Evidence for a group II intron–like catalytic triplex in the spliceosome

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To catalyze pre-mRNA splicing, U6 small nuclear RNA positions two metals that interact directly with the scissile phosphates. U6 metal ligands correspond stereospecifically to metal ligands within the catalytic domain V of a group II self-splicing intron. Domain V ligands are organized by base-triple interactions, which also juxtapose the 3′ splice site with the catalytic metals. However, in the spliceosome, the mechanism for organizing catalytic metals and recruiting the substrate has remained unclear. Here we show by genetics, cross-linking and biochemistry in yeast that analogous triples form in U6 and promote catalytic-metal binding and both chemical steps of splicing. Because the triples include an element that defines the 5′ splice site, they also provide a mechanism for juxtaposing the pre-mRNA substrate with the catalytic metals. Our data indicate that U6 adopts a group II intron–like tertiary conformation to catalyze splicing.

Introns are removed from pre-mRNAs by the spliceosome—a dynamic ribonucleoprotein machine composed of 80 conserved proteins and five small nuclear RNAs (snRNAs)1. Although proteins have key supporting roles in catalysis2–3, the catalytic core itself is composed of RNA4. Indeed, this RNA-based core catalyzes two sequential phosphotransesterifications that are identical to the reactions performed by group II intron RNAs, which self-splice in the absence of proteins. Specifically, in both systems an intronic 2′-hydroxyl first attacks the 3′ splice site to form a branched lariat structure5, and then the 5′ exon attacks the 3′ splice site to form mRNA. These two reactions were proposed to be catalyzed by a general two-metal mechanism6, in which one divalent metal stabilizes the nucleophile, and the second divalent metal stabilizes the leaving group. Indeed, crystal structures of group II introns have revealed that ligands in the catalytic domain V position two divalent metals within 4 Å, the preferred distance for the two-metal mechanism, and that these metals interact with the 3′ splice site7–10. Supporting a catalytic role for these metals, divalent metals stabilize the leaving groups during group II intron splicing, thus promoting catalysis9,10. Indicating a two-metal mechanism for pre-mRNA splicing as well, we have recently demonstrated that ligands in U6 snRNA (Fig. 1a) bind two distinct divalent metals that catalyze splicing by interacting with the leaving groups during both chemical steps4.

In the group II intron catalytic core, the conserved AGC triad of domain V together with nucleotides in the upper portion of the stem-loop, including a conserved bulged position, bind two distinct metals7,11 (Fig. 1b,c). The ligands that form the two metal sites are brought together by base-triple interactions between the AGC triad and the bulge in a configuration stabilized by a conserved distal element termed the J2/3 linker12,13 (Fig. 1b,c). By organizing domain V, the triple helix positions the two catalytic metals with the 4-Å spacing preferred for phosphoryl transfer catalysis6,8,10. Additionally, the J2/3 linker functions in both steps of splicing12,13 and recognizes the 3′ splice site14, thereby promoting docking of the 3′ splice site into the catalytic core. Thus, in the group II intron the triple helix effects catalysis both by positioning catalytic-metal ligands and by recruiting the 3′ splice site. In the spliceosome, however, the mechanism for catalytic-metal positioning and substrate docking has remained unclear.

Nonetheless, the RNA structures at the heart of the spliceosome share several similarities to the catalytic core of group II introns. Like RNA domains of group II introns, the snRNAs define and juxtapose the chemically reactive sites in the substrate, through U2–U6 helix Ia and adjacent interactions15–17 (Fig. 1a). Additionally, similarly to the catalytic domain V of group II introns, U2–U6 helix Ib and the intramolecular stem-loop (ISL) of U6 adopt a secondary structure in which a conserved AGC triad is situated 5 bp away from a conserved bulge (Fig. 1a,b); both the triad and the bulge are important for each step in splicing15,17–20. Moreover, the U6 catalytic-metal ligands, situated in the triad and the bulge, correspond directly and stereospecifically to the domain V metal ligands4,7,8.

The functional and structural similarities between group II introns and the spliceosome led to the prediction that a group II–like triple helix may form in U6 snRNA to similarly position analogous metal ligands. This spliceosomal triplex would join the AGC triad, the bulge and the terminal GA of the conserved ACAGAGA sequence11 (Fig. 1a). Because the 5′ end of the ACAGAGA sequence base-pairs with the 3′ splice site11,22, such a triple helix would also provide a structural
mechanism for docking of the 5′ splice site into the catalytic RNA core, a process required for both transesterifications. Recently, three-dimensional modeling of the spliceosomal active site revealed that such a triplex is consistent with current experimental data. To determine whether the U6 snRNA positions catalytic metals in a manner similar to that of domain V of group II introns, we therefore investigated whether a group II intron–like catalytic triplex forms in the U6 snRNA.

Through molecular genetics in vivo and in vitro, in combination with site-directed cross-linking, we present evidence that a triple helix equivalent to that seen in domain V forms in U6 snRNA during catalytic activation of the spliceosome. Our data define a structural mechanism by which the spliceosome positions two metals in a catalytic configuration and juxtaposes these metals with the substrate reactive groups within a single catalytic core for both steps of splicing.

**RESULTS**

**Genetic evidence for the U6 triplex**

The AGC triad of U6 snRNA can base-pair with U6 downstream, to form an extended ISL, with U4, to form U4–U6 stem, and with U2, to form U2–U6 helix Ib. However, genetics implicates a function for the AGC triad beyond these roles. For instance, a thorough in vivo compensatory analysis that repaired all of these predicted base-pairing interactions was unable to alleviate the lethality of three mutations in the AGC triad. To determine whether the residual function of the AGC triad reflects formation of a catalytic triplex (Fig. 1), we exhaustively tested whether the growth defects of the AGC triad could be suppressed in an allele- and position-specific manner by compensatory mutations in the predicted base-triple partners (Supplementary Note 1). A similar mutational approach has been applied previously in the study of triple-helix interactions in other RNAs26,27 and subsequently validated by direct structural observation with NMR or X-ray crystallography.27,28

The final position of the AGC triad, C61 of U6, has been predicted to interact with the bulged U6 ISL residue U80, which binds catalytic metals4,7; blue dashed lines with question marks, base-triple interactions tested in this study. The pre-mRNA in configuration before branching. (e) Structure of the group II intron domain V, highlighting the catalytic triplex (PDB 4FAQ). Coloring is as in a. Residue numbers are shown in orange, green and blue for the group II intron, with the proposed corresponding residues in the U6 snRNA denoted below in black or magenta (for catalytic-metal ligands). Black dashed lines, Watson–Crick interactions; blue dashed lines, base-triple interactions. The catalytic metals (M1 and M2), their nonbridging oxygen ligands and the scissile phosphate at the 5′ splice site are colored magenta. This particular class of group II introns contains an unusual CGC triad rather than the canonical AGC.

