SUPPLEMENTARY NOTES

Supplementary Note 1: Affinity purification with Prp19 requires reconstitution with U6 snRNA and enhances the potential to detect rescue by thiophilic metal.

Reconstitution with U6 snRNA increased the amount of pre-mRNA associated with Prp19p by 5-fold compared to the no reconstitution control, validating the specificity of the immunoprecipitation (Extended Data Fig. 1c, lanes 4 vs. 2). Moreover, this requirement for U6 snRNA reconstitution indicates that the majority of the immunoprecipitated pre-mRNA has assembled into activated spliceosomes, given the dependence of Prp19p association with pre-mRNA on the activation of the spliceosome[36]. Therefore the calculation of branching efficiency, which includes the immunoprecipitated pre-mRNA, is not confounded by unassembled pre-mRNA.

Removal of ATP to inactivate proofreading mechanisms[35] generally increased the efficiency of splicing for sulfur-substituted spliceosomes that could be rescued by thiophilic metal. For example, the efficiency of branching in Cd^{2+} for G78-PS(S_p) spliceosomes was on average 3-fold lower in the presence of ATP (Extended Data Fig. 1d). Therefore assaying for splicing in the absence of ATP allowed greater sensitivity for detection of rescue by thiophilic metals. However, in the case of G60-PS(R_p) and A59-PS(S_p) spliceosomes spliceosomes, ATP did not significantly affect Cd^{2+} rescue of branching (data not

shown). This behavior was similar to that observed previously for U80-PS(S_p) spliceosomes[14,28] and likely reflects tighter metal binding at the M2 site compared to the M1 site (data not shown; Extended Data Fig. 7), which results in a faster rate of branching that competes efficiently with Prp16-mediated proofreading.

Supplementary Note 2: Limitations of metal rescue experiments

Metal rescue experiments in RNA can in some instances be limited by the chemical properties of the P-S bond. An oxygen-to-sulfur mutation alters the chemical properties of a metal binding site in more ways than just favouring thiophilic metals[31]. For example, the P-S and S-Cd^{2+} bonds are longer than their P-O and O-Mg^{2+} counterparts[62], so with some sulfur substitutions, the substitution may preclude metal binding[63], depending on the environment, and even if metal does bind it may bind with a geometry incompatible with catalysis. These effects could be further compounded by the fact that negative charge is localized differently in P-O and P-S bonds, with the sulfur generally thought to bear a full negative charge[62]. Consequently, some sulfur modifications are intractable to metal rescue analysis (or if the sulfur can be rescued, then a second sulfur may be incompatible for the same reasons).

For example, while five sulfur substitutions in U6 inhibit both branching and exon ligation (which is consistent with all five ligands binding metals in each step), only in branching were the defects rescued by thiophilic metal in all five cases. However, the three sulfur substitutions that were not rescued at the exon

ligation stage – (G78-PS(S_p), U80-PS(R_p) and G60-PS(R_p) – showed more stringent requirements at the branching stage, as compared to the substitutions that were rescued at both steps. Specifically, these three substitutions were only rescued at the branching stage by Cd^{2+} while the other two positions were rescued at the branching stage by Mn^{2+} or Cd^{2+} (Extended Data Fig. 2b). This difference not only highlights the vagaries of metal rescue but also provides a simple explanation for why these three positions are not rescued during exon ligation – metal rescue for exon ligation is more stringent than for branching.

Finally, sulfur is inert as a nucleophile with respect to attack on an RNA backbone phosphate[64-66]. Thus, a substrate with sulfur at the 2' position of the branch site adenosine would not undergo branching – even in the presence of thiophilic metals. The same is true for a substrate with a sulfur at the 3' position of the 5' splice site; a sulfur at this position is functional as the leaving group during the first step, but the sulfur cannot act as a nucleophile for the second step, just as we observed (Extended Data Fig. 4a). Further, sulfur at the leaving groups in both steps prevents reversal of either step, simplifying our analysis of catalysis. Therefore, while the chemical properties of sulfur permit an investigation of interactions between metals and the leaving groups, the chemical properties of sulfur do not permit an investigation of interactions between metals and the nucleophiles.

