THE EMBO JOURNAL

Manuscript EMBO-2015-92022

Protein localisation by electron microscopy reveals the architecture of the yeast spliceosomal B complex

Norbert Rigo, Chengfu Sun, Patrizia Fabrizio, Berthold Kastner, Reinhard Luehrmann

Corresponding author: Reinhard Luehrmann, Max Planck Institute for Biophysical Chemistry - Member

Review timeline:

Submission date:	12 May 2015
Editorial Decision:	10 June 2015
Revision received:	30 September 2015
Accepted:	19 October 2015

Editor: Anne Nielsen

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 10 June 2015

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see from the reports, all referees express interest in the findings reported in your manuscript, although they also raise a number of points - largely related to technical aspects of your work - that you will have to address in full before they can support publication of this manuscript in The EMBO Journal

The reports are in our view rather clear and consistent in their recommendations (with ref#1 and #3 sharing many of the same concerns) and it is clear that you will have to strengthen/clarify the criteria for particle sorting and epitope localization to make the overall interpretation of the data more objective and quantifiable. In addition, ref #1 asks for further validation that protein tagging and antibody presence does not disrupt the native spliceosome function per se. You will also see that both ref #2 and ref #3 find that further discussion of the absence of U1 in the complex is needed.

Given the referees' overall positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers in full. I should add that it is EMBO Journal policy to allow only a single round of revision, and rejection or acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

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://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

REFEREE REPORTS

Referee #1:

To shed light on the architecture of the yeast B complex the authors tried to map the positions of several U5, U4/U6 and U2 snRNP proteins by TAP-tagging them, immuno-labeling them using polyclonal anti-TAP antibody and visualizing them by negative-stain electron microscopy. This paper will be improved substantially if a few control experiments are included and data are presented in a more rigorous and objective way.

In the "main view" B complex exhibited a triangular shape with main body, head, stump and neck domains. The antibody will bridge two B complexes as each of the two Fab portions of the antibody react with the TAP-tag present in each B complex. The mapping of proteins in B complex relies on the antibody-mediated dimerization of B complex.

The mapping of a protein component would be convincing if the epitope is located on the perimeters of the "main view" and two particles are well separated as depicted in Figure 1E. However, it seems very difficult to locate the epitope if it is near the center of the "main view" and the two B complexes contact extensively with each other or overlap significantly in the EM image. The authors estimated that the two antigen binding sites of the Fab domains are 14 nm apart while the long straight axis of B complex is ~30nm and the two Fab domains are connected by a flexible linker. This would also make protein mapping by this method prone to errors.

As it stands the position of the epitope is determined quite subjectively (relying on the experience and the judgment of the authors). In order to convince general readers the authors should analyze the data by more objective and statistical methods. The method used by Häcker et al. (2008) is much more objective and relies less on human judgment.

There are points to consider.
(1) In some of the images the particle in the main view is attached to an object much larger than B complex. Since some of them have unrecognizable morphology we cannot exclude the possibility that B complex is bound to aggregates of partially disassembled particles. The authors should use particles in which two B complexes have easily recognizable morphologies (main view). In some figure panels the majority of the particles have recognizable features but in others the particles have little in the way of recognizable features. In the latter case it would be helpful to have cartoons showing how the authors think the two B complexes are interacting with each other. The authors should also indicate what fraction of the total particles were used for analysis for each tagged protein.
(2) It is possible that the attachment of the TAP-tag makes the particle less stable. For example if the C-terminus of the protein is at the interface between two proteins or between protein and RNA the attachment of TAP-tag could disrupt subunit interactions. Although all TAP-tagged strains are viable some could show growth defects. So the authors should carry out spot tests and show their growth phenotype. This is an easy experiment, which could demonstrate the integrity and stability of the particle, albeit indirectly.
(3) Ideally the authors should demonstrate that after immuno-labeling B complex still remains intact. Further addition of antibody to TAP-tag near the subunit interface could cause partial disassembly of the particle. If the authors can demonstrate that particles contain all the protein components even after antibody binding this would add great confidence in their results. If the

