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Structure and Assembly of the SF3a Splicing Factor Complex of U2 snRNP

Pei-Chun Lin and Rui-Ming Xu

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 12 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 in general positive regarding the study, but do require further experimental analysis regarding the RNA binding activity of SF3a, as outlined by both referee #2 and #3. Given the support from the referees and should you be able to address these concerns 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 REPORTS:

Referee #1:

In the manuscript 'Structure and Assembly of the SF3a Splicing Factor Complex of U2 snRNP' Lin and Xu present an important piece of structural information for understanding the spliceosome. They trimmed and crystallized the yeast SF3a complex consisting of the proteins Prp9, Prp11 and Prp21. By x-ray analysis they determined the 3D structure of the trimmed SF3a core complex to a final resolution of 3.1 A showing the protein folding and, in particular, the intriguing interactions of the Prp21 protein with Prp9 and Prp11. The high homology of the yeast SF3a subunits with the human SF3a proteins, SF3a60, SF3a66 and SF3a120, makes the yeast structure also a good template for the human SF3a complex structure. Finally, using synthetic small RNAs from U2 snRNA they tested the SF3a complex for RNA binding and found that the Prp9 protein of the complex is capable of binding RNA. All tested RNA fragments bound to some degree, but the stem-loop 2a RNA showed the highest binding activity. Mutation of a positively charged patch of the Prp9 protein inhibited binding to all the of the tested RNA fragments.
The manuscript is well written and the various features of the crystal structure are well presented. In particular, the quite complex binding modes of Prp9 and Prp11 with the bridging protein Prp21 is presented in a clear, understandable manner. The RNA binding study is also clearly presented but some of their conclusions are still a bit speculative, and the discussion gets a bit out of hand as the authors discuss extensively the binding results even though their conclusions are not supported by very hard facts. Foremost, it is not clear if stem-loop 2a is indeed the native binding partner of the positive patch of the Prp9 protein in the 15S U2 snRNP (to which it would normally have to bind during U2 snRNP assembly), as binding was analyzed in a very reduced system. Furthermore, binding of all of the tested RNA fragments depended on this patch. The authors also discuss extensively an apparent discrepancy between their binding results and the cross linking data of intact, in vivo assembled human U2 snRNPs. In fact, possible crosslinks of the Prp9 ortholog SF3a60 to stem-loop 2a were described in Dybkov et al. (2006) as well, but in this study an indirect interaction of SF3a60 via another protein could not be excluded. Thus, I recommend to condense the discussion of the RNA binding section.
Additional minor points:
1. At several positions (pages 4, 18, 19) a wrong character is used for the 'ä' in the name "Krämer".

2. For easier identification of the figure content, it would be very helpful if the protein names were placed directly in the figures (in particular in figures 3, 4, 5, 6, 7C) and not only in the legend.

3. In Fig. 2A, αC and α4 should be labeled in order to more readily understand the text on page 9, last paragraph.

4. In Fig. 3A NTD and CTD should be labeled.

5. In Fig. 3B the Arg24 label is missing.

6. In Fig. 4B sheet 1 and 2 should be labeled.

7. The information in Fig. 5 would be easier to understand if it were presented as stereo views.

8. In Fig. 5B, α3 should be labeled to allow easer identification of the a2 and a3 connecting loop. Here, also labeling of αD would be helpful. Additionally, the labeling of the positions of the ten Met mutations would also be quite helpful. 9. In Fig. 6A the amino acids L184 and C188 of Prp21 are described but not shown in the referred figure.
10. In Fig. 6B the amino acid labeled C140 probably is amino acid C240.
11. In the legend to Fig. 7B the length of the Prp21 fragment should be 87-176 and not 87-196.

Referee #2:

In this manuscript, Lin and Xu describe a 3.1≈ crystal structure of the Prp9-11-21 hetero-trimeric SF3a component of U2 snRNP. Physiologically, the SF3a complex joins a 15S U2 snRNP to form the fully assembled 17S U2 snRNP, and thus is important for U2 maturation and function in the spliceosome. The manuscript clearly describes the architecture of each subunit and the physical bases for their interactions.