Figure 1 Base-triple interactions in the group II intron catalytic core and their proposed counterparts in the spliceosome, (a,b) Secondary-structure model of key RNA structures present in the spliceosome (a) and group II intron (b) catalytic cores. Orange, residues in the catalytic triad; green, their base-pairing partners; blue, residues involved in base-triple interactions in domain V and their proposed counterparts in U6; circles, U6 and domain V residues that bind catalytic metals4,7; blue dashed lines with question marks, base-triple interactions tested in this study. The pre-mRNA in configuration before branching. (c) Structure of the group II intron domain V, highlighting the catalytic triplex (PDB 4FAQ). Coloring is as in a. Residue numbers are shown in orange, green and blue for the group II intron, with the proposed corresponding residues in the U6 snRNA denoted below in black or magenta (for catalytic-metal ligands). Black dashed lines, Watson–Crick interactions; blue dashed lines, base-triple interactions. The catalytic metals (M1 and M2), their non-bridging oxygen ligands and the scissile phosphate at the 5′ splice site are colored magenta. This particular class of group II introns contains an unusual CGC triad rather than the canonical AGC.

Figure 2 Genetic evidence for a base-triple interaction between U80 and C61 in the U6 snRNA. (a,b) Spot assays showing growth on selective medium of equivalent numbers of yeast cells containing combinations of alleles at U80 and either C61 (a) or A59 (b) of U6. The allele combinations for the U2–U6 helix Ib base pair mutated in each case are indicated above each panel. Alleles of U6 U80 present in each row are indicated on the left. (c) Diagrams of isomorphic base-triple interactions: CC·G (left, as in group II intron structure11), U·C·G (middle, predicted for the spliceosome) and G·C·G (right, predicted for the spliceosome). The location of the glycosyl bond is highlighted with a circle: open black, major groove–interacting residue; filled black, catalytic triad; gray, U2 or equivalent residues in group II intron. WT, wild type. Uppercase represents wild-type allele; lowercase represents mutant allele.
fall between the 5′ splice site–binding site of U6 and U2–U6 helix 1a, the latter of which is immediately adjacent to the catalytic core, that such triplex formation would promote docking of the 5′ splice site into the catalytic core11 (Fig. 1a). All three point mutations at the central position of the AGC triad, G60, are lethal, and only the conservative substitution G60A can be suppressed by restoring base-pairing in U2–U6 helix 1b15,19. Nevertheless, we found that a mutation of the predicted base-triple residue, G52, suppressed G60U, albeit mildly (Fig. 3a). To our knowledge, this marks the first observed suppression of G60U (Supplementary Note 3). Remarkably, suppression of G60U did not require restoration of base-pairing in U2–U6 helix 1b (Fig. 3a). As we observed for mutations at U6 C61 (Fig. 2), suppression of G60U was specific with respect to allele and position (Fig. 3a, b, Supplementary Fig. 1b and Supplementary Note 4), thus providing compelling evidence for an interaction between G52 of the ACAGAGA sequence and G60 of the AGC triad, in the context of a base triple that includes helix 1b base-pairing.

Mutations at U6 A59 showed a wider range of phenotypes, thus allowing for multiple tests of suppression at this position. Although restoration of U2–U6 helix 1b suppresses A59C and A59G almost completely, A59U is not suppressed at all by restoration of U2–U6 helix 1b, U4–U6 stem 1 or both19 (Fig. 3c). Remarkably, a mutation at the predicted base-triple partner, U6 A53C, suppressed not only the sick phenotype of A59C and the lethality of A59G but also the lethality of A59U—all without U2–U6 helix 1b repair (Fig. 3c and Supplementary Fig. 1c). To our knowledge, this marks the first observed suppression of A59U (Supplementary Note 4). Although A53C suppressed all three alleles at position A59, suppression was position specific (Fig. 3d and Supplementary Fig. 1d). Additionally, only the A53C allele suppressed all three A59 point mutations (Fig. 3c), thus suggesting a specific mechanism of suppression (described below). Together, these data, especially regarding the positional specificity, provide evidence for a base-triple interaction between A53 of the ACAGAGA sequence and A59 of the AGC triad.

If the suppression of AGC-triad mutations is direct, via formation of compensatory base-triple interactions, as the allele specificity and position specificity of our data strongly imply, then suppressor combinations should be capable of forming similar base-triple interactions. Consequently, we assessed the structural similarity between potential suppressor base triples and wild-type base triples, modeled on the basis of the group II structure7–11. Where appropriate, to guide this comparison, we used published matrices enumerating the many possible base-base interactions observed crystallographically or predicted by modelling29. Two of the three group II–intron triples are unusual11, and the third triple, although resembling analogous triples, is not strictly isosteric with any of these interactions29. Two base-base interactions are generally considered to be similar if the configurations conserve the distance between their linkages to the sugar-phosphate backbone. We focused specifically on the C1′–C1′ distance between the AGC-triad residues and their predicted base-triple partners (Figs. 2c and 3e, f) because this distance probably influences catalytic metal positioning by the AGC triad4.

For each of the predicted base triples, we discovered a suppressor base triple that could form interactions that resembled the configuration of the wild-type base triple, predicted from the group II structure (Figs. 2c and 3e, f and Supplementary Note 5). For example, with suppression of the U2–U6 G21C C61G double mutant by U6 U80C (Fig. 2a), O6 of C61G could interact with N4 of U80C to form a similar base triple (Fig. 2c). Similarly, for the base triple involving G52 and G60, the C1′–C1′ distance for our proposed suppressor base triple is remarkably similar to that of the predicted wild-type base triple (within 0.4 Å, Supplementary Table 2). For the final base triple, we can in some cases model a plausible suppressor base triple (for example, Fig. 3f), but the capacity of the A53C mutation to suppress generally suggests that it might do so by forming a base-neutral interaction with the phosphate backbone of A59, just as the equivalent residue of the group II intron interacts with the backbone11 (Fig. 1c and Supplementary Notes 5 and 6). This analysis suggests that the base-triple suppressor combinations probably act directly by restoring a configuration of the bases similar to that observed in the wild-type context.