authors can demonstrate that splicing activity is unaffected even after addition of antibody that would be even better.
(4) The authors fixed B complex with glutaraldehyde after it reacted with antibody. In Häcker et al. (2008) the authors added antibody after the Grafix procedure. In this case the integrity of the particle is more likely to be maintained by cross-linking. Why did the authors run Grafix after the addition of antibodies in this study? If they fixed prior to antibody addition they could demonstrate the integrity of the particle by adding Fab fragment as the image would look simpler (no dimerization) and by addition of intact antibody they could still have carried out the same analysis.
(5) As far as I can find out the polyclonal antibody used by the authors was raised against peptide representing the C-terminus of the TAP construct after TEV cleavage (i.e. anti-CBP antibody). The authors did not say that they digested their TAP-tagged complex with TEV protease. If they did not digest it with TEV protease the Fc portion of the antibody (polyclonal antibody is predominantly IgG) will also bind to the protein A moiety of the TAP-tag. This would complicate the situation. The authors should clarify this point.
(6) In Figure 8 the authors show by circles the approximate position of each protein. Considering the relative size of the antibody and B complex the authors could have done statistical analysis (presentation) with more data points to show their locations. It is a dangerous practice to select particles that can be interpreted easily as this could bias the outcome. The authors should indicate estimated positions of each protein (epitope) as a dot as well as their average position on each cartoon and their standard deviation obtained from statistical analysis should also be mentioned. Three U2 snRNP proteins are located in three overlapping circles but how significant are these relative positions? How do the authors estimate systematic errors arising from the size and flexibility of the antibody?
(7) The distance between the C-termini of Brr2 and Prp8 is known precisely from the work of the authors and others (Mozaffari-Jovin et al. 2013; Nguyen et al., 2013). So the distance between the estimated positions of the C-termini of Brr2 and Prp8 serve as an excellent internal control. Are they in good agreement with the known distance?

When these points are properly addressed this paper could make an important contribution to the splicing field.

Referee #2:

Rigo et. al set out to map the location of all of the major subcomplexes that together form the "B complex" of the spliceosome. This complex has been previously purified and imaged by the same lab. Using a clever dimerization method they localize specific proteins in the complex using negative stain EM. By forming dimers of the spliceosome they are able to identify the location of the comparatively small and otherwise invisible antibody tag. Multiple proteins from each complex were individually tagged, the complexes purified, and their locations identified. By combining their results with their previous characterization of the tri-snRNP complex they are able to identify how the U2 snRNP fits into the B complex. They are not however able to identify U1 or NTC component locations. This work is a step toward structural characterization of the pre-assembled spliceosome and should be accepted for publication pending minor revisions.

In this lab's previous work by Fabrizio et. al (2009) components of the NTC seemed to be maintained when the B complex was purified. However, in this work the authors describe the NTC as underrepresented and cannot map its location. The authors must describe more clearly if/what the difference between the current study and their previous work is. If the NTC is only present in some of the spliceosomes, are there particles detected by EM that are larger or have extra features suggestive of where the NTC might be? If so, these features should be noted and explored in the discussion section of the paper.
The authors should also comment as to why they believe U1 was not retained.

The labels in Figure 8 are difficult to read due to small yellow labels on a yellow background. The contrast or color scheme needs to be improved for readability.

Referee #3:

Rigo et al. use electron microscopy to map immunological tags on the surface of yeast spliceosomes. The method involves purification of complexes by RNA affinity chromatography, followed by cross-linking gradient centrifugation. The tags are detected not via the antibodies directly but by following dimerization in the presence of antibodies. This is a very neat solution to the problem of detecting the relatively small antibodies on such large particles. The authors mapped a number of important spliceosomal components on the B complex. The resultant map was then compared to the tri-snRNP, which at this stage would be expected to more or less remain in its original form when incorporated within the spliceosome. This is an incremental step, but in the case of such a large and complex entity such incremental steps are in themselves major achievements and they would be helpful to any other groups working on spliceosomal structure.

I have one major reservation only in regard to this work. The method involves identifying potential positions for the antibody at the dimer interface of each molecule. The region of maximal overlap between molecules is then taken as being the probable region of antibody binding. There is no description in the methods of the procedure by which potential sites, such as those shown in Figure 5, were identified. Bearing in mind that the inter-epitope distance on the antibody could be 14 nm, half the maximum dimension of the particle, it seems probable that many possible sites could be identified. The authors should describe how possible sites were identified on each particle (by intuition, algorithm, or a set of visual criteria). Clearly, rule-based algorithms would be most convincing. In addition, the oval drawn to describe the region the possible sites on each particle is then, apparently, subjectively overlapped with those on the other 100 or so particles to find a region of maximum probability. This appears to be highly subjective and not open to reproduction or validation. I suggest that the authors should compile a sum of possible sites for all the molecules in each set and display this as a contour map on the particle. This would have the advantage of enabling the reader to see whether there are other possible sites that occur with significant frequency. We are, in short, being required at present to trust the authors in regard to both the identification of possible sites and the position of the overlaps. This should not be the case.

