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Cwc2 and its human homolog RBM22 promote an active conformation of the spliceosome catalytic center

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1st Editorial Decision 02 September 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. I have now received the final report from the three referees who evaluated your study and I enclose their comments below. As you will see they are generally positive regarding the study and require that several issues are addressed in a revised manuscript. As a consequence given the support form the referees I would like to invite you to submit a revised version of the manuscript.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees’ comments. Please note that when preparing your letter of response to the referees’ comments that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal
REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The manuscript by Rasche et al. characterizes the previously unknown role for Cwc2, a Nineteen Complex protein, in the first step of pre-mRNA splicing. They use glycerol gradient sedimentation to demonstrate that the spliceosome can still fully assemble on pre-mRNA in yeast extracts immuno-depleted of Cwc2. However, denaturing gels show that splicing does not occur in the absence of Cwc2. Using UV crosslinked samples analyzed by primer extension they are able to map Cwc2 at the U6 ISL, near the U6 ACAGAGA box, and downstream of the 5' splice site. These sites are further supported by the chemical protection data and place Cwc2 near the catalytic center of the spliceosome. They further show similar results with the human homolog, RBM22, although the splicing defect is not as severe in RBM22 immuno-depleted HeLa extract as in the yeast extract. The experimental data suggest a role for Cwc2 in positioning the pre-mRNA and spliceosomal RNA that is essential for yeast and highly important in humans.

The experiments are performed and described well and the analysis is thorough. The discussion nicely ties in previous literature while hypothesizing about Cwc2's mechanistic role.

I have only one minor question, what do the authors make of the fact that splicing can still proceed in the absence of RBM22 and not in the case of Cwc2? They nicely show RBM22 also shares homology with part of another yeast NTC protein and I might have hypothesized that this would have made its absence more deleterious, especially considering the previous reference to Ecm2's role in U6/U2 pairing. Is the discrepancy in splicing efficiency merely due to the different temperatures the reactions were performed at or different pre-mRNAs, or do the authors think the more complex human spliceosome is able to compensate for the loss of RBM22?

Referee #2 (Remarks to the Author):

The manuscript "Cwc2 and its human homolog RBM22 promote an active conformation of the spliceosome catalytic center" by Rasche, et al explores two critical problems for the field of pre-mRNA splicing: 1) What are the roles of the different spliceosome associated proteins? and 2) What structures the RNA in the active site of the complex? They focus on an essential protein but previously uncharacterized protein, Cwc2, and take a biochemical approach to answering these questions using an in vitro spliceosome purification system, which they have pioneered. Using a depletion/add-back experiment, they demonstrate that Cwc2 and it's human homolog is required for first-step splicing chemistry, but not for assembly of the spliceosome to the point where the protein Prp2 functions to "activate" the spliceosome for catalysis. This result is important, because it shows that something more is required for first-step chemistry than the action of Prp2, and that Cwc2 must place a role in that "something more".

In the remaining portion of the paper, the authors then use a combination of crosslinking and chemical modification mapping to examine Cwc2 interactions with RNAs in the active site of the spliceosome prior to and following the first step of splicing chemistry. They find crosslinks to U6 snRNA and pre-mRNA that can be pulled down by Cwc2 (and its human homolog) at both assembly stages. With Cwc2, they also see changes in protection from chemical modification of U6 snRNA when the protein is not present in the complex. However, while these data are very clear, and point to the presence/function of Cwc2 near the catalytic center of the spliceosome, I believe that the authors over-interpret the crosslinking data, in particular, to conclude that Cwc2 is directly responsible for all crosslinks. It is possible, even likely, that some crosslinks represent RNA/RNA or RNA/protein interactions that are induced upon spliceosome assembly, and that Cwc2 pulls these crosslinks down via its crosslink at another point within the RNA. The authors argue that the loss of protections in the absence of Cwc2 indicate that it is contacting a good portion of the RNA in the likely active site of the spliceosome. Again, the data do not distinguish between this possibility, or whether Cwc2 stabilizes some sort of closed conformation of the active site from a distance. With these two caveats in mind, the somewhat lengthy discussion placing Cwc2 at the heart of the
spliceosome is unwarranted.