Overall, our analysis is consistent with a mechanism of suppression in which the new suppressor combinations permit growth by forming base-triple interactions structurally similar to those predicted for the wild-type context. Further, as has been observed previously for other triple-helix interactions27, we observed that a mutation that suppressed disruption of one base triple exacerbated disruption of another base triple (in 75% of the tests, Supplementary Table 1)—a result that is consistent with triplex formation, because these tests would disrupt two, rather than one, of the three triples. Thus, although our data are not sufficient to define base-triple interactions at atomic
resolution, which is currently not feasible for the spliceosome, our data establish evidence that the residual function of the AGC triad, not accounted for by pairing with U2, U4 or U6, is to form a triplex interaction, in the context of helix Ib, with the bulge of the U6 SISL and the ACAGAGA region of U6, the latter of which binds the 5′ splice site.

Physical evidence for the U6 triplex
As a result of triple-helix formation in domain V, two of the base-triple partners (C377 and G288) stack through their base rings (Fig. 4a). To investigate whether the corresponding positions in U6 (U80 and G52) form a similar stacking interaction, we designed a cross-linking assay. We reconstituted U6-depleted extract with a synthetic U6 (U6 4SU80) containing 4-thiouridine at U80 and a single radioactive label at G52 (Fig. 4a and Supplementary Fig. 2a). If these two bases stack, UV irradiation should induce formation of a covalent linkage between U80 and G52 (ref. 30), with the site-specific radiolabel facilitating identification of such a linkage.

We detected three major cross-links that were dependent on both UV irradiation and 4-thiouridine (Fig. 4b and Supplementary Fig. 2b), including an extract-independent cross-link (X2) and the previously reported U4-U6 cross-link involving U80 (X3; ref. 31 and Fig. 4b; characterization of X2 and X3 in Supplementary Fig. 2c–e). Most importantly, we observed extract-dependent and pre-mRNA-stimulated formation of a cross-link migrating closely to un–cross-linked U6 (X1, Fig. 4b and Supplementary Fig. 2c). To determine whether this reflected intramolecular cross-linking, we performed P1 nuclease digestion, which degrades RNA to single nucleotides with 5′-monophosphates (Fig. 4c). Whereas un–cross-linked U6 digested to radiolabeled mononucleotide, reflecting formation of 5′-pG (p, phosphate; Fig. 4c,d), X1 digested to a species migrating more slowly than the mononucleotide and close to a dinucleotide standard (Fig. 4d), thus providing evidence that G52 cross-linked to U80. In contrast, when we performed RNA hydrolysis, which yields single nucleotides with cyclic 3′-monophosphates (Fig. 4e), both un–cross-linked U6 and X1 yielded radiolabeled mononucleotide, thus reflecting formation of Ap-3′ (Supplementary Note 7 and Supplementary Fig. 2f,g) and thereby ruling out that U80 cross-linked to A51. We conclude that X1 results from cross-linking between G52 and U80. Given an analogous cross-link between equivalent positions in the group II intron, which stack in the domain V triplex, and the chemical mechanism of 4-thiouridine cross-linking (Supplementary Note 8), the simplest explanation for our observed cross-link is that U80 and G52 stack in the context of a triplex structure. Thus, this provides physical evidence for a group II intron–like U6 triplex in the spliceosome.

The NTC promotes U6-triplex formation
The cross-linking of U80 to G52 allowed us to investigate when the U6 triplex forms during the splicing cycle. To do so, we induced cross-linking of U6 4SU80 splicesomes after stalling at defined stages through the use of dominant-negative mutations of DEABox ATPases known to promote conformational rearrangements during

Figure 5 The NTC promotes formation of the U6 triplex. (a) Denaturing PAGE analysis of radiolabeled U6 4SU80 extracted from a splicing reaction after UV irradiation and immunoprecipitation via Prp19p. 5% of the input (I) and all of the beads (B) were analyzed. (b) Denaturing PAGE analysis of radiolabeled ACT1 pre-mRNA after in vitro splicing in extracts depleted of the NTC (NTCA) with or without addition of purified yeast NTC (NTCA). (c) Denaturing PAGE analysis of U6 4SU80 recovered from in vitro splicing reactions after UV irradiation. Splicing of un–cross-linked ACT1 pre-mRNA was performed in extracts depleted of the NTC (NTCA) and reconstituted with radiolabeled U6 4SU80. In a and c, rPrp2p-K252A was added to stall splicesomes immediately after NTC binding and before Prp2p-dependent activation. In a and c, the efficiency of X1 formation is quantified below the gel; in c, the X1 efficiency was normalized to the mock-depleted reactions for individual experiments; in b, the splicing efficiency, normalized to the mock–depleted reaction, is quantified below the gel. Error bars, s.d. from three technical replicates. Full gels are in Supplementary Figure 8c–e.
in vitro splicing. Unexpectedly, we detected X1 at very high levels in B\(^{\text{act}}\) spliceosomes, which are stalled by a dominant-negative Prp2p mutant (rPrp2p-K252A) at the final ATP-dependent step in spliceosome activation (Fig. 5a and Supplementary Fig. 3), thus providing evidence that the U6 triplex forms before the final stage in catalytic activation. Strikingly, immunoprecipitation via Prp19p revealed that more than 50% of U6 in B\(^{\text{act}}\) spliceosomes formed the X1 cross-link in extract (Fig. 5a). Such high cross-linking efficiency requires stacking of the residues involved\(^{33-35}\) and suggests that the U6 triplex is a defining feature of spliceosomes poised for final activation.

Given triplex formation in B\(^{\text{act}}\) spliceosomes, we asked whether triplex formation required the Prp19p-associated complex (NTC), which is necessary for stabilization of B\(^{\text{act}}\) spliceosomes after release of U4 snRNA\(^ {36}\). Depletion of the NTC from splicing extracts resulted in a strong reduction in X1, and purified NTC rescued the cross-link (Fig. 5b), thus defining a role for the NTC in stabilizing the U6 base-triple interactions (additional information in Supplementary Fig. 4 and Supplementary Note 9). Further, given the involvement of the ACAGAGA sequence in the U6 triplex, our data indicate that the 5’ splice site docks into the catalytic core at the stage of NTC binding (described in Discussion).

