A conformational switch in PRP8 mediates metal ion coordination that promotes pre-mRNA exon ligation

Matthew J Schellenberg1,4,5, Tao Wu1,5, Dustin B Ritchie1,4, Sebastian Fica2, Jonathan P Staley2, Karim A Atta1, Paul LaPointe3 & Andrew M MacMillan1

Splicing of pre-mRNAs in eukaryotes is catalyzed by the spliceosome, a large RNA-protein metalloenzyme. The catalytic center of the spliceosome involves a structure comprising the U2 and U6 snRNAs and includes a metal bound by U6 snRNA. The precise architecture of the spliceosome active site, however, and the question of whether it includes protein components, remains unresolved. A wealth of evidence places the protein PRP8 at the heart of the spliceosome through assembly and catalysis. Here we provide evidence that the RNase H domain of PRP8 undergoes a conformational switch between the two steps of splicing, rationalizing yeast prp8 alleles that promote either the first or second step. We also show that this switch unmasks a metal-binding site involved in the second step. Together, these data establish that PRP8 is a metalloprotein that promotes exon ligation within the spliceosome.

The spliceosome is a large RNP particle consisting of the U1, U2 and U4–U5–U6 small nuclear ribonucleoprotein particles (snRNPs), with each containing a unique snRNA and associated proteins bound to the pre-mRNA substrate1–2. Spliceosome assembly is a complex process that includes formation of a U2–U6 snRNA structure that has been shown to be essential for catalysis of splicing transesterifications4. Although pre-mRNA splicing is therefore believed to be intrinsically RNA catalyzed, there is considerable evidence suggesting an intimate interaction between spliceosomal proteins and the active site of the spliceosome. The highly conserved U5 snRNP protein PRP8 has been shown to be essential for catalysis of splicing transesterifications4. PRP8 rearrangement unmasks a conserved metal-binding site We and others recently solved the X-ray structures of the human and yeast domain IV of PRP8 (refs. 14–16; hereafter referred to as PRP8 RH). This domain is C terminal to reverse transcriptase–derived and endonuclease-like domains and N terminal to a Jab-PMN domain within PRP8 (ref. 17; Supplementary Fig. 1a,b). Here, we report our detailed structural analysis of PRP8 RH and a panel of domain mutants, highlighting a conformational change that unmasks a Mg2+-binding site that promotes splicing (Fig. 1, Table 1 and Supplementary Tables 1 and 2). Our previously described crystals of human PRP8 RH (amino acids (aa) 1769–1990) included two monomers in the asymmetric unit. A salient feature was the presence of an N-terminal RNase H domain that included a 17-aa insertion (aa 1787–1803) between two adjacent β-strands of the fold. Within the crystal, in what we describe as the closed conformation, this insertion forms a two-stranded antiparallel β-hairpin, whereas in the second, open conformation this hairpin is disrupted to form a displaced loop (Supplementary Note 1). The translation of this loop back by ~45° pulls aa 1782–1784 to extend the 5’ splice site and U6 snRNA during spliceosome assembly5–7. Mutant prp8 alleles in yeast also suggest interactions of this factor with both substrate and snRNA catalytic structures in the spliceosome8–12. Therefore, the catalytic center of the spliceosome may include protein as well as RNA components13.

A large number of mutant prp8 alleles related to spliceosome activation and possibly catalysis have been characterized in yeast, which suggests a central role for this factor in both processes, including a proposed conformational change between the two steps of splicing8,13. The clustering of these alleles, along with cross-linking data, suggested the presence of a core domain within PRP8 that could be part of or interact with the spliceosomal catalytic machinery12,13. To test these hypotheses, we examined the role of this domain of PRP8 within the spliceosome by using a combination of X-ray structure determination and characterization of mutant prp8 alleles in yeast and yeast splicing extracts.

RESULTS

PRP8 rearrangement unmasks a conserved metal-binding site

We and others recently solved the X-ray structures of the human and yeast domain IV of PRP8 (refs. 14–16; hereafter referred to as PRP8 RH). This domain is C terminal to reverse transcriptase–derived and endonuclease-like domains and N terminal to a Jab-PMN domain within PRP8 (ref. 17; Supplementary Fig. 1a,b). Here, we report our detailed structural analysis of PRP8 RH and a panel of domain mutants, highlighting a conformational change that unmasks a Mg2+-binding site that promotes splicing (Fig. 1, Table 1 and Supplementary Tables 1 and 2). Our previously described crystals of human PRP8 RH (amino acids (aa) 1769–1990) included two monomers in the asymmetric unit. A salient feature was the presence of an N-terminal RNase H domain that included a 17-aa insertion (aa 1787–1803) between two adjacent β-strands of the fold. Within the crystal, in what we describe as the closed conformation, this insertion forms a two-stranded antiparallel β-hairpin, whereas in the second, open conformation this hairpin is disrupted to form a displaced loop (Supplementary Note 1). The translation of this loop back by ~45° pulls aa 1782–1784 to extend the 5’ splice site and U6 snRNA during spliceosome assembly5–7. Mutant prp8 alleles in yeast also suggest interactions of this factor with both substrate and snRNA catalytic structures in the spliceosome8–12. Therefore, the catalytic center of the spliceosome may include protein as well as RNA components13.

A large number of mutant prp8 alleles related to spliceosome activation and possibly catalysis have been characterized in yeast, which suggests a central role for this factor in both processes, including a proposed conformational change between the two steps of splicing8,13. The clustering of these alleles, along with cross-linking data, suggested the presence of a core domain within PRP8 that could be part of or interact with the spliceosomal catalytic machinery12,13. To test these hypotheses, we examined the role of this domain of PRP8 within the spliceosome by using a combination of X-ray structure determination and characterization of mutant prp8 alleles in yeast and yeast splicing extracts.

