N-terminal domain of Prp28. However, as the spliceosome complexes are highly dynamic during the splicing process, our biochemical approach might have captured a structurally dynamic, but so far undetected, intermediate state.

**Prp28’s contacts with key spliceosomal proteins are functional.**

To assess the physiological relevance of the Brr2 cross-linking data, we pairwise combined four cross-linking-compromised prp28 alleles, each containing a 10 amino-acid deletion flanking the K82, K27, K41, or K136 residues (prp28-K82Δ10, -K27Δ10, -K41Δ10, or -K136Δ10), with seven brr2 alleles. All double mutants exhibited synthetic-sick or -lethal phenotypes (Fig. 2a and Supplementary Fig. 5, Supplementary Table S1), suggesting that Prp28 and Brr2 are functionally interacting with each other. To analyze the physiological relevance of the observed cross-linking between Prp8 and Prp28, we performed a genome-wide genetic screen and uncovered prp8-501, a mutant allele of PRP8 that resulted in a lethal phenotype in the prp28-bypass background. In this finding prompted us to test a panel of 47 other prp8 alleles in a similar manner. Among them, 14 yielded lethality and one caused severe growth defect (Supplementary Data 6). The amino-acid changes inferred from all of these mutant alleles are localized to a ~300-amino-acid region (1574–1883) partially overlapping with the maintenance of 3′SS fidelity region (1385–1625) and the structurally defined RNase H domain (1833–1950). Because all of these prp8 alleles are recessive to the wild-type PRP8 allele, the simplest interpretation would be that Prp8 acts in concert with Prp28 to promote U1 snRNP’s departure. We note that genetic interaction between Prp28 and Snu114 was previously documented. We next examined how these crosslink-compromised prp28 alleles impact on spliceosome remodeling. Chromatin immunoprecipitation (ChIP) showed that U1 snRNP departure in the prp28-K82Δ10 strain is delayed during co-transcriptional splicing of the ACT1 pre-mRNA (Fig. 2b). Using an alternative in vitro strategy to monitor the U1 snRNP presence on assembled spliceosome, we found U1 snRNP accumulated at a higher level in K27Δ10, K41Δ10, K82Δ10, and K136Δ10 reactions than that in the wild-type reaction and, importantly, the addition of purified Prp28 rescued this defect (Supplementary Fig. 6b, c).
Prp28 also contacts the conserved GU dinucleotide at the 5′ splice site. To address whether Prp28 also makes specific contact with pre-mRNA, we irradiated the splicing reactions with UV (254 nm) to induce protein-RNA cross-linking. The cross-linked RNA was then immunoprecipitated under denaturing conditions. We found that Prp28′s contact with pre-mRNA peaked at 0.05 mM ATP (Fig.2c), consistent with the native immunoprecipitation data (Fig. 1a). Using oligonucleotide-directed RNase H cleavage followed by immunoprecipitation, we then assigned the contact point to a small region covered by oligonucleotides (oligos) D and E, the latter of which is downstream of 5′SS (Supplementary Fig. 7). To map the contact sites precisely, we use a downstream oligo for primer extension analysis. This analysis identified two strong reverse transcriptase stops. Sequencing ladder (lanes 1–4) allows assignment of the two stops to the high conserved GU dinucleotide (lane 8, triangles) that remain after denaturation (Denat) and immunoprecipitation with anti-Prp28 antibody. Source data are provided as a Source Data file.