My minor comments are as follows:

1. The U1 snRNP is sub-stoichiometric, apparently. In the 100-150 molecules used for locating the antibody bridges, there are presumably some that contain U1 snRNP. Some statement should be made as to the proportion and the location of the U1 snRNP shown.

2. It is implied that the other components are all present. How were the RNA bands in EV2B measured, and how closely do they fit the expectation of equal molecular representation?

3. Figure 1E shows a mirror image representation of the bridged dimer. I doubt very much that complex B has mirror symmetry in the x-y plane.

4. What is in fractions 11 and 12 of the 'spliceosomal' peak in Figure EV2A?

5. Is the TAP-tagged spliceosome functional? Might the complex isolated in some cases be not B but a complex whose assembly has been stalled because of interference by the tag?

1st Revision - authors' response 30 September 2015

Point-by-point response to the referees' comments

Referee #1

To shed light on the architecture of the yeast B complex the authors tried to map the positions of several U5, U4/U6 and U2 snRNP proteins by TAP-tagging them, immuno-labeling them using polyclonal anti-TAP antibody and visualizing them by negative-stain electron microscopy. This paper will be improved substantially if a few control experiments are included and data are presented in a more rigorous and objective way.
In the "main view" B complex exhibited a triangular shape with main body, head, stump and neck domains. The antibody will bridge two B complexes as each of the two Fab portions of the antibody

react with the TAP-tag present in each B complex. The mapping of proteins in B complex relies on the antibody-mediated dimerization of B complex.

General point:
The mapping of a protein component would be convincing if the epitope is located on the perimeters of the "main view" and two particles are well separated as depicted in Figure 1E. However, it seems very difficult to locate the epitope if it is near the center of the "main view" and the two B complexes contact extensively with each other or overlap significantly in the EM image. The authors estimated that the two antigen binding sites of the Fab domains are 14 nm apart while the long straight axis of B complex is ~30nm and the two Fab domains are connected by a flexible linker. This would also make protein mapping by this method prone to errors.
As it stands the position of the epitope is determined quite subjectively (relying on the experience and the judgment of the authors). In order to convince general readers the authors should analyze the data by more objective and statistical methods. The method used by Häcker et al. (2008) is much more objective and relies less on human judgment.

The methods used by Häcker *et al* 2008 for the tri-snRNP could not be applied to the B complex. The tri-snRNP has a Y-shaped structure in which hardly any point is far away from its outline. Wherever the antibody binds it can protrude from the particle's outline and can therefore be located directly. The same is true of the tDimer2 tag used by Häcker *et al*. Particularly problematic are proteins that are located in the centre of the particle when it displays the main view.
We agree that the description of our localisation method in the first version conveyed an impression that the method was subjective and correspondingly error-prone. We have therefore changed the presentation and description of the localisation method in the Results and Materials and Methods sections.
From negative-stain EM images we first collected all dimers in which at least one particle shows the B complex main-view (main-view dimers) until the number of main-view dimers was around 100. These 100 dimers represent all main-view dimers detected in the original micrographs. Then we identified the outline of the second particle at the dimerisation interface. We then applied the criteria that the distance between the antibody binding sites in the two connected particles has to be 14 nm or shorter. That is, due to their flexibility, the domains of the IgG molecule that are involved in dimer formation (see Referee #1 point (5)) and the possibility that the antibody binding site at the second particle of the dimer is not at the particle periphery, the antibody binding site in the main-view particle can maximally be 14 nm from the dimerisation interface. Therefore, we next identified the area in the main-view particle that is 14 nm or closer to the dimerisation interface but not further away. The antibody binding site definitely has to be inside this area and cannot be outside. This area includes all the possible binding sites in one dimer. We determined this area for all 100 dimers computationally. Then we computationally aligned all 100 dimers including their associated localisation area according to the main-view particle. The last step was to determine the overlapping region of all 100 areas. The resulting area of maximal overlap represents the region of the main-view in which the antibody binding site has to be located. Only when inside this area the location of the antibody binding site is compatible with all (or nearly all) 100 observed dimers. Therefore, the TAP tag of the main-view particle has to be somewhere inside this overlapping area or more precisely, it cannot be outside.
Thus this method does not include a subjective identification of binding sites or antibody positions and no subjective overlapping.
In the cases of Brr2-TAP and Prp3-TAP we collected up to 150 main-view dimers. All of them were analysed, however, to be comparable to the other protein locations we randomly selected 100 dimers (the first 100 found in the original electron micrographs) to produce the image of overlapping areas for Figures 4 and 6.
We changed the Results section and describe the new method in the chapter "The B complex main axis harbours the U5 snRNP proteins Snu114, Brr2 and Prp8". We also included a brief description of the localisation method in the Methods section.