The U6 triplex is present at branching and exon ligation

Biochemical data and crystal structures of the group II intron imply a role for the domain V triplex in both catalytic steps\(^{7,8,11,20}\). Further, we have shown in the spliceosomes that catalytic-metal ligands that would be organized by the triplex function in both catalytic steps\(^4\). Consequently, we tested whether the U6 triplex is present during each catalytic step.

To test for the triplex at the branching stage, we stalled spliceosomes just after branching by addition of a dominant-negative Prp16p mutant that remains bound to the spliceosome (rPrp16p-K379A) and isolated from glycerol gradients the resulting spliceosome complexes, which had catalyzed branching but had not undergone the Prp16p-dependent rearrangement (denoted B\(^{\text{bt}}\) (prp16)) (Fig. 6a). UV irradiation of these spliceosomes induced high levels of X1 (Fig. 6c), thus suggesting the presence of the triplex at the branching stage. To test this interpretation further, we used Prp16p as a specific immunoaffinity handle for X1 in genuine B\(^{\text{bt}}\) (prp16) spliceosomes, because Prp16p binds stably to this complex in a Prp2p-dependent manner just before branching\(^ {37}\), and dissociates just after branching\(^ {38}\), X1 immunoprecipitated efficiently from the B\(^{\text{bt}}\) (prp16) peak (Fig. 6c and Supplementary Fig. 5). By contrast, when we stalled spliceosomes before branching at the Prp2p stage, X1 did not efficiently immunoprecipitate from the B\(^{\text{bt}}\) peak, thus indicating that the immunoprecipitation of X1 via Prp16p was specific for the B\(^{\text{bt}}\) (prp16) complex (Fig. 6c and Supplementary Note 10).

To test for the triplex at the exon-ligation stage, we stalled spliceosomes just after exon ligation by addition of rPrp22p-K512A and isolated from glycerol gradients the resulting complexes (denoted P) that had not released the mRNA (Fig. 6a,b). UV irradiation of these complexes induced the X1 cross-link (Fig. 6d), thus suggesting the presence of the triplex at the exon-ligation stage. To test this implication further, we used Prp22p as a specific immunoaffinity handle for P-complex spliceosomes, because Prp22p binds stably to only this complex, in a manner dependent on Prp16p, Slu7p and Prp18p just before exon ligation\(^ {37,39}\), and dissociates just after exon ligation\(^ {40}\). When spliceosomes were stalled after exon ligation, we observed substantial immunoprecipitation of X1 from fractions of the P peak (Fig. 6d). By contrast, when spliceosomes were stalled just after branching, X1 immunoprecipitated three-fold less efficiently from equivalent fractions (Fig. 6d), even though the accumulating B\(^{\text{bt}}\) (prp16) complexes and associated X1 cross-link migrated in the same fractions (Fig. 6b); thus, the immunoprecipitation of X1 via Prp22p was specific for the P complex. We conclude that U80 and G52 also stack at the exon-ligation stage. These results indicate that the U6 catalytic triplex is present during both catalytic steps of splicing and provides a structural framework for the organization of the U6 ligands for the catalytic metals that function during both reactions\(^4\).
Figure 7 The U6 triplex promotes catalytic-metal binding during branching. (a) Diagram of the 3′S-PS(3′P) pre-mRNA. (b) Denaturing PAGE analysis of splicing of the 3′S-PS(3′P) pre-mRNA catalyzed by affinity-purified spliceosomes in the presence of the indicated combinations of metals. In b, the band marked with an asterisk results from exonucleolytic degradation that stops at the sulfur. (c) Quantification of Mn2+-potentiation of Cd2+-dependent rescue of branching of the 3′S-PS(3′P) pre-mRNA by spliceosomes containing the indicated U6 variants. Mn2+ potentiation is quantitated relative to Mg2+. Bars indicate ranges of two technical replicates. (d) Titration curves showing efficiency of Cd2+-mediated rescue of branching of the 3′S-PS(3′P) pre-mRNA by affinity-purified spliceosomes reconstituted with the indicated U6 variants. Hill fits to the data, assuming one rescuing metal, are shown (solid lines). The apparent transition midpoints are indicated below the label of each U6 variant. Error bars, s.d. of three independent replicates. (e,f) Diagrams showing predicted catalytic-metal binding by spliceosomes containing U6 WT (e) or U6 G52A (f). Relevant U6 ligands and the nucleophile are colored red. Metals are colored magenta (Cd2+) and blue (Mn2+), and their interactions with specific U6 ligands are depicted as dashed lines, with differential shading intensity illustrating differences in the expected strength of interaction with oxygen versus sulfur, as inferred from studies with model compounds.37 Shading of metals bound at M1 and M2 is further adjusted to reflect experimental observations. Br. A, branch-site A nucleophile.

The U6 triplex promotes catalytic-metal binding

The U6 triplex provides a structural mechanism for positioning the two catalytic metals4 in a proper configuration for phosphoryl transfer (Fig. 1). To determine whether the U6 triplex promotes catalytic-metal binding, we tested whether mutations predicted to disrupt the triplex impair catalytic-metal binding. The mutations G52A and A53U in U6 confer lethality or a growth defect in vivo (Fig. 3) and cause exon-ligation defects in vitro (Supplementary Fig. 6 and Supplementary Note 1); we infer that these phenotypes result from disruption of their base-triple interactions with the AGC triad. We assessed the impact of these mutations on catalytic-metal binding during branching by using a pre-mRNA substrate that bears sulfur substitutions at both the leaving group and the nonbridging pro-Rp phosphoryl oxygen at the 5′ splice site (3′S-PS(Rp)), which disrupts interactions with both catalytic Mg2+ ions4 (Fig. 7a). In a background of Mg2+, these interactions can be rescued by binding of two thiophilic Cd2+ ions at high concentrations.4 In a background of Mn2+, which is more thiophilic than Mg2+, Cd2+ can rescue at lower concentrations, because Mn2+ can populate one site (M2) and enable high-affinity binding of Cd2+ to the other site, M1 (ref. 4; Fig. 7c). Thus, at limiting Cd2+ concentrations (for example, 10 μM), Mn2+ potentiates the efficiency of branching for the 3′S-PS(Rp) substrate (compared to the efficiency observed in Mg2+; Fig. 7b,c). Remarkably, in a background of Mn2+, mutations in U6 predicted to destabilize the base-triple interactions (G52A and A53U) compromised Cd2+-dependent rescue of the 3′S-PS(Rp) substrate (Fig. 7b,c). Indeed, compared to U6 wild type, U6 G52A increased the apparent transition midpoint for Cd2+ rescue of the 3′S-PS(Rp) substrate by more than ten-fold (Fig. 7d). These data indicate that destabilization of the U6 G52-U6 G60–U22 base triple disrupted metal binding at the catalytic core (Fig. 7f). In agreement with this interpretation, the strong G52A-induced defect in branching at low Cd2+ concentrations, relative to branching in wild type, was almost completely suppressed at high Cd2+ concentrations (Fig. 7d). In contrast, U6 U80C, predicted to maintain the proper configuration of the U6 triplex,11 did not compromise Cd2+ rescue of the 3′S-PS(Rp) substrate at limiting Cd2+ (Fig. 7c) and did not affect the transition of these mutations on catalytic-metal binding during branching by using a pre-mRNA substrate that bears sulfur substitutions at both