Supplementary Note 3: Identification of a sulfur substituted pre-mRNA substrate sensitive to thiophilic metal binding at the 5' splice site during branching

As a first step towards determining whether the U6 metal ligands function by binding catalytic metals during branching, we identified catalytic metal interactions at the 5' splice site during branching in yeast. In a model substrate, sulfur substitution of the 3'-oxygen leaving group at the 5' splice site (3'S) compromised splicing in Mg^{2+} (Extended Data Fig. 4a), but after affinity-purification of stalled spliceosomes, the thiophilic metals Mn^{2+} and Cd^{2+} rescued branching 4-fold more than Mg^{2+} (Extended Data Fig. 4b); in contrast, thiophilic metals did not appreciably stimulate branching for the control 3'O substrate (Extended Data Fig. 4c,d; Supplementary Note 4). Thus, a metal catalyzes branching in yeast by binding and stabilizing the leaving group.

Unfortunately, the 3'S substrate was not sensitive to interactions between U6 metal ligands and the 5' splice site (data not shown) in part due to the need to include EDTA in the splicing buffer to observe specificity of metal rescue of the branching defect induced by the 3'S substitution. Therefore we investigated additional sulfur substitutions at the 5' splice site to find a substrate that was sensitive to interactions between the spliceosomal metal ligands and the 5' splice site during branching.

The two metal mechanism[22] predicts that the non-bridging *pro-*R_p oxygen of the scissile phosphate at the 5' splice site would also interact with catalytic

metals during branching (Fig. 1a). Consistent with this prediction, substitution of this oxygen with sulfur compromises branching in mammals[37], but rescue of this defect with thiophilic metals has not been observed. In *S. cerevisiae* splicing extracts, a sulfur substitution of this non-bridging *pro-*R_p oxygen (PS(R_p)), but not the *pro-*S_p oxygen (PS(S_p)), at the 5' splice site also repressed branching almost completely (Extended Data Fig. 3a, lanes 1, 5). When proofreading was disabled, Cd^{2+} rescued branching of the 3'O-PS(R_p) substrate but only marginally (Extended Data Fig. 3a, lane 2 vs. 4). In the *Tetrahymena* group I ribozyme, a block induced by an analogous sulfur substitution at the scissile phosphate was rescued robustly by Cd^{2+} only when the leaving group was also substituted with sulfur[50]. We therefore synthesized dithioate substrates, containing sulfur at both the leaving group and the non-bridging *pro-*R_p position (3'S-PS(R_p)) or, as a control, the *pro-*S_p position (3'S-PS(S_p)). Strikingly, the 3'S leaving group facilitated robust Cd^{2+}-mediated rescue of branching with the PS(R_p) substitution (Extended Data Fig. 3a, lane 12, Extended Data Fig. 3b). Thus, the 5' splice site *pro-*R_p oxygen binds a metal important for branching. The PS(R_p) substitution, but not the PS(S_p), restricted rescue of branching with the 3'S leaving group to Cd^{2+}, which prefers sulfur over oxygen, unlike Mn^{2+} (Extended Data Fig. 3a, compare lanes 11,12 to 15,16; ref. 38), providing evidence that the leaving group and the *pro-*R_p oxygen bind the same metal, as proposed in the two metal mechanism model.

Supplementary Note 4: Strategy for testing specific thiophilic metal stimulation of branching for the 3'S-PO substrate

Normally, complete branching of the *UBC4* 3'O substrate occurs in extract during the initial incubation prior to affinity purification of spliceosomes (Extended Data Fig. 4a). Thus, to rule out that thiophilic metals generally stimulate branching, we assayed splicing activity under conditions where spliceosomes are stalled just prior to branching, whether assembled on either the 3'S substrate or the control 3'O substrate. To do so, we assembled spliceosomes in extracts depleted of Cwc25p, which functions immediately prior to branching[59,60]. We then affinity purified these stalled spliceosomes and complemented them by adding rCwc25p, allowing us to assay the effect of thiophilic metals on branching of both the 3'S and the 3'O substrates.