Received 29 January; accepted 12 March; published online 19 May 2013; doi:10.1038/nsmb.2556
The possibility that a protein-bound metal could participate in catalysis of PRP8 associated with the spliceosomal catalytic core raised the question of MgCl₂ during crystallization, we did not observe metal ion coordination. This site is completely conserved in all PRP8 orthologs Mg²⁺ coordination by PRP8 in the spliceosome. These observations led us to investigate the possible coordination from Co²⁺-soaked crystals (R1865A PRP8, confirming this observation by anomalous scattering. The open, but not the closed, conformation of both wild-type and crystals. However, under these conditions we noted coordination of the PRP8 RH domain closed (yellow) and open (cyan) conformations, detailing the displacement of Thr1783 to allow Mg²⁺ coordination. The metal ion bound in the open conformation is not shown for clarity.

Coordination of a metal ion to the canonical RNase H site of PRP8 involves a two-metal mechanism in which divalent magnesium ions, bound at adjacent sites separated by ~4 Å, promote hydrolysis by activation of a water nucleophile combined with transition-state stabilization. Thus, the presence of the RNase H domain in a portion of PRP8 associated with the spliceosomal catalytic core raised the possibility that a protein-bound metal could participate in catalysis of splicing. Inspection of the PRP8 RH structure showed that one of two canonical RNase H metal-binding sites is present: two aspartates (Asp1781 and Asp1782) with coordinating side chains spatially conserved with respect to Mg²⁺-coordinating residues within the RNase H fold. This site is completely conserved in all PRP8 orthologs (Supplementary Fig. 1c). In our initial studies, despite the presence of MgCl₂ during crystallization, we did not observe metal ion coordination at this site, a result that we attribute to the high ionic strength (2.5 M NaCl) of the crystallization conditions. Additionally, the side chain of the conserved arginine residue Arg1865 positioned over this site possibly has a shielding role. In the work reported here, we crystallized and solved the 1.4-Å structure of PRP8 RH and the 1.15-Å structure of the R1865A mutant under low ionic strength but in the presence of divalent magnesium. Again, we observed both closed and open conformations of PRP8 RH in the asymmetric units of the crystals. However, under these conditions we noted coordination of a single magnesium ion at the conserved metal-binding site within the open, but not the closed, conformation of both wild-type and R1865A PRP8, confirming this observation by anomalous scattering from Co²⁺-soaked crystals (Fig. 1b, Supplementary Fig. 2a and Table 1). The electron density suggests that the Mg²⁺ ion is present at partial occupancy in the wild-type structure but is consistent with full occupancy in the R1865A structure. The overall features of the R1865A crystals and the resulting structure are markedly similar to those of wild type. A higher metal occupancy is consistent with a shielding role of the arginine side chain in the wild-type protein, which we propose could be relieved by rearrangement associated with RNA binding within the spliceosome.

Coordination of a metal ion to the canonical RNase H site of PRP8 includes inner-sphere contact with the side chain of Asp1781 and outer-sphere coordination through five ordered waters to the carboxylate of Asp1782, the carboxyls of Asp1782 and Leu1891, the amide carbonyl of Gln1894 and the hydroxyl of Thr1864. One consequence of the structural rearrangement involved in the closed-to-open transition is that Asp1782 moves closer to Asp1781 and Thr1863 is displaced by ~4 Å upward (Fig. 1c and Supplementary Movie 1). In the closed conformation, it is this positioning of Thr1783 that essentially blocks metal binding at this site; the substantial local reorganization of the PRP8 RH structure is essential to allow Mg²⁺ coordination. These observations led us to investigate the possible roles of both the conformational change and conformation-dependent Mg²⁺ coordination by PRP8 in the spliceosome.

**Table 1 Data collection and refinement statistics**

| Data collection<sup>a</sup> | WT, 300 mM MgCl₂ | R1865A, 300 mM MgCl₂ |
|-----------------------------|------------------|---------------------|
| Space group                 | P2₁2₁2₁          | P2₁2₁2₁             |
| Resolution (Å)              | 76.03, 78.02, 93.81 | 76.20, 78.04, 94.06 |
| R<sub>sym</sub>              | 0.069 (0.525)    | 0.038 (0.482)       |
| I / σI                      | 18.1 (2.3)       | 26.7 (2.5)          |
| Completeness (%)            | 99.5 (99.5)      | 98.9 (96.6)         |
| Redundancy                  | 4.9              | 4.3                 |
| Refinement                  | 60–1.40          | 60–1.15             |
| No. reflections             | 109,740          | 186,162             |
| R<sub>work</sub> / R<sub>free</sub> | 0.136 / 0.177   | 0.140 / 0.160      |
| No. atoms                   | 3,357            | 3,884               |
| Protein                     | 19               | 26                  |
| Ligand/ion                  | 709              | 735                 |
| Water                       | 34.6             | 35.9                |
| R.m.s. deviations           | 0.011            | 0.012               |
| Bond lengths (Å)            | 1.44             | 1.51                |

Each data set was collected from a single crystal.

<sup>a</sup>Values in parentheses correspond to the highest-resolution shell (10% of reflections).

WT, wild type.
second-step allele suppresses a defect in the second but enhances a
defect in the first step of splicing. Notably, the largest proportion of
first- or second-step suppressor alleles associated with the PRP8 RH
domain map to the two-stranded β-hairpin or rearranged loop
(Supplementary Fig. 1d). This suggests that the disposition of this
structure is critical to the function of this domain within the spliceo-
some. Because of this, we endeavored to determine whether first-
or second-step phenotypes associated with specific alleles could be
rationalized by reference to either of the two conformations observed
crystallographically.