Specific comments/questions:

1. The text refers to "lanes" in Figure 1C, which is a graph. It should refer to specific traces in the graph instead.

2. In figure 1C, why purify a spliceosome arrested by a Prp2 mutation to analyze the effect of depleting Cwc2? At what point does spliceosome assembly arrest in extracts simply lacking Cwc2, like those used for experiments shown in 1B?

3. Is it the author's interpretation that the gradient peaks in Figure 1C labeled +Prp2, Spp2, Δ/+Cwc2 represent B* complex? What happens if the ΔCwc2 complex is supplemented with Cwc25?

4. In Figure 2, it is surprising that the U6 snRNA in the IP is shifted upward, whereas it is not in the input. If the shift is due to incomplete digest of the Cwc2 protein, shouldn't that occur in both samples?

5. In Figure 3, it is unclear what the RNA only control represents. From the text, it appears that U6, U2 and U5 snRNAs as well as pre-mRNA are present. After phenol-chloroform extraction, how were these RNAs treated? Is it possible that the RNAs are base-paired with each other? What could the apparent U6 structure in the 10-30nt and 85-90nt regions represent?

6. In Figure 5, how do the authors ensure that equal amounts of splicing reaction are loaded in the different lanes? In the text, they call the loss of splicing in the absence of RBM22 significant. Can they quantify the difference? Also, do they know the identity of the band that appears above mRNA product when RBM22 is added back to the reaction? It is interesting that it comes and then goes during the reaction.

Referee #3 (Remarks to the Author):

Rasche and colleagues report that depletion of the spliceosomal protein Cwc2 from yeast extracts leads to the assembly on a pre-mRNA of catalytically inactive complexes that contain all the major spliceosomal components and that even undergo Prp2-mediated conformational rearrangements. UV-mediated crosslinking and chemical probing experiments indicate that Cwc2 contacts/protects regions of U6 snRNA at or near secondary structure elements known to be important for catalytic activation of the spliceosome. A parallel study with the human homologue of Cwc2, RBM22, provides evidence for a conserved function of these proteins in mammalian cells.

These results are important because they provide clear evidence that Cwc2/RBM22 perform an essential conserved function and support the notion that these proteins provide a critical set of contacts that coordinate the spatial orientation of reactive groups leading to catalytic activation of the spliceosome. The data are of the excellent quality and rigor characteristic of the Lührmann's group, and fully support the conclusions. The manuscript is clearly written and the discussion enriching. I recommend publication in EMBO J.

The authors may want to indicate whether Cwc2 depletion leads to co-depletion of other components of the NTC-related complex or even of the NTC complex itself (e.g. of the known interactor Isy1), an information that they may have available through their proteomic analysis of ΔCwc2 complexes. This information would be of interest even if the efficient rescue of depleted extracts with purified recombinant Cwc2 shows that these proteins are not essential for catalysis.

Reply to Referee Comments

1st Revision - authors' response 21 October 2011
At the outset we would like to express our appreciation of the referees' positive overall assessment of our manuscript and their constructive criticisms, which have certainly helped us to improve it. Below we reply to their points in detail.

Referee 1:

I have only one minor question, what do the authors make of the fact that splicing can still proceed in the absence of RMB22 and not in the case of Cwc2? They nicely show RMB22 also shares homology with part of another yeast NTC protein and I might have hypothesized that this would have made its absence more deleterious, especially considering the previous reference to Ecm2's role in U6/U2 pairing. Is the discrepancy in splicing efficiency merely due to the different temperatures the reactions were performed at or different pre-mRNAs, or do the authors think the more complex human spliceosome is able to compensate for the loss of RMB22?