Phosphorylated Npl3 potentiates Prp28′s ATPase activity. If Prp28 is indeed a canonical RNA helicase, a vexing question remains as to why it harbors only negligible ATPase activity. In our BPA experiments, we noticed Prp28-E326 BPA yielded a particularly strong cross-linked product (Supplementary Fig. 9a, b). Mass-spectrometry analysis indicated that this protein is likely Npl3 (Supplementary Data 7 and Supplementary Figs. 8, 9c), a multifunctional SR-like RNA-binding protein involved in splicing 29 and mRNA export30 and loosely associated with the purified U1 snRNP29,31. Immunoblotting analysis validated this cross-linked species as the phosphorylated form of Npl3 (p-Npl3) (Supplementary Figs. 9c and 10) 32. Genetic analysis shows that the prp28-E326Δ pneumonic pl3Δ double mutant exhibits a synthetic-let hal phenotype (Supplementary Fig. 9d). Immunoprecipitation analysis revealed that p-Npl3 associates with ACT1 transcript in an ATP-independent fashion throughout the course of the splicing reaction (Fig. 3a). ChIP analysis reveals that U1 snRNP′s recruitment was dramatically reduced in npl3Δ mutant (Fig. 3b and Supplementary Fig. 13), whereas in prp28-E326Δ3 mutant U1 snRNP′s departure is

**Fig. 2 Prp28 genetically interacts with Brx2 and crosslinks to pre-mRNA 5′SS in the pre-B complex.** a Growth phenotypes of yeast strains harboring various combinations of prp28-K82Δ10 and brx2 alleles spotted in a dilution series to YPD medium at 30 °C. Asterisk represents stop codon. b ChIP analysis revealed that U1 snRNPs′ departure is delayed in the prp28-K82Δ10 strain. Short lines in the top panel represent the amplicons used for monitoring U1 snRNP recruitment. In the bottom panel, the amplicon numbers are denoted on X axis. Y axis represents relative enrichment of U1 signal to that of the amplicon 1. Error bars are ± SEM; n = 3 biological repeats; ****P < 0.0001, unpaired two-tailed t-test. c Splicing reactions using radiolabeled ACT1 transcript were done in wild-type extracts at 0.02, 0.05, 0.2, or 2 mM ATP and subjected to 254-nm UV irradiation (even-numbered lanes 6–20). Immunoprecipitations after denaturation were done with anti-Prp28 (lanes 5–12) or with negative-control anti-V5 antibody (lanes 13–20). Relative loadings are 1:1000 for splicing reactions alone (lanes 1–4) vs. immunoprecipitated reactions (lanes 5–20). The experiment was repeated three times with similar results. d Primer extension revealed two strong reverse transcriptase stops. Sequencing ladder (lanes 1–4) allows assignment of the two stops to the high conserved GU dinucleotide (lane 8, triangles) that remain after denaturation (Denat) and immunoprecipitation with anti-Prp28 antibody. Source data are provided as a Source Data file.
Npl3 may correspond to the metazoan-specific N-terminal region of Prp28. The hPrp28 has an N-terminal region, consisting of an RS domain (residues 1–221) and an anchor domain (residues 286–356). A recent human Pre-B complex structure shows that this anchor domain, which is immediately N-terminal to the RecA1 domain, docks at the interface between Prp8 N terminus and Snu114 (Supplementary Fig. 2c). Intriguingly, although this anchor domain is well conserved in metazoans but it is completely absent in yeast (Supplementary Fig. 7). Significantly, the yeast Npl3 also contains an RS domain and a functional counterpart of the hPrp28 N-terminal region. The latter is especially important, because an inopportune ATP hydrolysis may negatively impact on the reported Prp28’s proofreading activity. Our finding that phosphorylation at S411 activates Npl3 to promote Prp28’s ATPase activity also raises a broader implication that a similar strategy may apply to other DEAD/H-box helicases for spatial and temporal regulation within splicing complexes.
a complex environment. Finally, our data show that, through two different regulatory routes, the function and behaviors of Prp28 are described in Supplementary Data 2 and 3, respectively.

Methods

Yeast strains. Strains used in this study are described in Supplementary Data 1.

Plasmids and oligonucleotides. Plasmids and oligonucleotides used in this study are described in Supplementary Data 2 and 3, respectively.

Antibodies and reagents. The following antibodies were used in this study: anti-Prp8 (ref.34; 1:2000), anti-Snu114 (ref.34; 1:2000), and anti-Brr2 antibodies. All antibodies and reagents are described in Supplementary Data 2 and 3, respectively.