Figure 8 The U6 triplex promotes exon ligation in vitro. (a) Configuration of the C288•C358-G385 base triple in the group II intron (PDB 4FAQ), equivalent to the spliceosomal U6 A53•U6 A59–U23 base triple. Left, side view; right, top view. (b) Denaturing PAGE analysis of splicing of ACTJ pre-mRNA in extracts reconstituted with the indicated U6 variants. Uppeercase indicates wild-type allele. Lowercase indicates mutant allele. (c) Quantification of exon ligation for the indicated U6 variants, normalized to wild-type U6; exon ligation was calculated as mRNA/ariat intermediate15. Error bars, s.d. of two independent experiments and two technical replicates for each experiment. The efficiency of branching was within 15% of wild type for all U6 variants (quantification not shown). Full gel is in Supplementary Figure 8i.
The U6 triplex promotes exon ligation

Because our cross-linking experiments implied that the triplex is present during exon ligation (Fig. 6d), we tested whether exon ligation required the triplex. Of the bases involved in the triplex, only A59 shows a specific defect in exon ligation when mutated18. Our in vivo results suggest that A59 forms a base-triple interaction with A53, similar to that observed for the equivalent positions in the group II intron (Figs. 3c and 8a). Therefore, we asked whether the exon-ligation defects conferred by U6 A59 mutations could be suppressed by mutations at U6 A53. Strikingly, the A53C mutation strongly improved exon-ligation efficiency for both A59U and A59G (Fig. 8b,c). Suppression was allele specific, because A59G was enhanced by rather than suppressed by other A53 mutations, and A59U was only mildly suppressed by other A53 mutations. Importantly, the observed suppression was also position specific, because neither A59G nor A59U was substantially suppressed by a mutation at the neighboring G52 (Fig. 8b,c), which nevertheless suppressed the exon-ligation defect conferred by a mutation at its base-triple partner G60 (Supplementary Fig. 7a,b). A53C also suppressed the exon-ligation defect of compromised pre-mRNA reporters both in vitro and in vivo (Supplementary Fig. 7d,e). Overall the allele- and position-specific suppression of exon-ligation defects that we observed in vitro explicitly paralleled the suppression that we observed in vivo (Fig. 3a,c). We infer that the in vitro suppression, as that in vivo, reflects the formation of a group II–like base triple that includes an interaction between A53C and the backbone of A59 (Fig. 8a). Together, these results indicate that the U6 triplex not only forms at the exon-ligation stage (Fig. 6d) but also functions at this catalytic stage both in vivo and in vitro.

DISCUSSION

Here we provide evidence for the formation of a catalytic triplex in the spliceosome, analogous to the metal-binding catalytic triplex of group II introns (Fig. 1). Specifically, despite the dynamic nature of the AGC triad4,5, the role for the triad in catalysis5 and the unusual and discontinuous nature of the group II intron triplex7,8, U6 mutations within the ACAGAGA and ISL bulged elements suppressed growth defects of AGC-triad mutants in the context of U6–U6 helix Ib in an allele- and position-specific manner at all three triplexes, thus providing independent and corroborative evidence for tertiary interactions between these highly conserved U6 residues (Figs. 2 and 3). By site-directed cross-linking, we revealed physical evidence for the U6 triplex (Fig. 4) and showed that, strikingly, the triplex forms upon NTC binding (Fig. 5), before the last ATP-dependent activation step, thus suggesting that the catalytic core is already formed at the precatalytic stage. Finally, we established that the catalytic triplex is present at both catalytic stages of splicing (Fig. 6), promotes both branching and exon ligation and has an essential role in positioning catalytic metals (Figs. 7 and 8). These data establish clear evidence that a catalytic triplex forms at the heart of the active spliceosome. The triple helix provides a mechanism by which the spliceosome structures U6 snRNA in a conformation competent to bind the two divalent metals that catalyze splicing4. Furthermore, the triplex provides a rationale for how the spliceosome recruits the pre-mRNA reactive phosphates to this metal-binding core for catalysis (described below). Finally, our results extend the deep mechanistic parallels between the spliceosome and group II introns and further support the likelihood of a common evolutionary precursor5.

The AGC triad was identified early on as a strictly conserved element highly sensitive to mutation15 and was later shown to base-pair with U4 (ref. 25) in assembling spliceosomes and with U2 in activated spliceosomes15,17,19. With this work, we show that in activated spliceosomes the triad interacts not only with U2 but also with distal regions in U6 to yield base triples (Figs. 2–4). Indeed, the triplex, with the AGC triad at the backbone, may explain the asymmetric phenotypes in U2–U6 helix Ib—the strong phenotypes in U6 and the weak phenotypes in U2 (refs. 15,19)—an asymmetry that is mirrored in the U6–U6 triplex interactions.

Because the two metal ions required for splicing catalysis are bound by the AGC triad and the bulge of the U6 ISL4, the catalytic triplex provides a mechanism to juxtapose these two metal–ion–binding sites in U6 for two-metal-ion catalysis. Indeed, our results indicate that the integrity of the U6 triplex is essential for proper metal positioning by the catalytic core, as observed during branching (Fig. 7).