We first generated mutant yeast strains to characterize the effect
on splicing of a series of mutations within the PRP8 RH-β-hairpin,
comparing these to both wild-type and known strong first-step
(E1960K) and second-step (V1870N; Supplementary Fig. 1d and
Supplementary Table 2) alleles. We assayed growth by using a
copper-resistance reporter system in which the branch-site A
of the ACT1-CUP1 intron was replaced with G (BSG), a change
that inhibits the second step of splicing. Three mutant alleles
(E1960K, V1860D and T1872E) caused less resistance to copper,
consistent with a first-step phenotype, whereas three alleles
(V1870N, T1861P and V1862Y) caused more resistance to copper,
consistent with a second-step phenotype (Fig. 2a). We directly
assayed steady-state pre-mRNA splicing efficiencies of the reporter
gene in these mutant PRP8 backgrounds, comparing splicing of the
BSG mutant ACT1-CUP1 intron with an RNA containing the
branch-site C mutation (BSC), known to inhibit both steps of splic-
ing. Consistent with the results of the growth assay, E1960K,
V1860D and T1872E acted as first-step alleles, enhancing the first
step in splicing of the BSG introns (Fig. 2b).

We next crystallized and solved the X-ray
structures of four human PRP8 RH mutants
corresponding to the yeast alleles mapping to
the β-hairpin under conditions such that
the asymmetric unit featured both the
closed and open conformations (Fig. 3a,b,
Supplementary Fig. 3a,b and Supplementary
Table 1). In all cases, a bound metal ion
was observed at the canonical RNase H site in
the open conformation but was occluded in the
closed conformation.

We solved the structure of human PRP8 RH mutants representing
the V1788D PRP8 (yeast V1860D) and T1800E (yeast T1872E) first-
step alleles; these revealed that both mutations introduce an addi-
tional hydrogen-bonding interaction within the closed conformation.
Replacement of the Val1788 side chain with an aspartate results in a
formation of a hydrogen bond to Tyr1786 at the base of the β-hairpin,
whereas mutation of the β-branched side chain of Thr1800 to a glutama-
mate allows sufficient freedom for the backbone peptide to rotate
and also form a water-mediated hydrogen bond to Tyr1786 (Fig. 3a).
These extra hydrogen bonds effectively anchor the β-strand in position,
stabilizing the closed conformation.

We also determined the structures of two mutants I1790Y (yeast
V1862Y) and T1789P (yeast T1861P) shown to be second-step sup-
pressors on the basis of splicing of the ACT1-CUP1 reporters in yeast.
We solved the 1.55-Å structure of the human I1790Y PRP8 RH mutant
and observed the formation of a new hydrogen-bonding interaction
between Tyr1790 and Asn1797 in the open conformation, in which
the aromatic side chain of Tyr1790 spans the displaced loop (Fig. 3b).
In the closed conformation, the side chains of Asn1797 and Tyr1790
project from opposite faces in the β-hairpin region. Therefore, the
isoleucine-to-tyrosine mutation appears to stabilize the open con-
formation relative to the closed.

We crystallized and solved the 1.65-Å structure of the human
T1789P and the 1.95-Å structure of the T1789P R1865A double mutant
(Supplementary Note 3). The T1789P mutation lies in the β-hairpin,
and as expected this structure is disrupted in the closed conformation
but with no discernible effect on the open conformation (Fig. 3b).
We performed a crystallographic mixing experiment wherein equal concentrations of R1865A and the T1789P
R1865A double mutant were mixed in the crystallization conditions
Figure 3  
PRP8 mutant alleles favor distinct conformations within the PRP8 RH domain. Shown are superpositions of X-ray structures of wild-type (gray) with mutant closed (yellow) or mutant open (cyan) conformers. (a) Top, detail showing formation of an additional hydrogen-bonding interaction to Tyr1786 in the closed conformation of the V1789E structure. Bottom, detail showing formation of a hydrogen bond across the loop with Asn1797 in the open conformation of the I1790Y structure. (b) Top, detail showing disruption of the β-hairpin of the closed conformation in the T1800E structure. (c) X-ray structural analysis from a crystallographic mixing experiment with T1789P R1865A and R1865A proteins. Shown are models for structures of the R1865A (gray) and T1789P R1865A protein for the closed (yellow, left) and open (cyan, right) conformation. A 2Fo − Fc map contoured at 1σ, calculated from the data set of the T1789P R1865A–R1865A mixture crystal, is shown in dark blue.

(Supplementary Note 3). The 1.8-Å structure of the resulting crystal shows that only wild-type protein, which contains a threonine at position 1789, is visible in the β-hairpin closed conformation. In contrast, within the loop of the open conformation a mixture of threonine and proline is observed at this position (Fig. 3c). This suggests that the T1861P mutation favors the open conformation by destabilizing the closed conformation.

The results of these structural studies complement and rationalize the analysis of mutant prp8 alleles in yeast. They suggest that two first-step alleles (V1860D and T1872E) result in the stabilization of the closed conformation of PRP8 RH and imply that this conformation is associated with the first step of splicing. The second-step allele V1862Y stabilizes the open conformation, whereas T1861P appears to destabilize the closed and thus favor the open conformation. The structure of the second-step allele L1798N (yeast V1870N) can also be modeled to suggest a hydrogen-bonding interaction stabilizing the open conformation (Supplementary Note 4 and Supplementary Fig. 3c). These observations suggest that the open and metal-binding conformation of PRP8 RH is associated with the second step of splicing.

PRP8 metal binding is coupled to the second step of splicing
To examine the role of metal binding by PRP8 in the context of the spliceosome, we created a panel of five yeast mutants, modifying the identity of the inner-sphere ligand at position 1853 (Fig. 4a). These are all (with the exception of D1853E) predicted to impair, to varying degrees, magnesium coordination in the open conformation (Supplementary Fig. 2b and Supplementary Note 5). Moderate effects on growth were observed for a number of these mutants, although both D1853S and D1853N exhibited a strong temperature-sensitive phenotype at 37 °C. The D1853C mutant was impaired in growth at both 16 °C and 30 °C and did not grow at 37 °C. Mutation of Asp1853 to a hydrophobic residue (leucine or alanine), which would be incapable of either directly or indirectly coordinating Mg2+, or to a positively charged residue (lysine) was lethal (Supplementary Note 6).