Co-immunoprecipitation assay. Protein A Mag Sepharoses Xtra (10 µl) were pre-bound with 1 µl of polyclonal anti-p-Npl3 or with 2 µl of polyclonal anti-Npl3 complemented with 500-µl NET2 buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 0.05% NP40), which was then placed on a rotating platform for 1–2 h at 4 °C. After binding, the resin was washed three times with 1-mL ice-cold NET2 buffer and stand on the ice. p-Npl3 (1.2 µg) or Npl3 (1.2 µg) was mixed with Prp28 (1.2 µg) under splicing condition without RNA transcript, incubated at 35 °C for 30 min, and then mixed with IgG-bound beads at 4 °C for 1 h. Beads were washed with 1-mL cold NET2 three times. Bound proteins were separated by 4–20% gradient gel for probing with either anti-Prp28, anti-p-Npl3, or anti-Npl3 antibody. In the reciprocal experiments, anti-Prp28 antibody (2 µl) was bound to the beads.

Immunoblotting. Standard immunoblotting analysis was used to detect Prp28 and its covalently cross-linked products. In a typical Western procedure, anti-Prp28 polyclonal antibody (1:2000 dilution) and HRP-conjugated anti-rabbit IgG (1:10,000 or 1:40,000 dilution, depending on the HRP substrate used subsequently) were employed. Alternatively, mouse monoclonal anti-HA11 antibody (1:2000 dilution) and HRP-conjugated anti-mouse IgG (1:10,000 or 1:40,000 dilution) were used. PVDF blotting membranes were used. Immobilon Western Chemiluminescent HRP Substrate (Millipore), SuperSignal West Femto Maximum Sensitivity Substrate (Thermo), Polyvinylidene difluoride membrane (AmershamTM Hybond 0.45 mm PVDF, GE), Protein A-Sepharose (PA) was obtained from GE Healthcare Life Sciences, Protein A Mag Sepharose Xtra (10 µl) were pre-bound to 1 µl of polyclonal anti-Npl3 or with 2 µl of polyclonal anti-Npl3 complemented with 500-µl NET2 buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 0.05% NP40), which was then placed on a rotating platform for 1–2 h at 4 °C. After binding, the resin was washed three times with 1-mL ice-cold NET2 buffer and stood on the ice. p-Npl3 (1.2 µg) or Npl3 (1.2 µg) was mixed with Prp28 (1.2 µg) under splicing condition without RNA transcript, incubated at 35 °C for 30 min, and then mixed with IgG-bound beads at 4 °C for 1 h. Beads were washed with 1-mL cold NET2 three times. Bound proteins were separated by 4–20% gradient gel for probing with either anti-Prp28, anti-p-Npl3, or anti-Npl3 antibody. In the reciprocal experiments, anti-Prp28 antibody (2 µl) was bound to the beads.

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Splicing extracts, radioactively labeled RNA, and splicing assays. Yeast whole-cell extracts were prepared according to the liquid-nitrogen-grinding method.

Actin precursor RNA substrates were synthesized in vitro as runoff transcripts using SP6 RNA polymerase and labeled with [α-32P]UTP at 20 Ci/m mole, which was defined as 1× specific activity for the ACT1 pre-mRNA substrate. The 10× specific activity was defined as substrates labeled with [α-32P]UTP at 200 Ci/mole. The full-length transcript is purified from 5% polyacrylamide (acrylamide: bisacrylamide 29:1)/8 M urea) gels. Standard splicing assays were carried out for 30 min at 25 °C unless otherwise indicated.