In our view, the data show a very substantial inhibition of splicing when the reaction mixture is depleted of RBM22, and we cannot immediately see why the referee believes that the opposite is the case. The manuscript states that the depletion depresses the level of splicing. To make this clearer and to provide a supporting argument, we have now calculated the efficiency of splicing, defined for this purpose as the ratio of the mRNA signal (at a given time point) to the initial pre-mRNA signal (at time 0). The residual splicing activity in the absence of RBM22 was usually 10–20% of the activity in the mock-depleted extract. Specifically, in the gel presented in Figure 5B, the splicing activity of the DRBM22 extract was 15% of that of the mock-depleted extract (lane 5 versus lane 2). This quantification has been added in the revised manuscript (legend of Figure 5B and Supplementary Methods, p. 5, para. 1). The remaining splicing activity of the depleted extract is presumed to be due to minor residual amounts of endogenous RBM22 that were at the lower limit of detection by the antibody in the immunoblot.

Referee 2:

i) However, while these data are very clear, and point to the presence/function of Cwc2 near the catalytic center of the spliceosome, I believe that the authors over-interpret the crosslinking data, in particular, to conclude that Cwc2 is directly responsible for all crosslinks. It is possible, even likely, that some crosslinks represent RNA/RNA or RNA/protein interactions that are induced upon spliceosome assembly, and that Cwc2 pulls these crosslinks down via its crosslink at another point within the RNA.

In principle, the alternative possibilities raised by the referee are plausible. Nevertheless, a consideration of all the evidence leads us to believe that our interpretation of our Cwc2–RNA crosslinking data is correct (i.e., does not involve over-interpretation of these data) for the following reasons:

1. First of all, the efficiency of UV-induced protein–RNA crosslinking is quite low – in our experience it amounts at most to a few per cent and is usually about 1 to 5%. It is therefore, for statistical reasons, very unlikely that immunoprecipitated species containing Cwc2–U6 snRNA also contain significant quantities of a non-Cwc2 protein that crosslinked simultaneously to the same U6 snRNA molecule and was co-precipitated. Furthermore, our read-out for protein–RNA crosslinks (namely, reverse transcriptase stops) is not especially sensitive, so that any theoretically possible non-Cwc2–U6 snRNA crosslinks – assuming that the non-Cwc2 protein is crosslinked to U6 snRNA with an efficiency 10 to 100 times less – would be co-precipitated in a yield of only 0.1-0.01% based on U6 snRNA-linked Cwc2, and a contamination level as low as this would not be detected.

2. Quite apart from the theoretical consideration given in point (1), we can virtually exclude the possibility that our Cwc2–U6 snRNA crosslinked species are partly due to other, simultaneously crosslinked proteins by reference to the following control experiment: After UV irradiation of the purified B act complex, we disrupted it by treatment with a detergent and pulled down specifically the U6 snRNA by using a complementary oligonucleotide immobilised on Sepharose beads. The protein(s) associated with the U6 snRNA attached to the beads were digested with trypsin and analysed by mass spectrometry; this revealed
Cwc2 – and no other protein – crosslinked to U6 snRNA in the Bα′ complex. Thus, any additional protein possibly crosslinked simultaneously with Cwc2 to U6 snRNA must have been present in quantities so small as to have been undetectable by MS under our conditions. This control experiment has now been mentioned in the revised manuscript (p. 12, para. 1).

3. The probability that the Cwc2–U6 snRNA crosslink positions are partly due to UV-induced crosslinks of U6 snRNA with an additional (non-U6) RNA is very small, for statistical reasons analogous to those described for additional protein–RNA crosslinks in point (1) above. Furthermore, if U6 snRNA were indeed crosslinked in significant quantities (5% or more) to another RNA, then we would have detected this in the Northern blot of the isolated Cwc2–U6 snRNA crosslinked species, which however we did not (see Fig. 2).