We prepared splicing extracts derived from all of the Asp1853 mutant strains and examined their splicing activity in vitro by using an ACT1 pre-mRNA substrate (Fig. 4b). Overall, we observed a general trend wherein residues that should impair Mg2+-ion binding impair splicing.

Figure 4  
PRP8 Asp1853 mutations selectively impair the second step of splicing. (a) Phenotypic analysis of PRP8 Asp1853 mutations. Photographs show serial dilutions of cells from strains containing the indicated PRP8 alleles, spotted onto YPD medium and incubated for 4 d at 16 °C, 2 d at 30 °C or 3 d at 37 °C. (b) Top, denaturing PAGE analysis of in vitro splicing of 32P-labeled ACT1 pre-mRNA in extracts prepared from strains containing the indicated Asp1853 PRP8 mutants. The positions of the mRNA, pre-mRNA, intron-lariat and exon-lariat intermediate in the gel are indicated. Bottom, quantification of first- and second-step efficiencies. In, input. (c) D1853C rescue by the second-step allele V1862Y. Growth of a dilution series of the D1853C V1862Y yeast strain at 16 °C, 30 °C and 37 °C. (d) Denaturing PAGE analysis comparing in vitro splicing of 32P-labeled actin pre-mRNA substrate in extracts prepared from wild-type, D1853C and D1853C V1862Y yeast strains. Error bars, s.d. from three independent experiments.
substantially, with the second step more strongly affected than the first. We confirmed that mutation of Asp1853 results in a specific second-step defect by preparing extracts from pseudodiploid strains and specifically immunoprecipitating epitope-tagged mutant PRP8 containing spliceosomes from splicing reactions (Supplementary Note 7 and Supplementary Fig. 4a–c). In all of these studies, the efficiency of the second step of splicing corresponds with the ability of an amino acid at position 1853 to coordinate or correctly position a Mg\(^{2+}\) ion at the canonical site (aspartate and glutamate > histidine; asparagine > serine >> cysteine).

Because D1853C was the most impaired viable mutant tested, we further examined the relationship of this mutation to the second-step conformation. We created a double mutant on the basis of the V1862Y second-step allele (D1853C V1862Y) and examined growth and splicing activity of extracts prepared from the mutant. Notably, V1862Y rescues the severe growth defect at 37 °C. Furthermore, as predicted for a second-step allele, V1862Y partially rescues the second-step splicing defect of D1853C in an in vitro assay with actin pre-mRNA (Fig. 4c,d).

One difficulty with mechanistic investigation of the second step of splicing is that a rate-determining conformational change may mask effects on the chemical step of splicing. An elegant approach to more directly observing the second step of splicing is the separation of the two steps in a bimolecular exon ligation reaction developed previously\(^{22,23}\). A pre-mRNA lacking a 3’ splice site undergoes the first but not the second step of splicing; subsequent addition of a 3’ splice site containing oligonucleotide in trans results in a bimolecular ligation that effectively separates the second chemical step from the first (Fig. 5a). This approach has been used successfully to investigate the effect, otherwise masked by a presumptive conformational change in the spliceosome, of substrate modifications on second-step splicing chemistry\(^{24}\).

We established a bimolecular exon ligation reaction in yeast extracts to directly observe the effects of Asp1853 PRP8 mutants on the second step of splicing (Fig. 5a, Supplementary Figs. 5 and 6 and Supplementary Note 8). We first synthesized a spliceosome substrate derived from the ACT1 pre-mRNA containing the 5’ exon, 5’ splice site, branch region and 40 downstream nucleotides (nt) but lacking a 3’ splice site. Additionally, we synthesized a 25-nt 3’ RNA substrate that included the last 5 nt of the intron (UUUAG) representing the 3’ splice site followed by 20 nt of 3’ exon sequence (Fig. 5b). Upon incubation of the 5’ substrate in wild-type extract, we observed an efficient first step of splicing but no second step. Chasing this reaction with the 3’ substrate resulted in bimolecular exon ligation. We then examined the splicing activity of extracts prepared from the panel of Asp1853 mutants by using this assay. All of these mutants carried out the first step of splicing with modest impairment corresponding to that observed for the full-length ACT1-substrate splicing assay (Supplementary Fig. 5a,b). An impairment of the exon ligation step matching the second-step defect observed with the full-length splicing substrate was also observed (Fig. 5c and Supplementary Fig. 5c). Notably, the efficiency of bimolecular exon ligation in extracts prepared from the D1853C mutant was ~3% of that observed in wild-type extract. This represents an order of magnitude further impairment with respect to the efficiency of exon ligation observed with the intact pre-mRNA. This observation suggests that the bimolecular exon ligation has revealed an effect of the D1853C mutation on a rate-limiting step and that this step involves the second transesterification reaction of splicing.

**DISCUSSION**

In the studies described here, we have observed crystallographically two distinct conformations of the PRP8 RH domain, one of which permits coordination of Mg\(^{2+}\) at a canonical and conserved metal-binding site (Fig. 6a). The structures of proteins corresponding to known suppressor alleles suggest both that the closed conformation of PRP8 corresponds to a first-step state and that the metal-binding open conformation corresponds to a second-step state. This is consistent with a model whereby PRP8 manifests conformations favoring or facilitating either the first or second transesterification steps of splicing (Fig. 6b).