The authors also present RNA-binding data, demonstrating that SF3a can bind to U2 SLIIa, and isolate the responsible protein components to the Prp9+21 domain of the complex. U2 SLIIa is thought to be mutually exclusive with another RNA structure, U2 SLIIc, and these two conformations are thought to toggle repetitively during spliceosome assembly and function. The binding analysis presented here would be more complete were the authors to also test the binding of SLIIc -- the results might point toward specific nucleotides important for the interaction and add an additional level of information about the biological role of SF3a binding to U2 snRNA and should be included based on the current thinking within the field.

Overall, the SF3a structure is highly informative and should be of broad interest to the RNA field.

minor points:
page 4: "SF3a120/Prp9" should be "SF3a120/Prp21".
page 6: "prp9 mutant" -- which prp9 mutant is being referred to? It would also be helpful for the authors to provide any known phenotypes of this mutant (e.g., heat sensitive).

Referee #3:

U2 snRNP, one of the important components of the spliceosome, contains two protein complexes known as splicing factors 3a and 3b in addition to U2 snRNA, seven Sm proteins and a complex of U2B"-U2A'. Previous biochemical studies on human U2 snRNP showed that SF3b binds to 12S U2 snRNP consisting of U2 snRNA, seven Sm proteins and the U2B'-U2A' complex forming a 15S U2 snRNP. This binding of SF3b requires the modification of the 5' end of U2 snRNA (Yu et al., EMBO J. 17, 5783-5795 (1998)). This will allow SF3a to bind forming a functional 17S U2 snRNP. SF3b consists of three proteins, PRP9, PRP11 and PRP21 in yeast Saccharomyces cerevisaie and in human their counterparts, SF3a60, SF3a66 and SF3a120. There are no reasons to believe that yeast and human U2 snRNPs are assembled differently but such hierarchical assembly process has been experimentally demonstrated for yeast U2 snRNP. Lin and Xu have now produced a ternary complex of yeast SF3a proteins. Although they were not able to crystallise the full-length yeast SF3a they crystallised and solved the structure of a ternary complex consisting of a large N-terminal fragment of Prp9 (Prp9 C, a.a. 1-389), a C-terminal fragment of Prp11 lacking the 49 N-terminal residues (Prp11 N, a.a. 50-266), and a middle fragment of Prp21 (Prp21M) spanning residues 87-237. This reveals the structure of these three proteins and how they interact with each other. Their EMSA result with various fragments of U2 snRNA shows that the yeast SF3a complex preferentially binds stem-loop IIa (SL2a). This binding pattern of the SF3a core domain is indistinguishable from the complex assembled from the full-length proteins. This crystal structure is of significant interest to those in the splicing field and would become significantly more interesting to more general readers of EMBO Journal after the following points are addressed.
(1) There are no specific functions or activities known to be associated with SF3a during the spliceosomal assembly or activation and hence this is not an easy paper to write. In introduction section the authors should refer to proteomic studies of splicing intermediates and explain in which intermediates SF3a is present (Bessonov et al., RNA. 16, 2384-2403 (2010); Bessonov et al., Nature 452, 846-850 (2008); Jurica & Moore, Mol Cell 12, 5-14 (2003)).
(2) When the spliceosome is activated the 5' end of U2 snRNA becomes involved in an intricate network of RNA-RNA interaction with U6 snRNA. Hence, SF3a and SF3b would not be able to interact with U2 snRNA in the same manner.
(3) In 12S U2 snRNP stem-loop is thought to be exposed as only the Sm protein binding site and stem-loop IV is bound by the proteins (Leung et al., 2011; Price et al., 1998). The authors now showed that SF3a binds to SL2a. Does SF3a bind to naked U2 snRNA? As far as I know the binding SF3a to U2 snRNA has not been reported. Why does the binding of SF3a require the prior binding of SF3b? Is SL2a masked in 12S U2 snRNP and does the binding of SF3b to 12S U2 snRNP expose SL2a? Is U2 snRNA folded differently in 12S U2 snRNP and does SF3b induces a different folding of U2 snRNA such that SL2a is formed? This is an important question to address?
(4) the authors should include a section (in the supplemental material?) to show they were able to fit the model unambiguously, perhaps show an overlap with the SeMet sites. Prp9 and prp21 are both mainly alpha helical so it must have been quite difficult to fit this into a 3.5A initial map with the right connections.