The catalytic triplex also provides a mechanism for recruiting the 5′ splice site to the metal-binding core. The 5′ splice site is defined through base-pairing interactions with the 5′ end of the ACAGAGA sequence of U6 (refs. 21,22). Because the 3′ end of the ACAGAGA sequence participates directly in the triplex (Fig. 1a), the triplex probably recruits the U6–5′-splice-site interaction to the catalytic core for branching. The timing of triplex formation supports this hypothesis. By site-directed cross-linking, we found that the triplex forms before the final catalytic activation step of the spliceosome, mediated by Prp2p; the triplex forms after the release of U4 snRNA from U6 and upon binding of the NTC and associated factors to the spliceosome (Fig. 5). Because the NTC consolidates interactions of the exonic region of the 5′ splice site with U5 and promotes correct pairing between the intronic region of the 5′ splice site and the ACAGAGA sequence of U6 (ref. 41), our results suggest coupling between binding of the 5′ splice site and formation of a catalytic configuration of the RNA core. Similarly, during the exon-ligation stage the integrity of the triplex may be coupled to recruitment of the 3′ splice site (Supplementary Note 12). Such coupling highlights the dependence of the catalytic core on substrate binding and may allow sampling and proofreading of the substrate through inspection of the catalytic core at subsequent steps in the splicing pathway. Indeed, downstream of NTC binding, both Prp2p and Prp16p have been implicated in proofreading, in part through destabilization of a weakly formed catalytic core17,42,43.

Before branching, Prp2p has been implicated in destabilizing the catalytic core, including the triplex, to promote on-pathway rearrangements41. Our in vitro analysis indicates that the U6 triplex must reestablish to promote branching (Figs. 6c and 8 and Supplementary Fig. 7). Cwc2p stabilizes the triplex before Prp2p activity (Supplementary Fig. 4), and after Prp2p action, when Cwc2p becomes essential for branching, Cwc2p interacts with the U6 ISL5. Thus, Cwc2p may act to restabilize the catalytic core for branching catalysis.

After branching, Prp16p has been implicated in destabilizing the catalytic core17, probably to accommodate substrate repositioning—the replacement of the branch site with the 3′ splice site at the catalytic metal–binding site4. We found that, consistently with a role in destabilizing the helix Ib component of the catalytic core17, mutations predicted to destabilize the U6 triplex (U6 A53C and U6 A53G) suppressed a hypomorphic allele of prp16 (Supplementary Fig. 7c). These data imply that Prp16p destabilizes the U6 triplex after the spliceosome has catalyzed branching. Interestingly, the transition from branching to exon ligation requires disruption of the U6–5′-splice-site pairing44. Thus, our data also suggest that disruption of the U6–5′-splice-site pairing after branching is coupled to destabilization of the U6 triplex.
As we have found for other elements of the catalytic core, here we found evidence that the triplex is destabilized only transiently during the catalytic stage; the triplex reforms at the exon-ligation stage (Fig. 6d and Supplementary Fig. 7) and is necessary for efficient exon ligation (Fig. 8). Interestingly, although the catalytic triplex of the group II intron similarly appears to function in both catalytic conformations, the group II triplex has also been suggested to disrupt transiently between the two steps of splicing, to allow substrate repositioning.

Overall our results indicate that the splicedecosomal catalytic core shares remarkable structural and functional similarity, if not also evolutionary origins, with the catalytic core of group II self-splicing introns. Thus, these data underscore evidence that splicing catalysis is performed by an RNA-based catalytic core stabilized by a complex protein scaffold.

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

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AUTHOR CONTRIBUTIONS
S.M.F., M.A.M., J.P.S. and J.A.P. designed the study; M.A.M. performed the in vivo genetics; S.M.F. performed the in vitro cross-linking and biochemical experiments as well as experiments in Supplementary Figure 7, which were initiated by M.A.M.; and S.M.F. and J.P.S. wrote the paper with input from M.A.M. and J.A.P.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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**ONLINE METHODS**

**Yeast strains and plasmids.** To test combinations of alleles in U2 and U6, we used strain yHM118, in which the endogenous genes encoding U2 and U6 were deleted and viability rescued with the URA3-marked plasmid pU2U6C15. To test, in addition, mutant versions of U4, we used strain yPJS628, containing p2U2U6U4 (ref. 19). The HIS3-marked plasmid pPJS216 encodes U2 (ref. 45); the TRP1-marked plasmid pPSX6 encodes U6 (ref. 15); and the ADE2-marked plasmid pPJS464 encodes U4 (ref. 19). Mutated U2, U6 and U4 plasmids, used for *in vivo* assays, were described previously15 or generated for this study by QuikChange mutagenesis (Stratagene) and verified by sequencing. For *in vitro* transcription, mutant U6 templates were generated by QuikChange mutagenesis (Strategene) from pPSJ888, which encodes wild-type U6 downstream of a T7 promoter49.

*In vitro* splicing was performed with extracts prepared from the following strains: yPJS1405 (PRP19 with a C-terminal biotin tag); yPJS1489 (CWC2-TAP; Open Biosystems); yPJS1492 (CWC25-HA, same as ySCC25 (ref. 51)); yPJS1510 (PRP19-HA, same as ySCC1 (ref. 36)).

**Plasmid-shuffle growth assays.** Variants of pPSJ216 and pPSX6 were cotransformed into yHM328 and selected on plates lacking histidine and tryptophan. To test for interactions with U4, variants of pPSJ216, pPSX6 and pPJS464 were cotransformed and selected on plates lacking histidine, tryptophan and adenine. Duplicate colonies were grown in liquid medium lacking the appropriate nutrients to an OD600 of approximately 0.8 and then spotted onto medium containing 5-fluoroorotic acid (5-FOA) to counter-select for the wild-type plasmid42. Cells were grown at 30 °C for 3–10 d for phenotypic analysis.

To test for a genetic interaction between PRP16 and the U6 triplex, variants of pPSX6 and wild-type pPJS216 were cotransformed into yHM187 (prp16-302 (ref. 45)) or yHM118 (PRP16) and selected on plates lacking histidine and tryptophan. Duplicate colonies were grown in liquid medium lacking histidine and tryptophan to an OD600 of approximately 0.4 and then spotted onto medium containing 5-FOA to counter-select for the wild-type plasmid42. Cells were grown at 30 °C and photographed after 6 d.