4. Finally, we should emphasize that in the assessment of the reverse transcriptase stops that we observed in the control experiment after UV irradiation of U6 snRNA prepared in vitro (such as the RT stops between nucleotides C85 and A90; Fig. 3), we did not consider these to be potential Cwc2–U6 RNA stops, as stated in our revised manuscript (p. 12, para. 2).

   ii). The authors argue that the loss of protections in the absence of Cwc2 indicate that it is contacting a good portion of the RNA in the likely active site of the spliceosome. Again, the data do not distinguish between this possibility, or whether Cwc2 stabilizes some sort of closed conformation of the active site from a distance. With these two caveats in mind, the somewhat lengthy discussion placing Cwc2 at the heart of the spliceosome is unwarranted.

We agree that the two caveats raised by the referee are valid ones; in fact we were aware of them and had endeavoured in the original manuscript to present the data and the conclusions drawn from them in a sufficiently cautious manner. We distinguish between two situations:

1. Enhanced accessibility of the nucleotides towards chemical probes in the immediate neighbourhood of a nucleotide of the U6 snRNA (or the pre-mRNA) to which we observed a Cwc2 crosslink (as was the case e.g. with U6-G39). In such cases we consider the most plausible possibility to be that Cwc2 is not only in contact with the crosslinked nucleotide, but also with further nucleotides in the immediate neighbourhood.

2. In the cases where we observed enhanced accessibility of nucleotides of a region of U6 snRNA in which no Cwc2 crosslink was detected (as for example the ACAGAGA box), we assessed the situation considerably more carefully and stated in our original manuscript, for example, that “the increased accessibility towards DMS and CMCT of the ACAGAGA box region in the absence of Cwc2 is consistent with the idea that Cwc2 may also interact with this area. However, in the absence of a Cwc2–ACAGAGA box crosslink, we cannot exclude the possibly that one or more additional proteins may contact the ACAGAGA box and that their recruitment to this site could be dependent on the presence of Cwc2” (p. 14, para. 2). A similar statement can also be found in our original Discussion (p. 20, para. 2). We believe that this statement is justified. As a further explanatory possibility, we have added the point raised by the referee (i.e., that “Cwc2 may stabilize some sort of closed conformation of the active site”) to the Discussion in the revised manuscript (p. 20, para. 2).

The text refers to "lanes" in Figure 1C, which is a graph. It should refer to specific traces in the graph instead

We have changed this in our revised manuscript and we have clarified the description of Figure 1C and 1D
In Figure 1C, why purify a spliceosome arrested by a Prp2 mutation to analyze the effect of depleting Cwc2? At what point does spliceosome assembly arrest in extracts simply lacking Cwc2, like those used for experiments shown in 1B? Is it the author's interpretation that the gradient peaks in Figure 1C labeled +Prp2, Spp2, DCwc2 represent B* complex? What happens if the DCwc2 complex is supplemented with Cwc25?

We have preferred to investigate the role of Cwc2 in the catalytic activation process using heat-inactivated splicing extracts from the prp2-1 strain because this procedure generally guarantees the isolation of biochemically highly homogeneous B* spliceosomes, which is important for meaningful subsequent reconstitution steps with recombinant step1 splicing factors. Using this system, we have also carried out the experiment requested by the reviewer, i.e. to complement purified ΔCwc2 B*ΔPrp2 spliceosomes which have undergone the Prp2 mediated remodelling step, with recombinant Cwc25 and have included this experiment as a new Figure 1E in the revised manuscript. As shown in Figure 1E, purified ΔCwc2 B*ΔPrp2 spliceosomes do not catalyze step 1 of the splicing reaction after incubation with Prp2, Spp2, ATP and Cwc25 (lane 7). As a control Cwc2 mock-depleted B*ΔPrp2 spliceosomes were active under the same conditions (lane 4). In conclusion, while the Prp2-remodeled ΔCwc2 B* (Prp2, Spp2, ΔCwc2) complex exhibits a similar S value as a wild type B* complex, it is nevertheless catalytically inactive. We have described this experiment appropriately in the revised version of our manuscript (p. 9, para. 2).