(5) Half of prp11 is missing from the model, can the authors confirm that residues 50-148 are actually present in the crystals. If yes could they comment on where they think this region would be and could it contact prp9? Did they not get a peak in the electron density for the zinc atom? It is not clear in the text which residues of prp11 N are replaced by the poly-alanine model. The interaction between prp11 and prp21 is very interesting with part of prp21 wrapping around prp11, but as the authors mentioned the density in this region was not very clear and residues 207-219 of prp21 are missing followed by a C-terminal short helix. Are the authors sure this density near prp11 belongs to prp21 and not to some of the 100 residues of prp11 which are missing in the model?
(6) In figure 2B the authors comment on a positively charged patch near the zinc finger of prp9. There seems to be another clear positively charged patch at the bottom of the structure why did they not comment on this and can it also be involved in RNA binding?
(7) From the EMSAs it seems that the specificity for SL2a is reduced by using the prp9-prp21 87-179 complex. Is this the case or an artefact of the gel? Could prp11-prp21 177-237 aid in the specificity?
(8) The last sentence of the first paragraph of the discussion is an overstatement and should be changed. It is a very interesting structure but how does it give insight into the assembly of the whole U2 snRNP?
(9) Legend of fig 4 last sentence: A ribbon diagram of prp21M should be prp11 N.

As it stands the manuscript does not provide much biological insight into U2 snRNP assembly or function. By considering the above points and carrying out a few key experiments the manuscript would become significantly more interesting and may become suitable for publication in EMBO J.

1st Revision - Authors' Response 30 November 2011

Referee #1:

In the manuscript 'Structure and Assembly of the SF3a Splicing Factor Complex of U2 snRNP' Lin and Xu present an important piece of structural information for understanding the spliceosome. They trimmed and crystallized the yeast SF3a complex consisting of the proteins Prp9, Prp11 and Prp21. By x-ray analysis they determined the 3D structure of the trimmed SF3a core complex to a final resolution of 3.1 A showing the protein folding and, in particular, the intriguing interactions of the Prp21 protein with Prp9 and Prp11. The high homology of the yeast SF3a subunits with the human SF3a proteins, SF3a60, SF3a66 and SF3a120, makes the yeast structure also a good template for the human SF3a complex structure. Finally, using synthetic small RNAs from U2 snRNA they tested the SF3a complex for RNA binding and found that the Prp9 protein of the complex is capable of binding RNA. All tested RNA fragments bound to some degree, but the stem-loop 2a RNA showed the highest binding activity.

Mutation of a positively charged patch of the Prp9 protein inhibited binding to all the of the tested RNA fragments. The manuscript is well written and the various features of the crystal structure are well presented. In particular, the quite complex binding modes of Prp9 and Prp11 with the bridging protein Prp21 is presented in a clear, understandable manner. The RNA binding study is also clearly presented but some of their conclusions are still a bit speculative, and the discussion gets a bit out of hand as the authors discuss extensively the binding results even though their conclusions are not supported by very hard facts. Foremost, it is not clear if stem-loop 2a is indeed the native binding partner of the positive patch of the Prp9 protein in the 15S U2 snRNP (to which it would normally have to bind during U2 snRNP assembly), as binding was analyzed in a very reduced system. Furthermore, binding of all of the tested RNA fragments depended on this patch. The authors also discuss extensively an apparent discrepancy between their binding results and the cross linking data of intact, in vivo assembled human U2 snRNPs. In fact, possible crosslinks of the Prp9 ortholog SF3a60 to stem-loop 2a were described in Dybkov et al. (2006) as well, but in this study an indirect interaction of SF3a60 via another protein could not be excluded. Thus, I recommend to condense the discussion of the RNA binding section.

The reviewer's positive opinion about our work is greatly appreciated! We share the same feeling that the binding of SF3a is best tested on the 15S U2 snRNP. In fact, we are actively trying to reconstitute the 15S and the intact 17S particles for biochemical and structural studies. However, it is a technically challenging task in the field and little progress has been made in the last two decades. We feel that using the reduced system in our manuscript represents a solid and significant step towards dissecting the complex problem.