As expected, spliceosomes affinity-purified from extracts depleted of Cwc25p were stalled prior to branching (Extended Data Fig. 4c, lane 1) and could catalyze branching when incubated with rCwc25p, HP, and metal (Extended Data Fig. 4c, lanes 2-4; Supplementary Methods). Spliceosomes assembled on the 3'O substrate catalyzed branching with equal efficiency in all metals. However, spliceosomes assembled on the 3'S substrate catalyzed branching 4-fold more efficiently in Mn^{2+} and Cd^{2+} than in Mg^{2+}, indicating that thiophilic metals specifically stimulated branching for the 3'S substrate. This stimulation was also observed when we analyzed the rate of branching rather than reaction endpoints. Although Cd^{2+} stimulates the rate of 5' splice site cleavage about 3-fold

compared to Mg^{2+} for complexes assembled on the 3'O substrate, Cd^{2+} has a significantly greater stimulatory effect (40-fold) for complexes assembled on the 3'S substrate (Extended Data Fig. 4d). These data indicate that spliceosomes assembled on the 3'S substrate and staged just before branching by depletion of Cwc25p exhibit a thiophilic metal rescue signature[31] – whether analyzed by endpoint or kinetics, strongly supporting a role for divalent metals in stabilizing the leaving group during branching.

Supplementary Note 5: The 3'S-PS(R_p) substrate did not improve rescue of U6 variants compromised for branching due to base mutations

To determine whether the 3'S-PS(R_p) substrate specifically improved branching for G78-PS(S_p), U80-PS(R_p), and U80-PS_2 spliceosomes, we asked whether 3'S-PS(R_p) substrate could improve branching for spliceosomes compromised due to base mutations in U6. These U6 variants contained the mutations U80g or U80g/C61g, which disrupt RNA-RNA interactions at the catalytic core by hyperstabilizing the ISL through induced pairing of the otherwise bulged U80 and/or by disrupting the secondary and tertiary structure of the U6 ISL and U2/U6 helix Ib (ref. 67). These mutations hindered branching but stalled spliceosomes at the catalytic stage (Extended Data Fig. 5c; data not shown), as reflected by the immunoprecipitation of pre-mRNA and the branching of the 3'O-PO substrate to a modest extent when the stalled spliceosomes were incubated in the absence of ATP (Extended Data Fig. 5d). However, in the presence of Cd^{2+}

the 3'S-PS(R_p) substrate did not improve branching relative to the 3'O-PO substrate (Extended Data Fig. 5d).

Supplementary Note 6: The tighter Cd^{2+} binding induced by U80-PS(S_p) for rescue of the 3'S-PS(R_p) substrate is specific

The apparent tighter Cd^{2+} binding (Fig. 3d) was only observed with sulfur substitutions at catalytically relevant oxygens in the substrate and U6. In contrast to the 3'S-PS(R_p) substrate, with the 3'S-PS(S_p) substrate, the U80-PS(S_p) substitution did not decrease the titration midpoint for rescue (Extended Data Fig. 6c). Similarly, in contrast to the U80-PS(S_p) substitution, a sulfur substitution at the G78 *pro-*R_p oxygen, which does not bind a metal important for branching (Fig. 2h), did not improve rescue of the 3'S-PS(R_p) substrate (Extended Data Fig. 6b,d).

Supplementary Note 7: Evidence for two distinct catalytic metals during branching

The data in Fig. 3e indicate that the 3'S-PS(*Rp*) substrate required Mn^{2+} for robust rescue at low Cd^{2+} concentrations, suggesting that the leaving group and the non-bridging *pro-*R_p oxygen interact with one of the metal sites (referred to as M1) and that one of these ligands also interacts with the other metal site (referred to as M2; see main text and Extended Data Fig. 7e,f). Importantly, the U80-PS(S_p) substitution did not require Mn^{2+} for robust rescue but rather enforced a

strict requirement for Cd^{2+} (Extended Data Fig. 7b-d,i,j). Indeed, the loss of Mn^{2+} stimulation implies that in the presence of U80-PS(S_p) both sites, not just M1, contain multiple sulfur substitutions, implicating the U80 *pro*-S_p oxygen as a ligand for the M2 site. In contrast, the U80-PS(R_p) and G78-PS(S_p) substitutions still required Mn^{2+} for rescue (Extended Data Fig. 7b,c,g,h), providing evidence that that the U80 *pro*-R_p and G78 *pro*-S_p oxygens interact with M1.