In Figure 2, it is surprising that the U6 snRNA in the IP is shifted upward, whereas it is not in the input. If the shift is due to incomplete digest of the Cwc2 protein, shouldn't that occur in both samples?

The difference between U6 signals in lanes 2 and 4 of Figure 2 may have at least two explanations. One possibility is that the efficiency of the proteinase K digestion might have been different in the input and IP samples because the input contains a much higher concentration of proteins compared to the IP reaction. In the input sample, incomplete digestion of Cwc2 crosslinked to U6 most probably led to the formation of the smear seen throughout the whole lane of the gel and in addition to the RNA band visible in the pocket of the gel (Figure 2 lane 4). A second reason is that in the input sample the fraction of U6 RNA crosslinked to Cwc2 is a minor fraction compared to uncrosslinked U6 RNA while in the IP sample the crosslinked U6-Cwc2 species (up shifted) was highly enriched. This behavior is more clearly observed in the crosslinking experiments performed with human complexes, where the amount of U6 RNA and thus also of the crosslinked U6-RBM22 species is much more abundant (compared to U6 and U6-Cwc2 crosslinked species in yeast extracts). In the input sample of Figure 6A lanes 1, two populations of U6 snRNA are visible and the U6 snRNA crosslinked to RBM22 was specifically enriched after IP (lane 3).

In Figure 3, it is unclear what the RNA only control represents. From the text, it appears that U6, U2 and U5 snRNAs as well as pre-mRNA are present. After phenol-chloroform extraction, how were these RNAs treated? Is it possible that the RNAs are base-paired with each other? What could the apparent U6 structure in the 10-30nt and 85-90nt regions represent?

Due to the lack of sufficient amounts of material in case of the yeast spliceosomal complexes, in vitro transcribed U6 snRNA was used as a naked RNA control. Therefore these lanes provide information only about potential intramolecular crosslinking events and UV-induced RNA damage of naked U6 snRNA (strand lesion, pyrimidine dimers, etc). The UV-induced stops in the 10-30nt and 85-90nt regions of in vitro transcribed U6 snRNA most likely represent crosslinks between adjacent nucleotides. On the other hand, for human complexes the RNA control was extracted with phenol-chloroform from purified B* and C complexes, respectively, including two subsequent chloroform extractions and then directly UV crosslinked in parallel to the RNP samples. We did not perform any sophisticated investigation of the RNA network prepared in this way, but we believe that core RNA-RNA interactions should be maintained in the deproteinized RNA network. We have
clarified this point in the revised manuscript (p. 26, para. 1). The extracted RNAs of the RNP samples were incubated for 1 min at 96 °C prior to primer-extension analysis, thus it is unlikely that the RNAs are base-paired with each other.

_In Figure 5, how do the authors ensure that equal amounts of splicing reaction are loaded in the different lanes?_

The reactions were set up in a very similar way using master mixes and quantified using a Cherenkov counter before loading the gel. In addition, equal volumes of each reaction were loaded into each pocket of the gel.

_In the text, they call the loss of splicing in the absence of RBM22 significant. Can they quantify the difference?_

See answer to reviewer 1

_Also, do they know the identity of the band that appears above mRNA product when RBM22 is added back to the reaction? It is interesting that it comes and then goes during the reaction_

The band mentioned above most likely represents the debranched lariat-intron. It often appears in our splicing reactions, including mock-depleted or non-depleted HeLa extracts, after 10-15 min incubation and then disappears at later time points, most probably due to complete degradation. The different behavior of this band between mock-depleted extracts and extracts supplemented with RBM22 shown in Figure 5B is likely due to the slightly different kinetics of accumulation and degradation of the spliced out lariat-intron.