Dybkov et al. (2006) reported the crosslinking of SF3a60 to stem-loop I and III, but not SL2a. As the reviewer pointed out, the crosslinks may be mediated by other proteins. They did point out in their discussion that some interactions may have escaped detection by this method, and that there were genetic evidences suggesting that the yeast SF3a complex interacts with SL2a. In our discussion section, we intended to clarify that the two results are not necessarily inconsistent with each other. The reviewer's suggestion is well taken, and we have deleted a few sentences at the end of 1st paragraph in page 14 to make the discussion more succinct.

Additional minor points:

1. At several positions (pages 4, 18, 19) a wrong character is used for the 'ä' in the name "ämer".

The PDF converter of the EMBO journal seemed to have messed up some of the "ä"s in the references to the papers coauthored by Angela Krämer in the submitted Word file. The error did not occur in my computer when I saved it as a PDF file. I apologize for the carelessness in approving the converted PDF file.

2. For easier identification of the figure content, it would be very helpful if the protein names were placed directly in the figures (in particular in figures 3, 4, 5, 6, 7C) and not only in the legend.

Fixed.

3. In Fig. 2A, αC and α4 should be labeled in order to more readily understand the text on page 9, last paragraph.

αC and αD of Prp21, and α4 of Prp9 are now labeled.

4. In Fig. 3A NTD and CTD should be labeled.

Fixed.

5. In Fig. 3B the Arg24 label is missing.

Arg24 hides behind helix α1 and it's difficult to see. Thus, it was not labeled. Now it's labeled with an arrow pointing to it.

6. In Fig. 4B sheet 1 and 2 should be labeled.

Now labeled.

7. The information in Fig. 5 would be easier to understand if it were presented as stereo views.

Suggestion taken.

8. In Fig. 5B, α3 should be labeled to allow easer identification of the α2 andα3 connecting loop. Here, also labeling of αD would be helpful. Additionally, the labeling of the positions of the ten Met mutations would also be quite helpful.

Fig. 5B remade to allow easier identifications of the said loops of Prp9 and αD of Prp21. The ten Met mutations are on Prp11, so they won't show up in this figure. Six ordered residues of the 10 that mutated to Met are now shown in Supplemental Fig. 2A.

9. In Fig. 6A the amino acids L184 and C188 of Prp21 are described but not shown in the referred figure.

They were not shown because helix αD would block the view. Their positions are now indicated with arrows.

10. In Fig. 6B the amino acid labeled C140 probably is amino acid C240.

You are right! Corrected.

11. In the legend to Fig. 7B the length of the Prp21 fragment should be 87-176 and not 87-196.

Corrected. Thank you!

Referee #2:

In this manuscript, Lin and Xu describe a 3.1Å; crystal structure of the Prp9-11-21 hetero-trimeric SF3a component of U2 snRNP. Physiologically, the SF3a complex joins a 15S U2 snRNP to form the fully assembled 17S U2 snRNP, and thus is important for U2 maturation and function in the spliceosome. The manuscript clearly describes the architecture of each subunit and the physical bases for their interactions.

The authors also present RNA-binding data, demonstrating that SF3a can bind to U2 SLIIa, and isolate the responsible protein components to the Prp9+21 domain of the complex. U2 SLIIa is thought to be mutually exclusive with another RNA structure, U2 SLIIc, and these two conformations are thought to toggle repetitively during spliceosome assembly and function. The binding analysis presented here would be more complete were the authors to also test the binding of SLIIc -- the results might point toward specific nucleotides important for the interaction and add an additional level of information about the biological role of SF3a binding to U2 snRNA and should be included based on the current thinking within the field.

Overall, the SF3a structure is highly informative and should be of broad interest to the RNA field.

Following the reviewer's suggestion, we have tested the binding of SF3a to SL2c and the result is shown in a new figure, Fig. 8. In brief, SF3a does not bind SL2c. This new result is presented at the end of the 1st paragraph of the "RNA-binding properties of SF3a" section in Results, and the result is also briefly discussed in page 14.

minor points:

page 4: "SF3a120/Prp9" should be "SF3a120/Prp21".