(1) In some of the images the particle in the main view is attached to an object much larger than B complex. Since some of them have unrecognizable morphology we cannot exclude the possibility that B complex is bound to aggregates of partially disassembled particles. The authors should use particles in which two B complexes have easily recognizable morphologies (main view). In some figure panels the majority of the particles have recognizable features but in others the particles have little in the way of recognizable features. In the latter case it would be helpful to have cartoons

showing how the authors think the two B complexes are interacting with each other. The authors should also indicate what fraction of the total particles were used for analysis for each tagged protein.

We agree that it is desirable to have dimers where both particles display the B complex main-view. However, we generally observe only very few dimers where this is the case and therefore cannot rely on only those. The low abundance of these "double main-view dimers" is very likely due to the restricted length and flexibility of the IgG molecule which prevent that both particles adsorb as they would in the monomeric state. In those dimers where one particle adsorbs to the carbon film in the standard orientation (thus displaying the main-view) the other particle is, in most cases forced into an orientation that leads to a 2D projection that does not display the B complex main-view. Locations at the periphery of the main-view should result in more "double main-view dimers" than sites located inside the particle. Indeed for Brr2, Prp9 and Prp3 that we located at the periphery, we observe more dimers with both particles displaying the main view.
Nevertheless, for all our B complex purifications that were used for analysis we measured the gradient tubes (including possible pellets) after fractionation and detected only 1-6% of the total radioactivity. From this we concluded that spliceosome aggregation that would lead to particles larger than dimers has not occurred. However, it is possibility that particles can adsorb to the EM grid in such a way that they touch each other although they are not dimerised in solution. To minimise these events we produced EM specimen in which the dimers are well separated from each other.

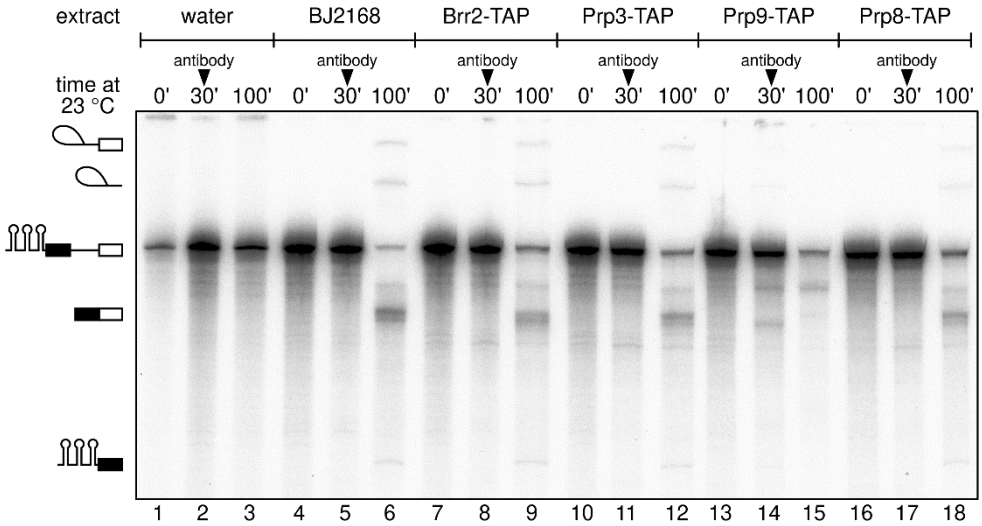
(2) It is possible that the attachment of the TAP-tag makes the particle less stable. For example if the C-terminus of the protein is at the interface between two proteins or between protein and RNA the attachment of TAP-tag could disrupt subunit interactions. Although all TAP-tagged strains are viable some could show growth defects. So the authors should carry out spot tests and show their growth phenotype. This is an easy experiment, which could demonstrate the integrity and stability of the particle, albeit indirectly.