Phosphorylated Npl3 add-back experiments. Two reactions (70 µl each), using either wild-type or npl3Δ splicing extract, were assembled and incubated. Aliquots (10 µl) were withdrawn at 5, 10, 15, 20, and 30 min and treated with proteinase K to stop the reaction for analyzing the splicing progression. Because at 5 min, a distinctive difference was observed between the two reactions, we, therefore, chose the 5-min incubation time for subsequent add-back experiments. To test whether p-Npl3 can rescue the delay of splicing in the npl3Δ reaction, purified p-Npl3 (see below) was added into the reaction mix without ATP and pre-incubated for 10 min. ATP was then added to start the splicing reaction for a total of 5 min.
Detection of splicesome-associated Prp28 by RNA pull-down experiment. Method for using MS2 loop-tagged transcript to pull down assembled splicesome has been presented in a step-by-step manner, except in this case, a splicing extract containing 5-tagged Prp28 was used and neither BPA incorporation nor UV irradiation was employed. Prp28 was detected by an anti-V5 antibody.

Mass-spectrometry analysis. After silver-staining the gel, regions corresponding to the locations of the cross-linked product in both lanes (with or without UV irradiation) were excised and subjected to standard mass-spectrometry methods (LC-MS/MS). The purified recombinant protein (1.2 μg) of p-Npl3 was resolved by SDS-PAGE and Coomassie-Blue stained, then the single band resolved and analyzed for phosphorylation position by MS analysis (Supplementary Fig 1G).

The phosphopeptide MS analysis presented was done from the same batch of phospho-tagged Npl3 that was used in the ATP hydrolysis assay and in the in vitro co-affinity studies. Samples were detected by Nano-LC-NanoESI-MS/MS on an Orbitrap fusion mass spectrometer (ThermoFisher Scientific, San Jose, CA) equipped with Easy-nLC 1200 system (Thermo, San Jose, CA, US) and ESI-spray source (Thermo, San Jose, CA, US). The digestion solution was injected (5 μl) at 1 μl/min with easy column C18, 0.075 mm × 150 mm, ID 3 μm (Thermo Scientific). Chromatographic separation was using 0.1% formic acid in water as mobile phase A and 0.1% acetic acid in 80% acetonitrile as mobile phase B operated at 300 nl/min. The gradient employed was 2% buffer B at 2 min to 40% buffer B at 40 min. Full-scan MS condition: mass range m/z: 35–1800, SCN: 6e5, with lock mass m/z 365, and maximum injection time of 50 ms. The MS/MS was run in top speed mode with 3-s fragmentation source with lock mass m/z 365. The digestion solution was injected (5 μl) was 0.5 Da (IT) or 0.01 Da (FT). Variable modifications (protein N-terminal) and modifications of phospho (STY) for the splicing reaction containing 2 nM of radiolabeled (10× specific activity; 200 Ci/mmol) [3H]-adenosine. Splicing reactions (25 μl each) were carried out in a 12-well culture plate with 10× specific activity of ACT1 pre-mRNA substrate (2 nM) for 30 min, quenched on ice, and then UV-irradiated (254 nm; 0.8 J/cm²) on ice in a CL-1000 Ultraviolet Crosslinker (UVP). The UV light source was placed ~5 cm above the sample. UV-irradiated samples were denatured in 1% SDS (w/v), 1% Triton X-100 (v/v), and 100 mM DTT, heated in boiling water for 90 s, then placed at 25 °C. The denatured samples were filtered in a microfuge at 16,363 × g for 1 min at room temperature. The supernatants were removed to new tubes. Before immunoprecipitation, the concentration of denaturants was reduced by 10-fold dilution with NET−2–300 (300 mM NaCl, 50 mM Tris-HCl [pH 7.4], 0.05% NP40) and RNA added at a final concentration of 200 μg/ml. The mixtures were incubated with 25 μl of antibody-conjugated protein A Sepharose (PAR) for 1.5 h at 4 °C. The precipitates were washed with 1 ml of cold NET−2–300 five times and 1 ml of room temperature NET−2 (150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 0.05% NP40) twice. After proteinase K treatment (1 mg/ml for 30 °C/30 min), RNAs were extracted for urea poly-acrylamide (8%) gel electrophoresis.