Referee 3:

_The authors may want to indicate whether Cwc2 depletion leads to co-depletion of other components of the NTC-related complex or even of the NTC complex itself (e.g. of the known interactor Isy1), an information that they may have available through their proteomic analysis of DCwc2 complexes. This information would be of interest even if the efficient rescue of depleted extracts with purified recombinant Cwc2 shows that these proteins are not essential for catalysis._

As we stated in our original manuscript (p. 7, para. 3 and p.18, para. 2), we could not identify any factor significantly co-depleted with Cwc2 by mass spectrometric analysis of purified DCwc2 complexes.
Referee #2 (Remarks to the Author):

The authors of "Cwc2 and its human homolog RBM22 promote an active conformation of the spliceosome catalytic center" have answered most of my questions about the work presented and I am by in large satisfied with their changes to the manuscript. However, I still do not agree with how they attribute crosslinks to Cwc2 in some cases. This issue is important because it underpins a good part of their current discussion, and because such attributions can take on a life of their own in future discussions of spliceosome structure.

In my mind, site-specific crosslinking with a modified nucleotide or RNase H mapping is the standard in the field for defining a protein/RNA crosslink. The argument that the chances of finding simultaneous crosslinks is negligible because of low crosslinking efficiency, seems to be at odds with them IPing presumable RNA/RNA crosslinks near U90. It is also in contrast to results they presented in their 2006 MCB paper examining U2 snRNA/protein crosslinks, where there found several cases of simultaneous crosslinks to different proteins in IPs. If the authors maintain that low crosslinking efficiency is sufficient to claim a specific RNA/protein interaction, they should spell out this argument very clearly in the text.

With that being said the mass spectrometry analyses of U6 crosslinked proteins, which identifies only Cwc2, is convincing for attributing the crosslinks in the Bact complex that they examined. It does not strictly rule out the possibility of U6/pre-mRNA crosslinks, but as they note, the G39 crosslink, which is the most efficient crosslink, is in agreement with previous data by Chan, et al, as likely being protein mediated. On another note, the authors need to add methods for the U6 pull-downs and mass spectrometry. It is somewhat surprising that they had enough material for the MS given the low crosslinking efficiency, and the field would benefit from knowing how to identify proteins crosslinked to RNAs in this manner.

One interesting point that arises from the MS of Bact results is it suggests that Cwc2 is the only protein that contacts U6 snRNA in the spliceosome at this stage. I might have expected that some U5 proteins shown previously to crosslink to U6 snRNA in tri-snRNP (Vidal, et al 1999), especially Prp8.

Finally, I do not see that it follows that because the U6 crosslinks in Bact are to Cwc2, then the ISL crosslinks in C complex and the pre-mRNA crosslinks in Bact also belong to Cwc2. More evidence is required to make that assertion.

Reply to the criticisms of Referee #2 on the revised version of our manuscript

The authors of "Cwc2 and its human homolog RBM22 promote an active conformation of the spliceosome catalytic center" have answered most of my questions about the work presented and I am by in large satisfied with their changes to the manuscript. However, I still do not agree with how they attribute crosslinks to Cwc2 in some cases. This issue is important because it underpins a good part of their current discussion, and because such attributions can take on a life of their own in future discussions of spliceosome structure.
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It is also in contrast to results they presented in their 2006 MCB paper examining U2 snRNA/protein crosslinks, where there found several cases of simultaneous crosslinks to different proteins in IPs. If the authors maintain that low crosslinking efficiency is sufficient to claim a specific RNA/protein interaction, they should spell out this argument very clearly in the text.

As discussed in our first reply to referee 2, we are aware that two or more proteins can be simultaneously crosslinked to the same RNA molecule. The question is rather that of the extent to which multiple crosslinks would be detectable after immunoprecipitation in the readout that we use for protein–RNA crosslinks (i.e., RT stops). This indeed emerges clearly in the earlier paper from our group (Dybkov et al., MCB 2006) which the referee mentions. In that work, we observed various crosslinks between U2 snRNA and protein following UV irradiation of isolated 17S U2 snRNPs, with differing efficiencies of crosslinking. For example, the Sm protein G was crosslinked to U2 snRNA with relatively high efficiency, proteins SF3b 49 and SF3a 60 with relatively low efficiency. In the immunoprecipitation of a less efficient protein–RNA crosslink (e.g. SF3b 49), small quantities of crosslinked complex with the efficiently crosslinking protein SmG were detected, but crosslinked complexes with other less efficiently crosslinked proteins (e.g. SF3a 60) were not seen. On the other hand, immunoprecipitation of crosslinked complexes containing a strongly crosslinking protein (Sm G) was not accompanied by any co-precipitating protein–U2 RNA crosslinked complex.