There is a rich and complex provenance to the prp8 suppressor alleles designated here and earlier\(^{8,15}\) as first or second step. For example, the V1870N second-step allele was isolated as a suppressor of the branch-site A-to-G mutation, but it also acts as a suppressor of 5’- and 3’-splice-site mutations\(^{8}\). This is reminiscent of previous observations with respect to a number of other prp8 suppressor alleles\(^{25}\), which were interpreted to suggest a role for PRP8 at the catalytic core of the spliceosome. A number of prp8 alleles relate to the transition from the U4–U6 snRNA base-pairing that precedes formation of the U2–U6
structure within the mature spliceosome. In a screen for suppressors of the effects of hyperstabilization of the U4–U6 duplex, one study identified alleles of PRP8 containing V1860D, T1861P or V1862Y that were able to suppress this cold-sensitive (c) defect. It was noted in several studies that prp8 alleles tend to suppress mutations either of the pre-mRNA splice sites or within spliceosomal components such as U4 snRNA. Although some prp8 alleles can suppress both types of defects, the general trend of segregation into two groups of prp8 alleles in the context of the results reported here suggests that the PRP8 RH domain plays a part in earlier assembly-precatalycytic steps within the spliceosome, which is distinct from the dynamics reported here. Beyond all of this is the identification of first- and second-step alleles outside of the PRP8 RH domain, which suggests a larger, concerted conformational change extending beyond that described for the PRP8 RH domain here.

The observation that PRP8 RH metal binding is only observed in the open conformation, combined with our structural and functional analyses of first- and second-step alleles, associates this state with the second step of splicing. Mutagenesis of the inner-sphere ligand Asp1853 combined with the effects of mutations at this site on splicing establish the importance of divalent-metal binding by PRP8 RH for the second transesterification step of splicing.

The recent structural analysis of a large fragment of yeast PRP8 in complex with the U5 snRNP–assembly factor Aar2 suggests the presence of a large cavity in PRP8, which on the basis of both mutational and cross-linking data has been proposed to encompass the active site of the spliceosome. This cavity is large enough to contain the second-step alleles outside of the PRP8 RH domain, which also contains the PRP8 sequence that cross-links to the 5′ splice site, faces into this cavity directly across from a disordered loop that cross-links to the pre-mRNA branch region before the second step of splicing. Thus, the conformational switch from closed to open conformations directly presents a metal ion, shown here to promote the second catalytic step of splicing, to the active site cavity of the spliceosome (Fig. 6a–d). These observations are consistent with a structural or catalytic role for the PRP8-bound metal ion in the spliceosomal active site for the second step of splicing and suggest that at this step the active site could contain both protein and RNA components. Notably, the C-terminal region of Aar2 lies across the PRP8 RH surface and stabilizes the β-hairpin structure by extending the β-sheet (aa 345–353). Mg2+-coordinating residues in RH are shown in red.

Figure 6 PRP8 RH undergoes a conformational switch to present a functionally important metal ion to the spliceosome. (a) Structures of closed and open conformations of PRP8 RH. Red, residues corresponding to first- and second-step alleles used in this analysis; red, Mg2+-coordinating ligands; purple, Mg2+; yellow, Thr1783; green, peptide that cross-links to the 5′ splice site. (b) Diagram showing first- and second-step alleles used in this analysis. (c) Modeling of the PRP8 RH domain closed (yellow) and open (blue) conformations on the overall PRP8 structure shown in gray. The Aar2 assembly-factor structure has been removed for clarity. (d) The switch from closed (yellow) to open (blue) conformation positions a Mg2+ ion that promotes the second transesterification reaction in the active site of the spliceosome. Closed and open structures of the PRP8 RH domain are modeled on the overall PRP8 structure shown in gray17. The Mg2+ ion is highlighted in purple. Ends of a disordered loop that cross-links to the branch region of the pre-mRNA before the second step are shown in green. (e) Aar2 sequesters the spliceosome active site cavity. The U5 snRNP assembly factor Aar2 (cyan) blocks the surface of the PRP8 RH domain (yellow), partially filling the active site cavity (aa 320–344, depicted as spheres) and stabilizes the PRP8 RH β-hairpin by extending the β-sheet (aa 345–353). Mg2+-coordinating residues in RH are shown in red.
The conformational change described here represents a specific switch mechanism between the two steps of splicing. Further, that this switch is coupled to the binding of a functionally important metal ion implicates it as a key regulatory mechanism in promotion of the second chemical step of splicing.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Coordinates and structure factors for the PRP8 RH domain x-ray structures have been deposited in the Protein Data Bank under accession codes 4JK7, 4JK8, 4JK9, 4JKA, 4JKB, 4JKC, 4JKD, 4JKE, 4JKF, 4JKG and 4JKH.

Note: Supplementary information is available in the online version of the paper.

ACKNOWLEDGMENTS

This work was supported by an Operating grant to A.M.M. from the Canadian Institutes of Health Research (CIHR) and a US National Institutes of Health grant to J.P.S. and J. Piccirilli (R01GM088656). We would like to thank M. Fris and M. Schultz for helpful advice, J. Beggs (University of Edinburgh, Edinburgh, UK) and C. Guthrie (University of California, San Francisco, San Francisco, California, USA) for providing yeast strains and plasmids and D. Brow (University of Wisconsin–Madison, Madison, Wisconsin, USA) for providing the PRP8 V1862Y plasmid. Research described in this paper was performed at the Advanced Light Source (Berkeley, California, USA; supported by the US Department of Energy under contract no. DE-AC02-05CH11231) and the Canadian Light Source (supported by the Natural Sciences and Engineering Research Council of Canada, the National Research Council Canada, CIHR, the Province of Saskatchewan, Western Economic Diversification Canada and the University of Saskatchewan).