UV cross-linking of Prp28 to pre-mRNA. Splicing reactions (25 μl each) were carried out in a 12-well culture plate with 10× specific activity of ACT1 pre-mRNA substrate (2 nM) for 30 min, quenched on ice, and then UV-irradiated (254 nm; 0.8 J/cm²) on ice at 1000 Ultraviolet Crosslinker (UVP). The UV light source was placed ~5 cm above the sample. UV-irradiated samples were denatured in 1% SDS (w/v), 1% Triton X-100 (v/v), and 100 mM DTT, heated in boiling water for 90 s, then placed at 25 °C. The denatured samples were filtered in a microfuge at 16,363 × g for 1 min at room temperature. The supernatants were removed to new tubes. Before immunoprecipitation, the concentration of denaturants was reduced by 10-fold dilution with NET−2–300 (300 mM NaCl, 50 mM Tris-HCl [pH 7.4], 0.05% NP40) and RNA added at a final concentration of 200 μg/ml. The mixtures were incubated with 25 μl of antibody-conjugated protein A Sepharose (PAR) for 1.5 h at 4 °C. The precipitates were washed with 1 ml of cold NET−2–300 five times and 1 ml of room temperature NET−2 (150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 0.05% NP40) twice. After proteinase K treatment (1 mg/ml for 30 °C/30 min), RNAs were extracted for urea poly-acrylamide (8%) gel electrophoresis.

Chromatin immunoprecipitation (ChIP) analysis. ChIP analysis was done exactly as described²⁵, except that anti-V5 (UIC-V5) and anti-Prp40 were used to precipitate the chromatin-bound U1 snRNP. Briefly, 50 ml of ~0.8 OD600 yeast cells were treated with formaldehyde (2%). Next, cells were resuspended in lysis buffer and disrupted by bead beater. The insoluble cross-linked fraction was subjected to sonication for shearing the DNA fragment to ~300 bp. Subsequently, immunoprecipitation of U1-V5 or Prp40 was performed by anti-V5-PAS or anti-Prp50-PAS beads at 4 °C overnight. After extensive washes, crosslinks were reversed and DNA was extracted. Primer pairs (Supplementary Table S3) covering the ACT1 gene were used in qPCR experiments. All data represent an average of three independent experiments. Error bars are ±SEM. The enrichment of the U1C-bound or Prp40–Prp8 association nor UV irradiation was employed. Prp28 was detected by an anti-V5 antibody.
formamide dye. An equal volume (5 μl) from all those samples was then analyzed by denaturing urea gel (8%) electrophoresis. To map Prp28 WT and Prp28 AAAD proteins, two plasmids were created under the pRSET-A (Addgene) vector backbone carrying wild-type yeast Prp28 WT and its mutant Prp28 AAAD protein—coding regions in frame with N-terminal 6-His tag. E. coli Rosetta cells (Stratagene) were employed and protein expression was induced by adding 0.3 mM IPTG at 37°C for 3 h. Cells were collected by centrifugation and resuspended in a buffer containing 50 mM KH2PO4, 50 mM Na2HPO4 (pH 7.4), 500 mM NaCl, 3 mM β-mercaptoethanol, 7.5% (v/v) glycerol, supplemented with complete EDTA-free protease inhibitor cocktail (Roche). The cells were harvested and lysed with a microfluidizer and then the lysates were centrifuged (Beckman Coulter) at 34,957 × g for 40 min at 4°C. Then, the supernatant was mixed with Ni-Sepharose 6 Superflow affinity resin (Qiagen) for 30 min at 4°C followed by a stepwise wash with buffer containing 25 mM and 50 mM imidazole. After that, Prp28 WT and Prp28 AAAD proteins were eluted with buffer containing 250 mM imidazole. Eluted proteins were pooled and dialyzed for 16 h at 4°C in a buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM dithiothreitol, and 7.5% (v/v) glycerol. After dialysis, protein samples were loaded into MonoS 5/50 GL column (GE Healthcare Life Sciences), equilibrated in a buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM dithiothreitol, and 7.5% (v/v) glycerol, and bound proteins were eluted with a linear gradient of NaCl concentration from 0 to 1 M. Protein-containing fractions were collected onto a HiLoad 200 10/300 GL column (GE Healthcare Life Sciences) equilibrated in a buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM dithiothreitol, and 7.5% (v/v) glycerol. Proteins fractionated (0.5 ml) from HiLoad 200 10/300 GL column (GE Healthcare Life Sciences) were collected and analyzed by Coomassie-stained SDS-PAGE (12%) and mass for spectrometry analysis. These two proteins were concentrated to 0.4 mg/ml in 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 50 mM KCl, 20% glycerol, 0.5 mM DTT and stored at −80°C.