As suggested, we performed a growth test for all strains used in this study and present the results in Appendix Fig S1of the revised version. The vast majority of the strains did not show any difference in growth compared to the strains that were used to introduce the TAP tag. The only two exceptions are the strains that express an N-terminally tagged Prp38 and C-terminally tagged Lsm4, respectively. Both strains show a temperature sensitive phenotype at 37 °C and the Lsm4-TAP strain grows slower at lower temperatures (16 °C and 25 °C) as well. However, at 30 °C, the temperature where the cells were grown for extract preparation both strains grow almost identical to the strain that does not express any tagged protein. We conclude that for all proteins except Lsm4 and Prp38 the addition of the TAP tag does not interfere with pre-mRNA splicing *in vivo* and thus cell growth is not affected. In the case of Lsm4-TAP the slower growth could indicate that the tag interferes with spliceosome assembly. The growth defect might as well have other reasons. To further investigate this question we prepared whole-cell extract for all strains and conducted splicing tests. The results are presented in Appendix Fig S2. All but three strains show an *in vitro* splicing activity identical to the extract prepared from BJ2168 cells where proteins carry no tag. In the strains that express Lsm4-TAP and TAP-Prp38, as well as Brr2-TAP, TAP-Prp8, Prp8-TAP, Snu114-TAP, Prp3-TAP, Prp9-TAP and Hsh155-TAP, *in vitro* splicing and thus B complex assembly (*in vitro*) was not affected. Thus, the growth defect observed for the strains expressing TAP-Prp38 and Lsm4-TAP, respectively, cannot be correlated with a splicing defect *in vitro*. Three strains, Prp11-TAP, Cus1-TAP and Bud13-TAP, respectively, show a lower splicing efficiency. Those three strains are based on the Prp2-1 strain that expresses a temperature-sensitive mutant of Prp2 that leads to stalling of the spliceosome at the B^{act} stage at 35 °C (Kim *et al*, 1996). The lower efficiency of pre-mRNA splicing probably occurs due to the temperature-sensitive mutation in Prp2 (although the splicing test was dome at 23 °C). Since Prp2 acts at the B^{act} stage, and it is not present in the B complex (Fabrizio *et al* 2009) the assembly of the B complex most likely is not affected. Anyway, for all the particles harbouring a TAP-tagged protein we observed the characteristic shape of the spliceosomal B complex in negative-stain images (Fig 7A, D and E).

(3) Ideally the authors should demonstrate that after immuno-labeling B complex still remains intact. Further addition of antibody to TAP-tag near the subunit interface could cause partial disassembly of the particle. If the authors can demonstrate that particles contain all the protein components even after antibody binding this would add great confidence in their results. If the

authors can demonstrate that splicing activity is unaffected even after addition of antibody that would be even better.

To demonstrate that the spliceosomal dimers are composed of intact B complexes, we purified B complexes, added antibodies to form dimers and analysed the protein content of these dimers by mass spectrometry. For practical reasons we could not perform this kind of analysis for all the tagged proteins used in this work. We chose B complex dimers that harboured Brr2-TAP and Prp3-TAP, respectively and compared the protein content of these two samples to that of untagged B complex monomers. The results are presented in Appendix Table S1, which shows that the protein composition of the respective dimers is almost identical to the composition of the monomers and shows a protein pattern characteristic for the spliceosomal B complex (Fabrizio *et al* 2009).



Response Fig R1: Effect of antibody labelling on the splicing activity of spliceosomes harbouring a TAP-tagged protein. Actin pre-mRNA was incubated under splicing conditions with extracts containing one TAP-tagged protein and ATP was limited to 50 µM to stall splicing at the B complex stage. B complexes were allowed to assemble for 30 min before a 4-fold molar excess (relative to the pre-mRNA concentration) of anti-TAP antibodies was added. After additional 30 min of incubation the ATP concentration was raised to 2 mM and splicing was allowed for 40 min. Aliquots were taken at 0 min, 30 min (before antibody addition) and at 100 min, digested with Proteinase K and analyzed by denaturing PAGE (8% acrylamide 29:1, 8.3 M Urea) followed by autoradiography. The extract used and the incubation time and temperature are given above each lane. The time point where antibodies were added is marked with a black triangle and the identity of the RNA molecules corresponding to the bands visible is given at the left as schematic drawings. Water marks the control where no extract was added.