Consistent with this model, with the 3'S-PO substrate having a sulfur exclusively at the leaving group, the U80-PS(S_p) substitution did not restrict the metal specificity to Cd^{2+} alone (Extended Data Fig. 8b), in contrast to the 5' splice site *pro*-R_p sulfur substitution (Extended Data Figs. 7b-d), indicating that each metal site contained only a single sulfur substitution (Extended Data Fig. 8e,f). These data support the conclusion that the U80 *pro*-S_p oxygen interacts with M2, rather than M1, and indicate that M2 binds to the *pro*-R_p oxygen of the 5' splice site, rather than the leaving group. In further support of this idea, a single Cd^{2+} was titrated during rescue of the substrate with U80-PS(S_p) spliceosomes whether rescue was measured in the presence or absence of Mn^{2+} (Extended Data Fig. 7d). In this case, the M2 site is likely filled by Cd^{2+} at the lowest Cd^{2+} concentration and rescue monitors titration of the M1 site (Extended Data Fig. 7i,j); in support of this interpretation, we have observed that U80-PS(S_p) increases the affinity of Cd^{2+} for the catalytic core (Fig. 3d).

Supplementary Note 8: The role of the identified interactions between U6 ligands and the splice sites in catalysis vs. substrate docking

Formally our data do not rule out a role for the ligands in substrate docking, distinct from a role in catalysis. Still, note that splicing is assayed, after immunoprecipitation of stalled spliceosomes and in the absence of ATP and soluble factors. Consequently, splicing is restricted beyond both the final ATP-dependent step and the final factor-binding step – Cwc25 binding for branching and Slu7/Prp18 binding for exon ligation, which in each case promotes substrate docking (ref. 68; data not shown). Thus, our results show that metal coordination by U6 contributes to splicing following all biochemically defined steps preceding branching and exon ligation, strongly implicating a direct role in catalysis, rather than docking. Indeed, by single molecule FRET, we have found at the branching stage that U80-PS(S_p) permits docking in the presence of Mg^{2+} that is comparable in efficiency to the docking observed in Cd^{2+} (D. Semlow, M. Blanco, S.M.F., N. Walter, J.A.P., and J.P.S., manuscript in preparation). Most importantly, because the interactions we have identified involve the reactive groups of the scissile phosphates, a role in docking but not catalysis is difficult to envision (in contrast to, for example, interactions between the spliceosome and functional groups on the branch site adenosine, which would be expected to impact docking independent of catalysis). For example, during exon ligation, our data indicate that the *pro*-S_p oxygen of U80 interacts with a divalent metal that in turn interacts with the 3' splice site leaving group, which is uncharged in the

docked, ground state but becomes charged in the transition state. Thus, even if such an interaction contributed to docking or a subsequent conformational rearrangement, the interaction would become even stronger during catalysis. For such an interaction to contribute to docking without contributing to catalysis, the spliceosome would have to disrupt the interaction after docking, which seems highly unlikely. Finally, whether the interactions we have discovered function in docking, catalysis, or both, the data establish for the first time direct interactions between the spliceosome and the scissile phosphates and, additionally, show that these interactions are mediated by U6, thereby defining a direct role for RNA in catalyzing the splicing reactions.