Purification of recombinant n-Npl3 proteins. We typically grew 101 of _E. coli_ culture for purifying recombinant n-Npl3 or Npl3 protein. Phosphorylation of Npl3 was carried out in Rosetta cells by co-expressing Npl3 and Sky1. Full-length DNA fragments of Npl3 and Sky1 protein-coding genes were PCR-amplified (0.5 ml) from HiLoad 200 10/300 GL column (GE Healthcare Life Sciences), equilibrated in a buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM dithiothreitol and 7.5% (v/v) glycerol. Proteins fractionated (0.5 ml) from HiLoad 200 10/300 GL column (GE Healthcare Life Sciences) were collected and analyzed by Coomassie-stained SDS-PAGE (12%) and mass for spectrometry analysis. These two proteins were concentrated to 0.4 mg/ml in 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 50 mM KCl, 20% glycerol, 0.5 mM DTT and stored at −80°C.

ATPase assay. Before assembling the reaction, the n-Npl3 or Npl3 was pre-mixed with Prp28 and incubated on ice for 30 min. The reaction (10 μl) contains 0.57 μM Prp28 WT or Prp28 AAAD protein or AAAD protein and 500 nM dATP and 1 mM DTT, together with 0.2 μl of [α-32P]ATP (3,000 Ci/mmole; Perkin-Elmer), 40 μM cold ATP, 40 μM Tris-HCl (pH 8), 40 mM KCl, 1.6 mM MgCl2, 0.08 mg/ml bovine serum albumin, and 0.8 mM dithiothreitol. The reaction was incubated at 37°C for 2.5 h and stopped with 4 μl of 100 mM EDTA. 2 μl of the reaction was spotted onto a TLC (analytic plate), which was developed with a buffer containing 0.375 M potassium phosphate at pH 3.5 for 1.5 h to separate ADP and ATP. The amount of ATP hydrolyzed was quantified using a PhosphorImager on a Typhoon scanner (GE Healthcare).

Statistical analysis and software. The cocrystal structure of the labeled human Prp28 WT (Protein Data Bank 6QX9) was displayed by PyMol 2.3.2 (Supplementary Fig. 2c and Supplementary Software 1). Gels and TLC (PEI) plate were exposed to GE Storage Phosphor Screen (GE Healthcare Life Sciences). RNA bands and the amount of ATP hydrolyzed were quantified with Typhoon FLA9000 (GE Healthcare Life Sciences) and analyzed with ImageQuant TL 7.0 (GE Health-care Life Sciences). All statistics were performed using GraphPad Prism 8 (Graphpad). Data are presented as the mean ± SEM. Statistical significance between the two groups was determined with an unpaired two-tailed Student’s t-test. ChiP experiments were carried out with n = 3 biological repeats (Figs. 2b, 3b, c, and Supplementary Fig. 1c), and n = 6 biological repeats (Fig. 3f). Prp28 ATPase assay was carried out with n = 3 biological repeats (Fig. 3e). Quantification of retained U1 snRNP from splicingosome was carried out (Supplementary Fig. 6b), n = 3 biological repeats.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The raw mass-spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifiers PXD024492 (Prp28-K136BPA)44; PXD024493 (Prp28-E326BPA)45; PXD024494 (p-Npl3)46. The uncropped gel or blot figures and original data underlying Figs. 1–3 and Supplementary Figs. 1–3 are provided as a Source Data file.47 All data supporting the findings of this study are available from the corresponding authors upon reasonable request. Source data are provided with this paper.

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