AUTHOR CONTRIBUTIONS

M.J.S., T.W., D.B.R. and A.M.M. designed the study; M.J.S. created mutant yeast strains, crystallized protein, collected X-ray diffraction data, solved the structures and performed in vivo and in vitro assays including development of the bimolecular exon ligation; T.W. created mutant PRP8 yeast strains, crystallized protein, collected X-ray diffraction data and carried out in vivo and in vitro assays; K.A.A. purified and crystallized proteins; S.F. and J.P.S. independently designed and tested mutants and critically analyzed data; D.B.R. designed, crystallized and analyzed purified proteins; S.B.R. designed, crystallized and analyzed mutants; P.L. provided technical expertise and helped design experiments in yeast; M.J.S., T.W. and A.M.M. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Jurica, M.S. & Moore, M.J. Pre-mRNA splicing: awash in a sea of proteins. Mol. Cell 12, 5–14 (2003).
2. Rappaport, J., Ryder, U., Lamond, A.I. & Mann, M. Large-scale proteomic analysis of the human spliceosome. Genome Res. 12, 1231–1245 (2002).
3. Zhou, Z., Licklider, L.J., Gygi, S.P. & Reed, R. Comprehensive proteomic analysis of the human spliceosome. Nature 419, 182–185 (2002).
4. Madhani, H.D. & Guthrie, C. A novel base-pairing interaction between U2 and U6 small nuclear RNA contributes to catalysis in the spliceosome. Nature 408, 881–884 (2000).
5. Adams, P.L., Staley, M.R., Kosek, A.B., Wang, J. & Strobel, S.A. Crystal structure of a self-splicing group I intron with both exons. Nature 430, 45–50 (2004).
6. Iken, S.L., Wuessnich, G., Termini, J. & Lin, R.J. Metal-ion coordination by U6 small nuclear RNA contributes to catalysis in the spliceosome. Nature 408, 881–884 (2000).
7. Kooidingh, P., Novak, T., Piccirilli, J.A. & Staley, J.P. The DEAH box ATPases Prp16 and Prp43 cooperate to proofread 5′ splice site cleavage during pre-mRNA splicing. Mol. Cell 39, 385–395 (2010).
8. Sontheimer, E.J., Sun, S. & Piccirilli, J.A. Metal ion catalysis during splicing of premessenger RNA. Nature 388, 801–805 (1997).
9. Kasital, D.G., Cornelscu, G., McManus, C.J., Brow, D.A. & Butcher, S.E. U2-U6 RNA folding reveals a group II intron-like domain and a four-helix junction. Nat. Struct. Mol. Biol. 11, 1237–1242 (2004).
10. Toor, N., Keating, K.S., Taylor, S.D. & Pyle, A.M. Crystal structure of a self-spliced group II intron. Science 320, 77–82 (2008).
11. Steitz, T.A. & Steitz, J.A. A general two-metal-ion mechanism for catalytic RNA. Proc. Natl. Acad. Sci. USA 90, 6498–6502 (1993).
12. Schmidt, B.H., Burgin, A.B., Deweese, J.E., Osheroff, N. & Berger, J.M. A novel and unified two-metal mechanism for DNA cleavage by type II and IA topoisomerases. Nature 465, 641–644 (2010).
13. Nakamura, T., Zhao, Y., Yamagata, Y., Hua, Y.J. & Yang, W. Watching DNA polymerase η make a phosphodiester bond. Nature 487, 196–201 (2012).
14. Le Du, M.H. et al. Artificial evolution of an enzyme active site: structural studies of three highly active mutants of Escherichia coli alkaline phosphatase. J. Mol. Biol. 316, 941–953 (2002).
15. Kim, E.E. & Wyckoff, H.W. Reaction mechanism of alkaline phosphatase based on crystal structures: two-metal ion catalysis. J. Mol. Biol. 218, 449–464 (1991).
16. Romier, C., Dominguez, R., Lahm, A., Dahl, O. & Suck, D. Recognition of single-stranded DNA by nuclease P1: high resolution crystal structures of complexes with substrate analogs. Proteins 32, 414–424 (1998).
17. Garcia, E.D. et al. DNA apurinic-apyrimidinic site binding and excision by endonuclease IV. Nat. Struct. Mol. Biol. 15, 515–522 (2008).
18. Shan, S., Yoshida, A., Sun, S., Piccirilli, J.A. & Herschlag, D. Three metal ions at the active site of the Tetrahymena group I ribozyme. Proc. Natl. Acad. Sci. USA 96, 12299–12304 (1999).

© 2013 Nature America, Inc. All rights reserved.
ONLINE METHODS

Protein expression, purification and crystallization. hPRP8(1796–1990) protein was grown and purified as previously described44; crystals were grown at 23 °C by using the hanging-drop vapor-diffusion technique. Crystals of native protein were grown by mixing 1 μl of 10 mg ml−1 protein solution (10 mM Tris, pH 8.0, 0.1 mM EDTA, 5 mM DTT and 0.02% NaN3) with 1 μl of precipitant (2.5 M NaCl, 100 mM Tris, pH 7.0 and 200 mM MgCl2 or 10–14% [v/v] PEG 4000, 100 mM Tris, pH 7.5, and 300 mM MgCl2). Crystals were transferred into cryoprotectant (25% [v/v] PEG 4000, 16% [v/v] glycerol and 100 mM Tris, pH 7.5) with the indicated concentration of divalent metal as a chloride salt and frozen in liquid nitrogen for data collection14.

Wild-type and mutant PRP16, PRP22 and PRP43 proteins were grown and purified as previously described42,43.

Data collection and processing. Data were collected at beamline 8.3.1 of the Advanced Light Source, Lawrence Berkeley National Laboratory, at beamline CMCF-1 at the Canadian Light Source, University of Saskatchewan, Saskatoon, and on an R-axis rotating Cu-anoide X-ray source. For the wild-type and R1865A PRP8 RH crystals grown in 300 mM MgCl2, a single-wavelength experiment was performed at 0.97949 Å at a temperature of 105 K. Data were processed and scaled by using HKL44; Friedel pairs were not merged when anomalous scattering maps were required. Anomalous scattering maps were calculated by using the CCP4 suite45.