Supplementary Note 9: Identification of a substrate sensitive to catalytic metal binding to the leaving group during exon ligation

To identify whether the U6 metal ligands function by binding catalytic metals during exon ligation, we first identified catalytic metal interactions at the 3' splice site during exon ligation. Our model substrate was constructed via splinted ligation and contained a mutation of the 3' splice site consensus sequence (UAG to UAc) that stalls spliceosomes prior to exon ligation[34]. In addition, we introduced a sulfur substitution at the 3' splice site leaving group (3'S). Direct rate measurements demonstrated that the rate of exon ligation in the UAc-3'S substrate, but not the UAc-3'O, is stimulated by Cd^{2+} (Extended Data Fig. 9a). We confirmed that exon ligation occurred at the correct junction in these

substrates by direct sequencing of the mRNA (Extended Data Fig. 9b, data not shown). We conducted analogous experiments with spliceosomes assembled on 3'O vs. 3'S wild-type substrates lacking a base mutation and instead having a photocaged G_{-1} residue at the intron terminus to stall spliceosomes (Extended Data Fig. 9c, ref. 69). (The synthesis of the 3'S-photocaged oligonucleotide will be described elsewhere.) Following photodeprotection, spliceosomes assembled on the 3'S, but not the 3'O, substrate catalyzed exon ligation faster and to a greater extent in the presence of Mn^{2+} or Cd^{2+}, relative to Mg^{2+} (Extended Data Fig. 9d-f). From this we conclude that the sensitivity to metals is due to the 3'S modification and not the base mutation.

Supplementary Note 10: Lack of rescue of substrates bearing double sulfur substitutions at the scissile phosphate during exon ligation

We have tested a substrate with a phosphorothioate substitution at the pro-Rp oxygen of the 3' splice site (ACT1-3'O-PS(R_p)). This substrate, but not the corresponding *Sp*-substituted substrate, conferred a strong block to exon ligation as expected from Moore and Sharp's previous work[37] (data not shown). However, the substrate failed to undergo exon ligation under any condition (data not shown), including the absence of ATP, the combination of U6-PS mutants, and the combination of a 3'S substitution (Extended Data Fig. 9h), which would yield the analog of the 3'S-PS(R_p) substrate used to assay for direct interactions between U6 metal ligands and the 5' splice site (Fig. 3). It is important to note

that a lack of rescue does not mean that the original position does not coordinate a functional metal (Supplementary Note 2). We note that analogous experiments for group I intron splicing using dithioate substrates (a combination of a 3'S leaving group and a non-bridging sulfur at the scissile phosphate) gave analogous results: a dithioate at the 5'-splice site undergoes rescue by Cd^{2+}, but a dithioate at the 3'-splice site does not rescue under any condition tested (unpublished data), in spite of the fact that a crystal structure shows that the metal at the 3'-splice site interacts with both the leaving group and the non-bridging oxygen of the scissile phosphate[70].

Supplementary Note 11: Exon ligation role of U6 positions where phosphorothioate substitutions could not be rescued during exon ligation

In addition to being tested with the UAc-3'S substrate (Fig. 4c), G60-PS(R_p) spliceosomes were tested with substrates having the 3'S and a sulfur substitution at either of the non-bridging oxygens; under no condition did the G60-PS(R_p) substitution permit rescue (Extended Data Fig. 9h; data not shown). Similarly, G78-PS(S_p) and U80-PS(R_p) spliceosomes were tested for exon ligation with the UAc-3'S substrate under a number of conditions; in no case did the sulfur substitutions permit rescue of exon ligation (Extended Data Fig. 9h; data not shown). While the lack of rescue precludes us from concluding definitively that these three ligands bind a metal at the exon ligation stage, the strong block to exon ligation conferred by these three sulfur substitutions is

consistent with each of them functioning as a ligand to a metal, if not a catalytic metal, during the exon ligation step of splicing as depicted in our model (Fig. 5d), especially given the established roles of G78-PS(S_p) and U80-PS(R_p) as catalytic metal ligands in the branching reaction (Fig. 3b). In particular, our data provide evidence that G78-PS(S_p) and U80-PS(R_p) stabilize the 5' exon leaving group, through M1 in the model, during branching, so the exon ligation defects of these substitutions are consistent with a deficiency in the same interaction during exon ligation, given that the 5' exon leaving group becomes the nucleophile in the second step (cf. Fig. 5a,b).