As suggested by the referee, in addition to the MS analysis we performed splicing activity tests in the presence of anti-TAP antibodies. To mimic our labelling experiment we first allowed assembly of B complexes at 50 µM ATP, then added anti-TAP antibodies and incubated further before raising the ATP concentration to 2 mM to allow splicing to occur. As can be seen in Response Fig R1 splicing is not inhibited by antibody binding when the TAP tag was present at the C termini of Brr2, Prp3 or Prp8, respectively. In these cases the efficiency of splicing is comparable to the control where no protein carried a tag (BJ2168). There is, however, an inhibition of splicing when antibodies are added to B complexes harbouring Prp9-TAP. With this extract, splicing in absence of the antibody is not inhibited (Appendix Fig S2). Since in EM images, we can identify dimers where at least one particle shows the characteristic shape of the B complex (Fig 7) with the usual abundance the observed inhibition very likely is a post-B complex event.

(4) The authors fixed B complex with glutaraldehyde after it reacted with antibody. In Häcker et al. (2008) the authors added antibody after the Grafix procedure. In this case the integrity of the particle is more likely to be maintained by cross-linking. Why did the authors run Grafix after the addition of antibodies in this study? If they fixed prior to antibody addition they could demonstrate the integrity of the particle by adding Fab fragment as the image would look simpler (no dimerization) and by addition of intact antibody they could still have carried out the same analysis.

Häcker *et al* 2008 purified the tri-snRNP using the TAP method, added anti-TAP antibodies to unfixed particles and afterwards cross-linked the immuno-complexes during gradient ultracentrifugation using a glutaraldehyde gradient parallel to the density gradient (GraFix; Kastner *et al* 2008). This is the same protocol that we used in this work. This approach leads to a stabilisation of the immuno-complexes (dimers or antibody-labelled monomers). We like to mention that in contrast to the endogenous tri-snRNP *in vitro* assembly and purification of a stage-specific spliceosome require much more effort, especially when the amount and the concentration of the B complex has to meet the requirements of EM of labelled complexes. Therefore, we had to apply an efficient method to obtain appropriate preparations of labelled complexes. Thus we had to add the antibody to affinity-purified B complexes and perform GraFix afterwards to obtain pure and stable antibody-labelled spliceosomes (dimers). Cross-linking the B complexes with glutaraldehyde before antibody addition reduces immune-complex formation, probably due to chemical modification of amino acids of the epitope.

(5) As far as I can find out the polyclonal antibody used by the authors was raised against peptide representing the C-terminus of the TAP construct after TEV cleavage (i.e. anti-CBP antibody). The authors did not say that they digested their TAP-tagged complex with TEV protease. If they did not digest it with TEV protease the Fc portion of the antibody (polyclonal antibody is predominantly IgG) will also bind to the protein A moiety of the TAP-tag. This would complicate the situation. The authors should clarify this point.

Indeed we did not digest the TAP-tagged complex with TEV protease. This means that the protein A domains are still present. We did not digest the purified spliceosomes with TEV protease because we did not want to introduce particle degradation during incubation. Three different scenarios of antibody binding might lead to dimer formation.

1. One Fab domain binds to the TAP tag of one particle and the other Fab domain binds to the TAP tag of the second particle. In this case the maximal distance of the TAP tags is 14 nm.
2. One Fab domain of the IgG molecule binds to the TAP tag of one particle and the TAP tag of the second particle is bound by the Fc domain of the IgG via the protein A domain. Both the Fc and the Fab domains have a length of ~7nm. The maximal distance between the TAP tags will be below 14 nm.
3. The Fc domain of the IgG binds the TAP tags of both particles via the respective protein A domain. In this case the distance between the TAP tags is lower than 14 nm.

Therefore, we can conclude with certainty that the distance of the TAP tags within one dimer is 14 nm or shorter. Our location method only relies on maximal distances; lower distances don't interfere. For clarification we incorporated this information in the chapter "The B complex main axis harbours the U5 snRNP proteins Snu114, Brr2 and Prp8" of the Results section of the manuscript.

(6) In Figure 8 the authors show by circles the approximate position of each protein. Considering the relative size of the antibody and B complex the authors could have done statistical analysis (presentation) with more data points to show their locations. It is a dangerous practice to select particles that can be interpreted easily as this could bias the outcome. The authors should indicate estimated positions of each protein (epitope) as a dot as well as their average position on each cartoon and their standard deviation obtained from statistical analysis should also be mentioned. Three U2 snRNP proteins are located in three overlapping circles but how significant are these relative positions? How do the authors estimate systematic errors arising from the size and flexibility of the antibody?