Model building and refinement. The structures were solved by molecular replacement by using Refmac46 to refine, with PDB 3ENB as a starting model. Water molecules were added to the model by using Refmac46. Iterative cycles of refinement in Refmac against the merged data set and manual model building in Coot were used to complete and refine the models46. Ramachandran statistics for the final refined models of wild type and R1865A are: wild type grown in 300 mM MgCl2, 98.4% favored, 0.0% outliers; R1865A grown in 300 mM MgCl2, 98.2% favored, 0.0% outliers. All additional Ramachandran statistics are given in Supplementary Table 1.

Creation of mutant S. cerevisiae strains and splicing extracts. Mutant prp8 genes were created by gap repair of plasmid pJU186 (ref. 49) containing a putative fragment into strain JDY8.06 (α ura3 his3 A1-3-122, ade2 his3-A1, trpl-289, prp8::LEU2, prp169 [PRP8, URA3, CEN, ARS]; gift from J. Beggs, University of Edinburgh, UK) containing wild-type PRP8 on a counterselectable URA3-marked plasmid47. After transformation with pJU186, cells were selected by growth at 30 °C on SDC plates lacking histidine and leucine. Transformants were streaked on medium lacking histidine and leucine and containing 5-fluoroorotic acid (5-FOA) to select for cells lacking the URA3 plasmid47. Cells from a single colony that survived on 5-FOA plates were grown in medium lacking histidine, and total DNA was extracted by using a DNeasy kit (Qiagen). All mutant PRP8 strains were verified by sequencing. Splicing extracts were prepared as described elsewhere48.

For creation of copper-resistant strains, the ACT1-CUP1 plasmid containing the wild-type sequence, BSC or BSG mutations was co-transformed along with pJU186 containing wild-type or mutant PRP8 into yJU75 (ref. 11) (MATa, ade2 cup1−1: ura3 his3 leu2 lys2 prp8::LYS2 trpl-1, prp169 [PRP8 URA3 CEN ARS]). Transformants were streaked on medium lacking histidine and leucine and containing 5-FOA to select for cells lacking the URA3 plasmid. Cells surviving on 5-FOA plates were grown in medium lacking histidine, and total DNA was extracted by using a DNeasy kit (Qiagen). All mutant PRP8 strains were verified by sequencing.

For the creation of pseudodiplotid strains, PRP8 was knocked out with a LEU2-marked fragment in yPsF662, the knockout confirmed by PCR and viability rescued with PRP8 on URA3-marked plasmid generating yPsF1481. To obtain pseudodiplotid strains, yPsF1481 was transformed with wild-type or mutant PRP8 on a HIS3 plasmid, and transformants that retained both the wild-type URA3 and the HIS3 PRP8 plasmids were selected on uracil-histidine dropout medium. Mutant PRP8 plasmids were generated from the wild-type plasmid pJU204 (PRP8-HA, HIS3, gift from C. Guthrie) by QuikChange PCR (Agilent) and verified by sequencing.

Growth assays. For spot-test analysis, yeast strains were inoculated in YPD medium (1% yeast extract, 2% peptone and 2% dextrose) and grown overnight at 30 °C with shaking. The next day, the OD at 600 nm was determined, and 10-μl serial dilutions from 1 × 106 to 1.6 × 102 cells ml−1 were spotted onto YPD plates. Plates were photographed after 2 d at 30 °C, 3 d at 37 °C and 4 d at 16 °C. For the copper growth assay, cultures containing the BSC ACT1-CUP1 and PRP8 mutant plasmids were grown overnight in SDC-leucine-histidine medium, diluted to 100 μl and spotted onto SDC-leucine-histidine agar plates containing the indicated concentrations of CuSO4 (ref. 32). Plates were photographed after 3 d at 30 °C.

Primer extension. RNA was extracted by using hot phenol53. Cultures containing the ACT1-CUP1 and PRP8 mutant plasmids were grown overnight in SDC-leucine-histidine medium, diluted to 5 ml (A600 = 0.2) and then grown in SDC-leucine-histidine medium for an additional 6 h to A600 = 1.0. The cells were spun down and resuspended in 400 μl of AE buffer with 10% SDS (50 mM NaOAc and 10 mM EDTA, pH 5.0). Hot phenol/AE solution (400 μl) was immediately added and incubated at 65 °C for 30 min. After centrifugation at 21,000g, the upper phase was transferred into clean tubes, and the hot phenol/AE treatment was repeated. The upper phase was extracted three or four times with phenol/ chloroform/isooamyl alcohol and chloroform followed by ethanol precipitation. The pellet was resuspended in 10 μl ddH2O.

Primer extensions were carried out by using the YAC6 primer complementarily to exon 2 of ACT1 (ref. 54). The primer was end-labeled with [γ-32P]ATP (3,000 Ci/mmol, PerkinElmer). Primer extensions were performed by using the RevertAid H minus First Strand cDNA Synthesis Kit (Fermentas). First, 12 μl of the mixture (1 μg total RNA and 2 pmol labeled primer) was heated to 70 °C for 10 min and slowly cooled down to 40 °C. The reaction was chilled on ice, supplemented to 20 μl (4 μl reaction buffer, 1 U RNase inhibitor, 1 mM dNTP mix and 10 U reverse transcriptase), then incubated for 5 min at 37 °C and 55 min at 42 °C. The reaction was terminated, and the RNA was degraded with 0.5 M NaOH at 70 °C. Extension products were extracted with phenol/chloroform/isoamyl alcohol and chloroform, then ethanol-precipitated. The pellet was resuspended, separated in gels containing 7% polyacrylamide and 8 M urea and visualized by autoradiography.

Construction of RNA substrates. The ACT1 and UBC4 pre-mRNAs were made by in vitro transcription with T7 RNA polymerase and [α-32P]ATP by using a PCR template amplified from the appropriate plasmid. The 25-μl reaction (40 mM Tris, pH 8.0, 6 mM MgCl2, 10 mM NaCl, 2 mM spermidine, 0.5 mM CTP, 0.5 mM GTP, 0.5 mM UTP, 60 mM ATP; 1.32 μM [α-32P]ATP (PerkinElmer), 5 ng DNA template, 10 mM DTT and 1 μl T7 RNA polymerase) was incubated at 37 °C for 4 h. The reaction was then separated on a PAGE gel containing 7% 19:1 acrylamide/bisacrylamide and 8 M urea.