Fixed.

page 6: "prp9 mutant" -- which prp9 mutant is being referred to? It would also be helpful for the authors to provide any known phenotypes of this mutant (e.g., heat sensitive).

The prp9 mutant, originally known as rna9, is a temperature sensitive mutant that lead to the discovery of the PRP9 gene. This information is now added in page 6 of the revised manuscript.

Referee #3:

U2 snRNP, one of the important components of the spliceosome, contains two protein complexes known as splicing factors 3a and 3b in addition to U2 snRNA, seven Sm proteins and a complex of U2B"-U2A'. Previous biochemical studies on human U2 snRNP showed that SF3b binds to 12S U2 snRNP consisting of U2 snRNA, seven Sm proteins and the U2B'-U2A' complex forming a 15S U2 snRNP. This binding of SF3b requires the modification of the 5' end of U2 snRNA (Yu et al., EMBO J. 17, 5783-5795 (1998)). This will allow SF3a to bind forming a functional 17S U2 snRNP. SF3b consists of three proteins, PRP9, PRP11 and PRP21 in yeast Saccharomyces cerevisaie and in human their counterparts, SF3a60, SF3a66 and SF3a120. There are no reasons to believe that yeast and human U2 snRNPs are assembled differently but such hierarchical assembly process has been experimentally demonstrated for yeast U2 snRNP. Lin and Xu have now produced a ternary complex of yeast SF3a proteins. Although they were not able to crystallise the full-length yeast SF3a they crystallised and solved the structure of a ternary complex consisting of a large N-terminal fragment of Prp9 (Prp9ΔC, a.a. 1-389), a C-terminal fragment of Prp11 lacking the 49 N-terminal residues (Prp11ΔN, a.a. 50-266), and a middle fragment of Prp21 (Prp21M) spanning residues 87-237. This reveals the structure of these three proteins and how they interact with each other. Their EMSA result with various fragments of U2 snRNA shows that the yeast SF3a complex preferentially binds stem-loop IIa (SL2a). This binding pattern of the SF3a core domain is indistinguishable from the complex assembled from the full-length proteins. This crystal structure is of significant interest to those in the splicing field and would become significantly more interesting to more general readers of EMBO Journal after the following points are addressed.

(1) There are no specific functions or activities known to be associated with SF3a during the spliceosomal assembly or activation and hence this is not an easy paper to write. In introduction section the authors should refer to proteomic studies of splicing intermediates and explain in which intermediates SF3a is present (Bessonov et al., RNA. 16, 2384-2403 (2010); Bessonov et al., Nature 452, 846-850 (2008); Jurica & Moore, Mol Cell 12, 5-14 (2003)).

Suggestion taken. A brief description and references added in the Introduction section of the revised manuscript at the end of page 3 and beginning of page 4.

(2) When the spliceosome is activated the 5' end of U2 snRNA becomes involved in an intricate network of RNA-RNA interaction with U6 snRNA. Hence, SF3a and SF3b would not be able to interact with U2 snRNA in the same manner.

U2 snRNA is extensively rearranged during the spliceosome assembly and splicing reactions. In pairing with the U6 snRNA, SL1 is unwinded and pairs with U6, but SL2a and SL2b are predicted to be intact. However, as the Reviewer #1 pointed out, SL2a was demonstrated to toggle between alternative conformations, namely SL2a and SL2c. SL2a is important for the prespliceosome assembly and the 2nd step of splicing reaction, while SL2c is important for the intermediate processes following prespliceosome assembly and the 2nd step of splicing reaction. Clearly, the binding mode of SF3a and SF3b cannot be the same throughout the steps of spliceosome assembly and splicing reactions. In fact, SF3a and SF3b will dissociate from the spliceosome before the first splicing reaction.

In the revised manuscript, we have provided data showing that SF3a will not bind SL2c (Fig. 8). Thus, it is conceivable that the direct binding of SF3a to SL2a we observed most likely represents the binding mode during prespliceosome assembly. When the spliceosome is activated by an ATP-dependent event mediated by Prp2, SL2a is switched to SL2c, triggering the release of SF3a from the spliceosome. The above data are presented at the end of the 1st paragraph of the "RNA-binding properties of SF3a" in Results, and also briefly in Discussion at the end of 1st paragraph in page 14.