As described above our localisation method does not intend to locate positions of single epitopes. Therefore we cannot depict them as dots or calculate a standard deviation. We understand that the initial presentation of our method suggests that we located individual epitopes because we presented schematic drawings with example positions of IgG molecules binding to two B complexes, which might have been misleading. We now present our method in a different, and in our opinion clearer way. We also do not select particles that can easily be interpreted, instead we interpreted all particles displaying the main-view. We now present our method for determining the protein locations in detail (see above).
The final area that we present for each protein presents the area in which the N or C terminus of the protein can be located in the B complex main-view. A position anywhere within this area is

possible. It might be suggestive that a position in the center of the area is more likely, however, we do not have solid measures for such an assumption.
The size of the final area of protein location indicates the degree of the localisation error.

(7) The distance between the C-termini of Brr2 and Prp8 is known precisely from the work of the authors and others (Mozaffari-Jovin et al. 2013; Nguyen et al., 2013). So the distance between the estimated positions of the C-termini of Brr2 and Prp8 serve as an excellent internal control. Are they in good agreement with the known distance?

The distance between the C termini of Brr2 and Prp8 is ~10 nm in the crystal structures. We determined that the distance of the C termini of Brr2 and Prp8 in a two-dimensional projection of the B complex has to be between 0 and 14 nm based on our location experiments. Since the areas where the C termini can be positioned based on our results partially overlap for Brr2 and Prp8 (Fig 8A) we cannot rule out a very low distance (in 2D). The points of the two areas that show the biggest distance to each other are 14 nm apart. Our measured distance fits to the distance of the C termini in the crystal structures. If we now chose the centres of the two areas as the positons of the respective C termini we measure a distance of approximately 6.5 nm which is consistent with the distance in the crystal structures. However, we prefer to avoid discussing in the manuscript the distances between arbitrarily chosen points within the areas that we find.

Referee #2

Rigo et. al set out to map the location of all of the major subcomplexes that together form the "B complex" of the spliceosome. This complex has been previously purified and imaged by the same lab. Using a clever dimerization method they localize specific proteins in the complex using negative stain EM. By forming dimers of the spliceosome they are able to identify the location of the comparatively small and otherwise invisible antibody tag. Multiple proteins from each complex were individually tagged, the complexes purified, and their locations identified. By combining their results with their previous characterization of the tri-snRNP complex they are able to identify how the U2 snRNP fits into the B complex. They are not however able to identify U1 or NTC component locations. This work is a step toward structural characterization of the pre-assembled spliceosome and should be accepted for publication pending minor revisions.

(1) In this lab's previous work by Fabrizio et. al (2009) components of the NTC seemed to be maintained when the B complex was purified. However, in this work the authors describe the NTC as underrepresented and cannot map its location. The authors must describe more clearly if/what the difference between the current study and their previous work is. If the NTC is only present in some of the spliceosomes, are there particles detected by EM that are larger or have extra features suggestive of where the NTC might be? If so, these features should be noted and explored in the discussion section of the paper.

In the MS analysis performed in the work by Fabrizio *et al* 2009 (Table 1) the peptide numbers of NTC and NTC-related proteins found in B complex purifications are significantly lower than the peptide numbers found for U2 snRNP and U5 snRNP proteins of comparable molecular weight. In B^{act} complex purifications the peptide numbers of NTC and NTC-related proteins are comparable to those of the U2 snRNP and U5 snRNP proteins. We obtained the same result for B complex monomers and dimers that we purified (Appendix Table S1). Therefore, we conclude that there is no difference regarding the abundance of the NTC between the previous work and our results. Moreover, in the previous work the peptide numbers of NTC proteins found in B^{act} complex purifications are 5- to 8-fold higher than the corresponding peptide numbers in B complex purifications whereas the numbers of peptides of U2 and U5 snRNP proteins are comparable (the same amount of complexes was used for analysis). From both studies we concluded that the NTC is not yet stably bound and therefore is underrepresented in the B complex (some particles contain NTC proteins, some don't). Moreover, it is possible to isolate B complexes from extracts which have been immune-depleted of endogenous NTC and which still have the characteristic B complex shape (data not shown).
Following this approach we consider the U2 snRNP, the U5 snRNP, the U4/U6 snRNP and the tri-snRNP specific proteins as present in stoichiometric amounts (as concluded already in Fabrizio *et al*, 2009). To judge the abundance of snRNPs and non-snRNP proteins we rely on mass

spectrometric analysis of the protein composition and not on measuring the RNA bands in silver stained gels, since silver staining is not quantitative.
In summary, we conclude that the majority of the main-view B complexes that we see in EM images do not contain the NTC. We did not search for particles with additional features and therefore, did not discuss the NTC.

(2) The authors should also comment as to why they believe U1 was not retained.