The 5′ ACT1 substrate used in the exon ligation assay was synthesized by in vitro transcription to yield the following sequence: 5′-CUUUAAUGAUUU UACCGCUUAUCGUUUUUCUCUCCAGAACUGCAAUUUACUGCA AAUAACAGUGUAGUUUUCUGACGCUUGCCGACACCCUUUUAUACGUAAGAAGAUUGACGCGUCCCAAUUGCGAGAAGAGUCUCUUUAUUCCUUUUUUCUUACUUUUUUACUCCCCUAAUACUGUAGAAGUUACGAGAAUACGAGAAGGUCGAGCAUACGAGAAGGUAAGGGGUGGACUUGAAGGAAAAAAGAAAAACUGAGAAUAUUUGUAGAGGAAG UACGAGAUAGAAGUUACUUCUUUUUUCUGACGCGUUGACGCGUAC UACACUGAGUUGUCACUUCUUGUUGCGUUAUAUAGUUAUU-3′. The 5′ ACT1 substrate used in the exon ligation assay was synthesized (IDT) with the following sequence: 5′-UUUAGAACCGUCGUCCUUUGUAAUU-3′.

Splicing assays. Splicing reactions (10 μl) were performed as described49 in reactions containing 2 mM ATP, 2.5 mM MgCl2, 3% [v/v] PEG 8000, 60 mM potassium phosphate, pH 7.0, 20 mM KCl, 8 mM HEPEs, 8% [v/v] glycerol, 80 μM EDTA, 0.2 mM DTT and 1 nM pre-mRNA. Reactions were quenched with 10 μl of stop solution (1 mg ml−1 proteinase K, 50 mM EDTA and 1% SDS) and then incubated at 65 °C for 15 min. Each sample was extracted with phenol/ chloroform/isooamyl alcohol, then chloroform, and was then ethanol-precipitated. Pellets were resuspended in loading dye, separated on PAGE gels containing 7% 19:1 acrylamide/bisacrylamide and 8 M urea, exposed to a phosphor storage screen and scanned with a PhosphorImage (Molecular Dynamics).

For the assay of extracts from pseudodiplotid strains, UBC4 pre-mRNA was preincubated for 20 min in 60-μl reactions. Mutant PRP43 protein was added
to block release of the excised intron and to allow accurate quantification of all splicing species associated with the HA-tagged PRP8. Intermediates associated with PRP8-HA were immunoprecipitated with anti-HA antibody conjugated to protein A-Sepharose (Sigma) (25 µl slurry in IPP150 (10 mM Tris-HCl, pH 8, 150 mM NaCl and 0.01% NP-40 substitute (Fluka))). Beads were incubated with the splicing reactions for 90 min at 4 °C and washed three times with ten volume equivalents of IPP150. RNA was extracted with phenol/chloroform/isooamyl alcohol and separated by 15% denaturing PAGE.

For the exon ligation assay, the labeled 5′ portion of the ACT1 pre-mRNA was preincubated for 30 min in 10-µl splicing reactions. Exon ligation was initiated by addition of the 3′ portion of the ACT1 RNA substrate to splicing reactions, and incubation then continued for 10, 20, 30 and 60 min. The exon ligation product was amplified by RT-PCR, and its identity was confirmed by sequencing. Quantifications were performed by using ImageQuant (Molecular Dynamics).

42. Tanaka, N. & Schwer, B. Mutations in PRP43 that uncouple RNA-dependent NTPase activity and pre-mRNA splicing function. Biochemistry 45, 6510–6521 (2006).
43. Mayas, R.M., Maita, H., Semlow, D.R. & Staley, J.P. Spliceosome discards intermediates via the DEAH box ATPase Prp43p. Proc. Natl. Acad. Sci. USA 107, 10020–10025 (2010).
44. Otwinowski, Z.M. & Minor, W. Processing of X-ray diffraction data collected in oscillation mode. Methods Enzymol. 276, 307–326 (1997).
45. Pöttersen, E., Briggs, P., Turkenburg, M. & Dodson, E. A graphical user interface to the CCP4 program suite. Acta Crystallogr. D Biol. Crystallogr. 59, 1131–1137 (2003).
46. Murshudov, G.N., Vagin, A.A. & Dodson, E.J. Refinement of macromolecular structures by the maximum-likelihood method. Acta Crystallogr. D Biol. Crystallogr. 53, 240–255 (1997).
47. Terwilliger, T.C. Automated main-chain model building by template matching and iterative fragment extension. Acta Crystallogr. D Biol. Crystallogr. 59, 38–44 (2003).
48. Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. Acta Crystallogr. D Biol. Crystallogr. 60, 2126–2132 (2004).
49. Umen, J.G. & Guthrie, C. A novel role for a U5 snRNP protein in 3′ splice site selection. Genetics 99, 855–868 (1995).
50. Brown, J.D. & Beggs, J.D. Roles of PRP8 protein in the assembly of splicing complexes. EMBO J. 11, 3721–3729 (1992).
51. Boeke, J.D., Trueheart, J., Natoufis, G. & Fink, G.R. 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. Methods Enzymol. 154, 164–175 (1987).
52. Lesser, C.F. & Guthrie, C. Mutational analysis of pre-mRNA splicing in Saccharomyces cerevisiae using a sensitive new reporter gene, CUP1. Genetics 133, 851–863 (1993).
53. Köhrer, K. & Domdey, H. Preparation of high molecular weight RNA. Methods Enzymol. 194, 398–405 (1991).
54. Lin, R.J., Newman, A.J., Cheng, S.C. & Abelson, J. Yeast mRNA splicing in vitro. J. Biol. Chem. 260, 14780–14792 (1985).