(3) In 12S U2 snRNP stem-loop is thought to be exposed as only the Sm protein binding site and stem-loop IV is bound by the proteins (Leung et al., 2011; Price et al., 1998). The authors now showed that SF3a binds to SL2a. Does SF3a bind to naked U2 snRNA? As far as I know the binding SF3a to U2 snRNA has not been reported. Why does the binding of SF3a require the prior binding of SF3b? Is SL2a masked in 12S U2 snRNP and does the binding of SF3b to 12S U2 snRNP expose SL2a? Is U2 snRNA folded differently in 12S U2 snRNP and does SF3b induces a different folding of U2 snRNA such that SL2a is formed? This is an important question to address?

Although implicated by genetic evidences, indeed, our observation is the first direct evidence that SF3a physically interacts with SL2a. The questions asked by the reviewer are extremely important for a comprehensive understanding of U2 snRNP assembly. We are pleased that our discovery opened the door for such deep, mechanistic curiosities, and it's our desire to tackle these questions in future studies.

(4) the authors should include a section (in the supplemental material?) to show they were able to fit the model unambiguously, perhaps show an overlap with the SeMet sites. Prp9 and prp21 are both mainly alpha helical so it must have been quite difficult to fit this into a 3.5A initial map with the right connections.

Prp9 and Prp21 was relatively easy to fit due to the SeMet and Zn anomalous signals, while we had to mutate several residues to methionines to aid the assignment of Prp11 sidechains. In the new Supplemental Fig. 1A, a section of the original 3.5 Å MAD-phased electron density map, together with the Se sites of Prp9 used for phasing, are shown with the Cα trace of Prp9.

(5) Half of prp11 is missing from the model, can the authors confirm that residues 50-148 are actually present in the crystals. If yes could they comment on where they think this region would be and could it contact prp9? Did they not get a peak in the electron density for the zinc atom? It is not clear in the text which residues of prp11ΔN are replaced by the poly-alanine model. The interaction between prp11 and prp21 is very interesting with part of prp21 wrapping around prp11, but as the authors mentioned the density in this region was not very clear and residues 207-219 of prp21 are missing followed by a C-terminal short helix. Are the authors sure this density near prp11 belongs to prp21 and not to some of the 100 residues of prp11 which are missing in the model?

The disordered segment (a.a. 50-148) is present in the crystal, as the SDS-PAGE result of the protein sample from the crystals showed no sign of degradation (Supplemental Fig. 2B). We did not see the anomalous Zinc signal from the N-terminal region of Prp11, which is consistent with our observation that the N-terminal region, including the putative Zinc finger, of Prp11 is disordered.

Amino acids located on α1 and β1 (36 a.a. in total) are modeled with alanines, as the weak electron density does not allow unambiguous assignment of side chains in this region. Thus, we can only say that a segment of 36 amino acids N-terminal to residue 149 of Prp11 is represented in a polyalanine model, which is shown in a yellow ribbon model in the new Supplemental Fig. 2A.

We are confident that the "orphan" short helix belongs to Prp21, as it has a methionine (Met 224) and the Se signal from the SeMet crystal is clear. It cannot be Prp11, as the two missing methionines in Prp11, Met 74 and Met76 are next to each other, but we only see one Se peak in the region.

(6) In figure 2B the authors comment on a positively charged patch near the zinc finger of prp9. There seems to be another clear positively charged patch at the bottom of the structure why did they not comment on this and can it also be involved in RNA binding?

We have now mutated a pair of two adjacent positively charged residues, Arg114 and Lys115, in the NTD of Prp9 (the bottom the structure in Fig. 2B and Supplemental Fig. 4A) to glutamic acids. The R114E/K115E mutant did not show reduced RNA binding (Supplemental Fig. 4B). Therefore, the distal positively charged area is not involved in RNA binding in our system.

(7) From the EMSAs it seems that the specificity for SL2a is reduced by using the prp9-prp21 87-179 complex. Is this the case or an artefact of the gel? Could prp11-prp21 177-237 aid in the specificity?