Supplementary Note 12: A metal specificity switch provides further support for a direct catalytic interaction between the 3' splice site leaving group and the U80 pro-S_p oxygen

An independent line of evidence supports the functional link between the metal that coordinates the 3' splice site leaving group and the U80 pro-S_p oxygen within U6. The U6 U80-PS(S_p) spliceosomes catalyzed exon ligation of the wild-type 3'O substrate efficiently in either Mn^{2+} or Cd^{2+} (Extended Data Fig. 8a). Similarly, U6 WT spliceosomes catalyzed exon ligation of the wild-type 3'S substrate efficiently in the presence of either Mn^{2+} or Cd^{2+} (Extended Data Fig. 8a). In contrast, U6 U80-PS(S_p) spliceosomes catalyzed exon ligation of the wild-type 3'S substrate efficiently only in Cd^{2+} (Extended Data Fig. 8a). Thus, while Mn^{2+} rescued each sulfur substitution when in separate splicing complexes, Mn^{2+}

failed to rescue the sulfur substitutions when together in a single splicing complex, but Cd^{2+}, a truly thiophilic metal, did rescue (Extended Data Fig. 8c,d), providing further evidence of an interaction between the *pro-*S_p oxygen of U80 and the 3' splice site leaving group. This switch in metal specificity is specific to exon ligation; as noted above, U6 U80-PS(S_p) spliceosomes catalyzed branching of the 5' splice site 3'O and 3'S substrates in either Mn^{2+} or Cd^{2+} (Extended Data Fig. 8b), indicating no interaction between the *pro-*S_p oxygen of U80 and the 5' splice site leaving group (Extended Data Fig. 8e,f). Thus, this metal specificity switch corroborates our evidence that the U6 U80 pro-S_p oxygen coordinates the same metal as the leaving group during exon ligation.

Supplementary Note 13: Evidence consistent with a single catalytic core during both steps of splicing

Although the limitations of metal rescue experiments precluded direct tests for metal interactions with the nucleophiles during each step of splicing (Supplementary Note 2), a range of our observations support a model for a single active site in the spliceosome, as described below:

(1) All five U6 ligands are defective for both the first and second chemical steps, when substituted with sulfur (Fig. 2, data not shown), and all five correspond stereospecifically to the five divalent metal ligands in the catalytic domain V of the group II intron (Fig. 2n; ref. 26). Indeed, we tested many other positions in the U6 snRNA in search of other possible ligands but found that no

other positions had defects.

(2) Three U6 ligands bind a metal that interacts directly with the 5' splice site. Importantly, we showed that one of these ligands, the U6 U80 *pro-Sp* oxygen, interacts with both the 5' splice site and the 3' splice site, a clear commonality between the first and second chemical steps that provides direct evidence to support the hypothesis of a single catalytic core. Our analysis of branching indicates that this U80 ligand binds a metal distinct from the metal that is coordinated by the G78 *pro-*S_p and U80 *pro-*R_p oxygens and that stabilizes the leaving group (see main text and Supplementary Note 7; Extended Data Figs. 7,8). In the two metal model (Fig. 5a,d), during branching this metal (M2) would coordinate the non-bridging oxygen of the scissile phosphate, as our data indicate (Supplementary Note 7; Extended Data Figs. 7,8), as well as the attacking group; during exon ligation this metal (M2) would coordinate the non-bridging oxygen of the scissile phosphate as well as the leaving group (Fig. 5b). Indeed, we show that the U80 *pro-*S_p oxygen interacts with a metal that stabilizes the leaving group during exon ligation. Further evidence that M2 switches function in the branching and exon ligation steps comes from analysis of rescue specificity for U80-PS(S_p) spliceosomes upon substitution of the leaving groups with sulfur. When the 5' splice site leaving group is substituted with sulfur, U80-PS(S_p) retains the ability to catalyze branching in both Mn^{2+} and Cd^{2+}. However, when the 3' splice site leaving group is substituted with sulfur, these spliceosomes lose the ability to catalyze exon ligation in Mn^{2+} and exhibit a strict