The growth spots presented reflect two related growth parameters. First, the spots reflect colony size and consequently colony growth. Second, the spots reflect the number of colonies growing in the spot. Before equal numbers of cells were spotted onto 5-FOA to counter-select against cells that retained the wild-type plasmid, so a large fraction of cells retained the wild-type plasmid, and consequently died on 5-FOA, thus leading to spots with reduced colony density.

**Copper growth assays.** yPJS1035 (ref. 45) was first transformed with ACT1-CUP1 reporter variants on LEU2-marked plasmids (derived from pPJS1920 (ref. 45)) and then cotransformed with pPSJ216 and pPSX6 variants and selected on plates lacking histidine, tryptophan and leucine. Cotransformants were streaked onto medium lacking leucine and containing 5-FOA52 and grown for 3 d at 30 °C. Colonies were then purified on medium lacking minimum leucine. Duplicate colonies were grown in liquid medium lacking leucine to an OD600 of approximately 0.2 and spotted on medium lacking leucine containing various concentrations of copper sulfate. Cells were grown at 30 °C and photographed after 3 d.

**Splicing extracts, U6 depletion and reconstitution, and in vitro splicing.** Splicing extracts were prepared from yPJS1405 unless otherwise noted. Preparation of splicing extracts, *in vitro* splicing, U6 depletion and reconstitution, affinity purification via Prp19p and incubation of affinity-purified splicesomes were performed essentially as described11. For cross-linking experiments, U6 was added back to a final concentration of 2–4 nM. *In vitro* splicing in extract was carried out for 25 min at 20 °C unless otherwise noted. Where noted, 20% of the standard splicing-extract volume was replaced with recombinant, dominant-negative Prp2p, K252A, Prp16p K379A or Prp22p K512A in buffer D, at a final concentration of approximately 40–80 ng/µL.

**Radiolabeled ACT1 pre-mRNA was synthesized by *in vitro* transcription.** Fluorescently labeled UBC4 pre-mRNA was synthesized by splint-mediated ligation, essentially as described13. Radiolabeled 3′-5′-S-P(γR) and 3′-O-PO4 pre-mRNAs were synthesized as described4.

In Figure 7b,c, splicesomes from extracts reconstituted with the indicated U6 variants were assayed on the 3′-5′-S-P(γR) substrate, affinity purified via Prp19p, and incubated in the absence of ATP in buffer PK (3% PEG, 60 mM KPO4, pH 7.0) with 1 mM MgCl2 or 1 mM MnCl2 in the presence of various amounts of CdCl2. In Figure 7d, splicesomes from extracts reconstituted with the indicated U6 variants were assayed on the 3′-5′-S-P(γR) substrate, affinity purified via Prp19p, and incubated in the absence of ATP in buffer PK (pH 7.0) with 1 mM MnCl2 in the presence of various amounts of CdCl2.

**Preparation of recombinant proteins.** Recombinant Prp16p, Prp22p and Prp2p were expressed in *Escherichia coli* Rosetta2 DE3pLysS cells (Novagen), essentially as described44. Recombinant Prp2p was expressed in the presence of 2% ethanol at 17 °C for 17 h. All proteins were purified by Ni2+–NTA affinity chromatography and subsequent glycerol gradient and were dialyzed against buffer D before use.

**Synthesis of U6 snRNA for *in vitro* reconstitution.** Synthetic U6, with modifications for cross-linking, was constructed by splint-mediated ligation with RNA oligonucleotides corresponding to the following U6 residues: 1–51, 52–79 and 80–112. Oligonucleotides 52–79 and 80–112 were purchased from Dharmacon, deprotected and used as supplied. U6 1–51 was generated from full-length, *in vitro*–transcribed U6 by DNAzyme-mediated cleavage after A51 with U6 A51 DNAzyme (5′-CTGATCTACCGCCGGAGCACTGTATTTG-3′, a modified 8–17 DNAzyme65). For a typical cleavage reaction, 2–4 nmol of gel-purified, full-length, transcribed U6 was mixed with a 1.2-fold molar excess of U6 A51-Dzyme (IDT) in cleavage buffer (100 mM Tris-Cl, pH 7.4, and 50 mM NaCl) and then annealed by heating to 90 °C and subsequent cooling to room temperature for 25 min. After hybridization, cleavage was performed overnight at 37 °C in cleavage buffer supplemented with 10 mM MgCl2. U6 1–51 product was gel purified, and the 3′-cylic phosphate resulting from DNAzyme cleavage was removed by incubation with polynucleotide kinase (NEB) at 37 °C for 2–4 h. The U6 52–79 oligonucleotide was 5′ phosphorylated with [γ-32P]ATP (PerkinElmer, 6,000 Ci/mmol) before ligation. Ligation of the three oligonucleotides and purification of ligated U6 was performed by splint-mediated ligation, as described46.

Wild-type and mutant U6 used for the *in vitro* splicing experiments were synthesized by *in vitro* transcription according to standard procedures with templates derived from linearization of pPSJ888 with Dral, as described48.

**UV cross-linking.** For cross-linking60, splicing reactions (20–40 µL) were transferred to a round-bottom 60-well plate (Fisher Scientific) on ice and irradiated with a 100-W UV lamp (FRUVLS-80, Fisher Scientific) set at 365 nm and placed at approximately 4 cm from the bottom of the well so that light would shine perpendicularly on the plane of the sample. Irradiation was performed under an aluminum-foil tent at 4 °C for 90 min. After cross-linking, RNA was analyzed by 8% denaturing PAGE.

**Analytical digestions.** For nuclease P1 digestion, gel-purified X1 RNA (3,000–10,000 c.p.m.) was incubated with nuclease P1 (1U/µL; US Biological) in a 4–µL reaction at 37 °C for 18–20 h. For alkaline hydrolysis, gel-purified RNA (3,000–10,000 c.p.m.) was incubated with 20 mM NaOH at 90 °C for 3 h. After digestions, RNA was analyzed by 20% denaturing PAGE.