It's probably a combination of both. In Figs. 7A & 7B, it appears that the non-specific bands with SL1 and SL2b are relatively more prominent for the Prp9-Prp21(87-176) mini complex than that for the trimeric SF3a core complex. We performed the experiment suggested by the reviewer, with the Prp21(177-237)-Prp11ΔN complex added to the Prp9-Prp21(87-176) mini complex in EMSA. The result (Supplemental Fig. 4C) shows that the non-specific band for SL1 appears to be reduced. We have noted this observation in the 1st paragraph of page 14 in Discussion.

(8) The last sentence of the first paragraph of the discussion is an overstatement and should be changed. It is a very interesting structure but how does it give insight into the assembly of the whole U2 snRNP?

We meant that the structure-guided discovery that SF3a binds to the SL2a element of U2 snRNA provided the first direct evidence of how SF3a is placed within the U2 snRNP. Obviously U2 snRNP is a large structure, the information obtained here is far from a complete picture of how SF3a is assembled into U2 snRNP. We have taken the reviewer's critique and modified the sentence in page 12.

(9) Legend of fig 4 last sentence: A ribbon diagram of prp21M should be prp11ΔN.

Fixed. Thanks!

2nd Editorial Decision 02 January 2012

Thank you for submitting your revised manuscript to the EMBO Journal. I apologise for the delayed response but I was out of the office the week before Christmas because of the flu. I have now the reports from two referees who find that you have satisfactorily addressed their original concerns and I will be happy to accept the manuscript for publication in The EMBO Journal once the additional sequence information has been added (see referee #2). It would be great if you could get the manuscript back to me before Thursday 5th Jan since this will be my last work day at the journal before I take up a position as Scientific Coordinator at the University of Würzburg.

When preparing your letter of response to the referees' comments, please bear in mind 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, 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 REPORTS:

Referee #2:

The authors have provided new RNA-binding data concerning SF3a-stem-II interaction, as requested by the Reviewers. The new data are compelling that SF3a can bind to U2 stem-IIa and not detectably to the alternative structure stem-IIc.

-minor point: The authors should describe in the Methods section the sequence of the IIc RNA used, as they do for other RNA structures tested. Although a schematic of IIc+loop2b is depicted in the figure, it is currently unclear exactly what IIc alone represents.

This result is biologically important, because it suggests that SF3a binding stabilizes the IIa conformation during spliceosome assembly, and only after the action of Prp2 ATPase -- which results in dissociation/destabilization of SF3a/b -- is it possible to allow an exchange of IIa for IIc, potentially enabling conformational change into the catalytically-active spliceosome.

Overall, I think that the manuscript is improved, the Figures are now more-clearly labelled, and that the work will be of broad interest to the RNA field.

Referee #3:

I have read the revised manuscript as well as the reviews on the original manuscript by all three referees. The authors responded adequately to all the comments by the referees and revised the manuscript accordingly. In my opinion the manuscript should now be accepted for publication in EMBO J.

2nd Revision - Authors' Response 03 January 2012

Referee #2:

The authors have provided new RNA-binding data concerning SF3a-stem-II interaction, as requested by the Reviewers. The new data are compelling that SF3a can bind to U2 stem-IIa and not detectably to the alternative structure stem-IIc.

-minor point: The authors should describe in the Methods section the sequence of the IIc RNA used, as they do for other RNA structures tested. Although a schematic of IIc+loop2b is depicted in the figure, it is currently unclear exactly what IIc alone represents.

This result is biologically important, because it suggests that SF3a binding stabilizes the IIa conformation during spliceosome assembly, and only after the action of Prp2 ATPase -- which results in dissociation/destabilization of SF3a/b -- is it possible to allow an exchange of IIa for IIc, potentially enabling conformational change into the catalytically-active spliceosome.

Overall, I think that the manuscript is improved, the Figures are now more-clearly labelled, and that the work will be of broad interest to the RNA field.

The sequences for the RNA oligos used for testing the binding of SF3a to the alternative Stem 2c structure are now explicitly stated in the Methods section (page 17, lines 10-13).

Referee #3:

I have read the revised manuscript as well as the reviews on the original manuscript by all three referees. The authors responded adequately to all the comments by the referees and revised the manuscript accordingly. In my opinion the manuscript should now be accepted for publication in EMBO J.

Thank you!