As the work described in our present manuscript involves the relatively efficient crosslink between Cwc2 and the G39 of U6 snRNA, we consider it reasonable that the further (weaker) stops that we see in Cwc2 pull-downs at positions U65, C68 and U74 of the U6 snRNA in the B* and C complexes also represent Cwc2–U6 RNA crosslinks: not ones that have formed on U6 snRNA molecule to which a Cwc2 is already crosslinked, but rather involving Cwc2 independently attached to other U6 snRNA molecules (see also below for additional evidence supporting the idea that the crosslinks to G39, U65, C68 and U74 are all due to Cwc2 crosslinks).

Concerning the possibility of RNA–RNA crosslinks near U90, we would like to emphasize again that the reverse transcriptase stops seen from nucleotides C85 to U90 are also observed (and are even more pronounced) when U6 snRNA, synthesized by transcription in vitro, was irradiated as a control (Fig. 3A–C, lane 11). As we have no evidence for crosslinks between distant nucleotides of U6 snRNAs (based on gel electrophoresis of the crosslinked in vitro transcribed U6 RNA, unpublished data), the observed RT stops are very likely due to RNA–RNA crosslinks between adjacent nucleotides or, alternatively, even strand cleavage. Thus, the reverse transcriptase stops observed in our spliceosomal complexes in the region near U90 of U6 snRNA are also very probably due to similar crosslinks occurring between adjacent nucleotides of the U6 snRNA. We have clarified this point in our manuscript (p. 13, para. 2).

With that being said the mass spectrometry analyses of U6 crosslinked proteins, which identifies only Cwc2, is convincing for attributing the crosslinks in the Bact complex that they examined. It does not strictly rule out the possibility of U6/pre-mRNA crosslinks, but as they note, the G39 crosslink, which is the most efficient crosslink, is in agreement with previous data by Chan, et al, as likely being protein mediated. On another note, the authors need to add methods for the U6 pull-downs and mass spectrometry. It is somewhat surprising that they had enough material for the MS given the low crosslinking efficiency, and the field would benefit from knowing how to identify proteins crosslinked to RNAs in this manner.

We are currently preparing a methodologically oriented manuscript which will include a detailed description of this method and its general applicability to the identification by MS of proteins that are crosslinked to an RNA molecule of interest.
One interesting point that arises from the MS of Bact results is: it suggests that Cwc2 is the only protein that contacts U6 snRNA in the spliceosome at this stage. I might have expected that some U5 proteins shown previously to crosslink to U6 snRNA in tri-snRNP (Vidal et al 1999), especially Prp8.