**NCTC depletion and complementation.** Splicing extracts were prepared from a strain containing Prp19p-HA (yPJS1510). For depletion, 200 µL of freshly prepared extract was incubated at 4 °C for 2 h with 150 µL of protein A-Sepharose
Preparation of B\textsuperscript{44}, B\textsuperscript{P} (prp16) and P complexes. To isolate B\textsuperscript{44} or B\textsuperscript{P} (prp16) complex splicing reactions, splicing reactions (80 µL) were performed with UBC4 pre-mRNA, labeled fluorescently with Cy3 at the seventh residue of the 5′ exon[25], and extracts from yPS1492. The splicing reactions were supplemented with rPrp2p K522A, to accumulate B\textsuperscript{44}, or rPrp16p K379A, to accumulate B\textsuperscript{P} (prp16), and were loaded on 11-mL 15–40% glycerol gradients in buffer G50 (20 mM HEPES, pH 7.9, 50 mM KCl and 0.5 mM EDTA). The gradients were centrifuged at 234,325 g in a Beckman SW 41 rotor for 1.5 h. Fractions were collected by hand from the top of the gradient; RNA was extracted from the fractions and analyzed by denaturing PAGE. The splicing species were visualized by detection of the Cy3 label with a Typhoon Trio phosphorimager (Amersham Biosciences).

Affinity pulldowns. For affinity purification of splicing complexes associated with biotinylated Prp19p, splicing reactions were diluted 1-fold to 2-fold in buffer D and incubated for 1–3 h at 4 °C with 0.1–0.2 volumes of streptavidin–agarose slurry (Thermo Scientific) prewashed twice with 25–50 volumes of IPP\textsubscript{150} (10 mM Tris–HCl, pH 8, 150 mM NaCl, and 0.1% NP-40 substitute (Fluka)). Use of Streptavidin–agarose for immunoprecipitation of biotinylated Prp19 was verified previously[4]. After incubation, beads were washed at 4 °C twice with 50 volumes of buffer DK\textsubscript{150}, supplemented with 0.01% NP-40 substitute.

For immunoprecipitation of Prp22p-associated splicing complexes, peak glycerol-gradient fractions were diluted 1:1 in buffer DK\textsubscript{150} and incubated for 1 h at 4 °C with 0.1–0.2 volumes of protein A–Sepharose slurry conjugated to anti-HA antibodies (12CA5, University of Chicago Fitch Monoclonal Antibody Facility, 2 mg/mL of bead slurry)[35]. After centrifugation at 800g for 4 min, the supernatant was used as NTc-depleted extract.

For complementation, the NTc complex was isolated from yeast splicing extracts prepared from yPS1510, as follows. The splicing extract was precipitated with 40% ammonium sulfate to obtain the 40P fraction, as described[37]. The 40P fraction (280 mg) was resuspended in 1 mL of buffer D supplemented with 60 mM potassium phosphate, pH 7.0 (buffer DK), and was incubated at 4 °C for 2 h with 200 µL slurry of protein A–Sepharose conjugated to anti-HA antibodies (12CA5, 2 mg/mL of bead slurry; cross-linked with DMP (Sigma))\textsuperscript{38}. Beads were then washed three times at 4 °C with 6 mL of buffer DK supplemented with 0.01% NP-40 substitute (Fluka), brought to room temperature and washed with an additional 6 mL of the same buffer. The bound NTc was eluted with 300 µL of 0.6 mM HA peptide at room temperature with rotation for 30 min. The eluate was concentrated ten-fold with a Vivaspin-500 column (Sartorius), flash frozen and stored at –80 °C. Typically 1–2 µL of the concentrated NTc was used for complementation of a 10-µL splicing reaction.

Affinity pulldowns. For affinity purification of splicing complexes associated with biotinylated Prp19p, splicing reactions were diluted 1-fold to 2-fold in buffer D and incubated for 1–3 h at 4 °C with 0.1–0.2 volumes of streptavidin–agarose slurry (Thermo Scientific) prewashed twice with 25–50 volumes of IPP\textsubscript{150} (10 mM Tris–HCl, pH 8, 150 mM NaCl, and 0.1% NP-40 substitute (Fluka)). Use of Streptavidin–agarose for immunoprecipitation of biotinylated Prp19 was verified previously[4]. After incubation, beads were washed at 4 °C twice with 50 volumes of IPP\textsubscript{150}.

For immunoprecipitation of Prp16p-associated splicing complexes, peak glycerol-gradient fractions were diluted 1:1 in buffer DK\textsubscript{150} (20 mM HEPES, pH 7.9, 150 mM KCl and 60 mM potassium phosphate, pH 7.0), and incubated for 1 h at 4 °C with 0.1–0.2 volumes of protein A–Sepharose slurry conjugated to affinity-purified anti-Prp16p antibodies (4–6 mg/mL of bead slurry; gift from C. Guthrie[2]). After immunoprecipitation, beads were washed at 4 °C twice with 50 volumes of buffer DK\textsubscript{150} supplemented with 0.01% NP-40 substitute.

For immunoprecipitation of Prp22p-associated splicing complexes, peak glycerol-gradient fractions were diluted 1:1 in buffer DK\textsubscript{150} and incubated for 1 h at 4 °C with 0.1–0.2 volumes of protein A–Sepharose slurry conjugated to affinity-purified anti-Prp22p antibodies (1.5 mg/mL of bead slurry; gift from B. Schwer[49]). After immunoprecipitation, beads were washed at 4 °C twice with 50 volumes of buffer NET-2 (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.05% NP-40 substitute).

Cwc2 depletion and complementation. Splicing extracts prepared from yPS1489 (Cwc2-TAP) were depleted of Cwc2p essentially as described[2].

For expression of rCwc2p, the Cwc2 sequence was amplified from a plasmid containing the Cwc2 ORF (bJPS2509, Open Biosystems) and cloned into pET15b (bJPS81) with the Ndel and BamHI cloning sites to give bJPS2621. The cloned plasmid was verified by sequencing. Expression of His\textsuberscript{6}-rCwc2p was performed in E. coli Rosetta2 DE3 pLysS cells. Cells were grown at 37 °C to OD\textsubscript{600} = 0.8, and expression was induced with 0.5 mM IPTG at 30 °C for 3 h. Cells were lysed with a French press, and His\textsuberscript{6}-rCwc2p was purified by Ni\textsuperscript{2+}–NTA affinity chromatography, but binding and washing were performed by gravity flow. After elution from the Ni\textsuperscript{2+}–NTA resin, the protein was further purified by glycerol-gradient centrifugation to more than 95% purity (as estimated by Coomassie blue staining) and dialyzed against buffer D. For complementation of Cwc2p-depleted extracts, rCwc2p was added back at a final concentration of 2–4 µM.

Original images of gels and autoradiographs used in this study can be found in Supplementary Figure 8.