requirement for Cd^{2+} (Supplementary Note 12; Extended Data Fig. 8). Given that Mn^{2+} is expected to bind one sulfur efficiently but not two[31,50], our results support a model in which the U80 *pro-*S_p oxygen-coordinated M2 activates the nucleophile during branching and stabilizes the leaving group during exon ligation. Thus, we were able to detect an interaction between U80-PS(S_p) and a metal in both the first and second steps – in the first step, by virtue of an interaction with the non-bridging *pro-*R_p position of the 5' splice site (and a lack of an interaction with the 5' splice site leaving group) and in the second step by virtue of an interaction with the 3' splice site leaving group. The simplest explanation of these data is that U80-PS(S_p) interacts with the same metal (M2) in both steps, providing compelling direct evidence for a single catalytic core.

(3) The A59 *pro-Sp* oxygen, like the U80 *pro-Sp* oxygen, stabilizes the leaving group during exon ligation (Figs. 4, 5b) and this same ligand also binds a metal important at the catalytic stage of branching (Fig. 2c), consistent with a role for this ligand, parallel to U80 *pro-*S_p, in stabilizing the attacking group in branching and leaving group during exon ligation (Fig. 5d).

(4) We show conversely that G78 *pro-*S_p and U80 *pro-*R_p oxygens interact with the 3'O of the 5' exon leaving group during branching (Fig. 3b) and that sulfur substitution of both ligands blocks exon ligation (data not shown); the simplest, compelling explanation for this defect in exon ligation is that the function of the ligands in binding the terminal 3'O of the 5' exon persists during exon ligation, especially given the role of this 3'O as the nucleophile during exon

ligation.

(5) While different metal ligands in U6 have been linked to the leaving group in the two chemical steps of splicing, these results are precisely what is predicted by a single catalytic core utilizing a reversible two metal mechanism – that is, the leaving group is stabilized by M1 in the first step and by M2 in the second step (Fig. 5a,b, ref. 22).

(6) We have found compelling physical and functional evidence for the formation of a catalytic triplex in U6 snRNA, analogous to the metal-binding catalytic triplex in domain V of the group II intron (ref. 71; M. Mefford, S.M.F., J.A.P., and J.P.S., manuscript in preparation).

Together, these broad and diverse observations are robustly consistent with a single catalytic core, which is also the simplest explanation for the entirety of the data.

Our data provide no evidence for an alternative model in which the catalytic core is different between the two chemical steps; specifically, our data are not consistent with any model in which any of the five U6 metal ligands have a functional role in one chemical step but not in the other.

Supplementary Note 14: Relationship of our spliceosomal functional data to group II intron structural data: implications for group II intron catalysis

While the parallel between metal coordination in the spliceosome and the group II intron is striking, our studies of U6 were largely independent of the group

II structure and indeed serve to inform group II mechanism. First, with the exception of G78-PS(S_p), the five metal ligands in U6, although parallel to those in domain V of the group II intron, were identified independently of the group II structure[26,27] and were discovered in tests at twenty sites in U6 and based on previous phosphorothioate interference data[12-14]. Second, our linkage of G78 *pro*-S_p, U80 *pro*-R_p and U80 *pro*-S_p oxygens to the scissile 5' splice site phosphate is independent, although parallel, to the linkage of the equivalent ligands in domain V to the scissile 5' splice site in the group II intron structure. In particular, our data link the G78 pro-S_p and U80 *pro*-R_p, and not U80 *pro*-S_p, to the leaving group at the 5' splice site and U80 *pro*-S_p to the *pro*-R_p oxygen of the scissile phosphate (Supplementary Note 7), as oriented for the equivalent ligands in the structure of domain V. Third, we provide the first functional evidence for binding of two different metals to the *pro*-R_p non-bridging oxygen at the 5' splice site during branching (Supplementary Notes 7,8). While the group II structure similarly implicates binding of two equivalent metals to the *pro*-R_p non-bridging oxygen at the group II 5' splice site, no data yet support a functional role for these interactions in hydrolysis of the 5' splice site or branching. Fourth, our linkage of U80 pro-S_p and A59 pro-S_p to the 3' splice site leaving group during exon ligation is novel; although equivalent interactions are predicted in the group II introns, there is as of yet no pre-second step structure that demonstrates equivalent physical linkages in group II introns[72]. Fifth, we have found evidence not only that the ligands are conserved but also that their structural configuration is conserved.