We have addressed this question experimentally by carrying out the following additional control experiments, which we have included in the revised version of the manuscript (p.11, para. 2, Supplementary Figure S1) We have used extracts from yeast strains that expressed TAP-tagged U5 proteins Prp8 or Snu114, or the TAF-tagged NTC-related proteins Ecm2 or Yju2 for the assembly of B\textsuperscript{act} complexes on the radiolabelled pre-mRNA construct M3ActD6. In each case, the spliceosomal B\textsuperscript{act} complexes formed were affinity-purified, UV-irradiated for 1 min and then subjected to denaturing conditions in order to disrupt protein–protein and non-covalent RNA–protein interactions. An aliquot was retained, and the remainder of the sample was incubated with IgG Sepharose, allowing the selective immunoprecipitation of RNA species crosslinked to the selected TAP-tagged protein (as described for the Cwc2 crosslink experiments). After proteolytic digestion, co-precipitated snRNAs were identified by Northern blotting using probes that hybridise to U2, U5 and U6 RNA. The outcome of these experiments is now shown in the Supplementary Fig. S1. In these controls, Prp8 showed crosslinks to U5 snRNA and to pre-mRNA (Fig. S1). Snu114 showed crosslinks to U5 snRNA, to the pre-mRNA and, interestingly, in significant quantities also to U2 snRNA. In contrast, Ecm2 was only crosslinked to the pre-mRNA, while Yju2 showed no RNA–protein crosslinks at all in the B\textsuperscript{act} complex. For this discussion it is decisive that none of the proteins of the B\textsuperscript{act} complex becomes crosslinked to the U6 snRNA. The contacts between the U6 snRNA and Prp8 and Snu114 (respectively) observed by Vidal et al. thus seem to be restricted to the stage of the U4/U6.U5 tri-snRNP complex and to be disrupted in the activation of the spliceosome – a process in which the displacement of the U4 snRNA is followed by a major restructuring of the U6 snRNA.

Moreover, the extremely selective crosslinking behaviour of the five spliceosomal proteins tested here (including Cwc2) provides independent evidence in support of our assumption that the RT stops that we observe at nucleotides G39, U65, C68, and U74 of U6 snRNA and at U+15 of the pre-mRNA after pull-down, do indeed arise from crosslinks to Cwc2 and not from crosslinks between the RNA and proteins other than Cwc2 – or from crosslinks between U6 snRNA and other RNAs – that were co-precipitated in Cwc2 pull-down experiments. If the latter were the case, it would be difficult to explain why no U6 snRNA was precipitated in any of the cases shown in Fig. S1.

Finally, I do not see that it follows that because the U6 crosslinks in Bact are to Cwc2, then the ISL crosslinks in C complex and the pre-mRNA crosslinks in Bact also belong to Cwc2. More evidence is required to make that assertion.

To obtain additional, independent experimental evidence that the crosslinks detected to the U6-ISL in the C complex are due to Cwc2 and not to a different protein, we performed the following experiment (unpublished data). Using yeast splicing extracts that contained Cwc2-TAP, we assembled C complexes on the radiolabelled pre-mRNA construct M3ActΔ31. The spliceosomal C complexes formed were affinity-purified, UV-irradiated for 1 min and then subjected to denaturing conditions as described above. The U6 snRNA was specifically cut in two by using DNA-oligo-directed RNase H cleavage and an oligodeoxyribonucleotide that hybridized to nucleotides 42–61 in the middle region of U6 snRNA. An aliquot was retained, and the remainder of the sample was incubated with IgG Sepharose, allowing the selective immunoprecipitation of the 5’ and/or the 3’ portion of U6 RNA (e.g. containing the U6-ISL), crosslinked to Cwc2-TAP. After proteolytic digestion, co-precipitated RNAs were identified by Northern blotting using a probe that hybridises to U6 snRNA. The contacts between the RNA and proteins other than Cwc2 – or from crosslinks between U6 snRNA and other RNAs – that were co-precipitated in Cwc2 pull-down experiments. If the latter were the case, it would be difficult to explain why no U6 snRNA was precipitated in any of the cases shown in Fig. S1.

As far as the the crosslinking site of Cwc2 to the pre-mRNA in B\textsuperscript{act} is concerned, we regard this as a genuine crosslink of Cwc2 to the pre-mRNA, since it is specifically enriched after pull-down (Figure 3D). In the input lane, in addition to the crosslinking signal at U+15, several bands are
visible; however, after the pull-down of Cwc2 the major crosslinking signal at U+15 stands out over the other stops, which are significantly reduced. In the same way, we concluded that the majority of the crosslinks to U6 snRNA shown in the input lanes of Figure 3A–C must be attributed to Cwc2, since they were even enhanced (Figure 3C), or were at least equally visible after the pull-down (Figure 3A and B).