In domain V, as in U6, the metal binding centers localize to the triad at the 5' end of a stem loop and a conserved bulge on the 3' side (Fig. 1c). These two centers are brought together for two-metal catalysis by three base triples that form a catalytic triplex[71]. In vivo and in vitro molecular genetics studies combined with site specific X-linking indicate that a similar triplex forms in the U6 snRNA (M. Mefford, S.M.F., J.A.P, J.P.S, manuscript in preparation). Finally, it is important to note that while the group II structure implicates these specific ligands in catalysis there are no functional data yet supporting such a role for these ligands, so our data provide the first functional evidence of linkage in catalysis for either system.

Supplementary Note 15: A test for the possible role of the Prp8 RNaseH-like domain in binding catalytic metals during exon ligation

Based on partial conservation in Prp8 of a metal binding motif characteristic of RNase H enzymes[73-75], it has been suggested that Prp8 might directly promote metal-mediated catalysis by the spliceosome[73,76]. To test this hypothesis, we mutated the most conserved residue of this motif (D1853), to a cysteine. This aspartate residue coordinates catalytic metals in RNaseH enzymes, as shown crystallographically[77]. Functional relevance of metal binding has been established in several cases by mutation of this aspartate to cysteine or asparagine and consequent observation of a metal specificity switch[77-79]. Indeed, D1853 binds a metal *in crystallo*[42]. The Prp8-D1853C mutation slowed growth of *S. cerevisiae* at 30°C and *in vitro* reduced by 2-fold the efficiency of exon ligation

of an *ACT1* pre-mRNA substrate (Extended Data Fig. 10a). Although not rescued alone by thiophilic metal, given this specific exon ligation defect, we sought to test the possibility that D1853 plays a role in positioning the metal that stabilizes the leaving group during exon ligation. To do so, we asked whether the D1853C mutation affects the specificity of thiophilic metal rescue of exon ligation for the sensitized UAc-3'S ACT1 substrate containing a sulfur substitution at the 3' splice site leaving group. We would expect that, if D1853 interacted with the same metal that stabilizes the leaving group during exon ligation, then the D1853C mutation might shift the specificity for rescue of the ACT1 UAc-3'S substrate to more thiophilic metals, e.g. by abolishing or severely impairing exon ligation in Mg^{2+} and/ or Mn^{2+}, without compromising splicing in Cd^{2+}, as was observed for U80-PS(S_p) spliceosomes assembled on the UAc-3'S substrate, as compared to the UAC-3'O substrate (Extended Data Fig. 8a). However, the D1853C mutation, as for Prp8 WT, permitted exon ligation of the UAc-3'S substrate with similar efficiencies in Mn^{2+} and Cd^{2+} (Extended Data Fig. 10b). Additionally, while Prp8 D1853C, relative to Prp8 WT, caused a general 2-fold reduction in the efficiency of exon ligation for the UAc-3'S substrate (Extended Data Fig. 10b), this decrease was similar to the reduction in exon ligation for the UAc-3'O substrate, suggesting that this effect was due to a general impairment of exon ligation and not to a specific effect on catalytic metal binding. With the UAG-3'O and UAG-3'S substrates, D1853C still failed to switch metal specificity (Extended Data Fig. 10a), although the mutation still reduced exon ligation for the 3'S substrate,

suggesting that Prp8 D1853 does play some role in exon ligation. In conclusion, we found no evidence that D1853 is important for stabilizing the leaving group during exon ligation, although we cannot exclude such a role.

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