Mass spectrometric analysis of the spliceosomal B complex (Fabrizio *et al* 2009 and Appendix Table S1 in our manuscript) show significantly lower peptide numbers for U1 snRNP proteins (and NTC proteins; see above) than for proteins associated with the other snRNPs (U2, U5 and U4/U6). The U1 snRNA band in silver stained gels is always weaker than the other snRNA bands. The presence of the U1 snRNP in the gradient fractions probably originates in part from a minor contamination with A complexes (see Referee #3 point (4) for more evidence) since a low number of peptides of early splicing factors are also found (Prp5 and Urn1; Appendix Table S1 and Fabrizio *et al* 2009). Moreover, we generally observe that the low amounts of U1 snRNPs present in B complex preparations are only loosely associated with the spliceosomes and readily dissociate at higher salt concentration, indicating that they are no longer base-paired to the 5' SS of the pre-mRNA.

(3) The labels in Figure 8 are difficult to read due to small yellow labels on a yellow background. The contrast or color scheme needs to be improved for readability.

The colours have been changed and yellow has been replaced by blue. We think that this has improved the readability substantially.

Referee #3

Rigo et al. use electron microscopy to map immunological tags on the surface of yeast spliceosomes. The method involves purification of complexes by RNA affinity chromatography, followed by cross-linking gradient centrifugation. The tags are detected not via the antibodies directly but by following dimerization in the presence of antibodies. This is a very neat solution to the problem of detecting the relatively small antibodies on such large particles. The authors mapped a number of important spliceosomal components on the B complex. The resultant map was then compared to the tri-snRNP, which at this stage would be expected to more or less remain in its original form when incorporated within the spliceosome. This is an incremental step, but in the case of such a large and complex entity such incremental steps are in themselves major achievements and they would be helpful to any other groups working on spliceosomal structure.

General point:
I have one major reservation only in regard to this work. The method involves identifying potential positions for the antibody at the dimer interface of each molecule. The region of maximal overlap between molecules is then taken as being the probable region of antibody binding. There is no description in the methods of the procedure by which potential sites, such as those shown in Figure 5, were identified. Bearing in mind that the inter-epitope distance on the antibody could be 14 nm, half the maximum dimension of the particle, it seems probable that many possible sites could be identified. The authors should describe how possible sites were identified on each particle (by intuition, algorithm, or a set of visual criteria). Clearly, rule-based algorithms would be most convincing. In addition, the oval drawn to describe the region the possible sites on each particle is then, apparently, subjectively overlapped with those on the other 100 or so particles to find a region of maximum probability. This appears to be highly subjective and not open to reproduction or validation. I suggest that the authors should compile a sum of possible sites for all the molecules in each set and display this as a contour map on the particle. This would have the advantage of enabling the reader to see whether there are other possible sites that occur with significant frequency. We are, in short, being required at present to trust the authors in regard to both the identification of possible sites and the position of the overlaps. This should not be the case.

As already described in the answer to the general point of referee #1, we changed the presentation of the location method and changed Fig 5 that illustrates the method. We now show the areas that

represent the sum of all possible sites for one dimer in Fig 4-7 and do not draw any ovals subjectively. We further included a chapter in the Methods section that explains our localisation method. We now show 100 overlapping areas for each protein so that the reader can follow our method. We are very thankful for this point of criticism and trust that the changes we have made have significantly improved our manuscript.

(1) The U1 snRNP is sub-stoichiometric, apparently. In the 100-150 molecules used for locating the antibody bridges, there are presumably some that contain U1 snRNP. Some statement should be made as to the proportion and the location of the U1 snRNP shown.

This point is identical to the point (2) raised by referee #2 and has been discussed in detail there.

(2) It is implied that the other components are all present. How were the RNA bands in EV2B measured, and how closely do they fit the expectation of equal molecular representation?

As already discussed in the answer to point (1) of referee #2 we did not quantify the silver-stained RNA bands, since silver staining is not quantitative. We rely on the number of peptides sequenced by LC-MSMS to provide a readout for the abundance of snRNPs and non-snRNP proteins in our purified B complexes. Based on this approach we concluded that the U2 snRNP, the U5 snRNP, the U4/U6 di-snRNP and the tri-snRNP specific proteins are present in stoichiometric amounts. The RES complex seems to be underrepresented but in those particles where it is present it seems to be bound stable enough for the localisation of Bud13.

(3) Figure 1E shows a mirror image representation of the bridged dimer. I doubt very much that complex B has mirror symmetry in the x-y plane.

Fig 1E should only explain our strategy that involves B complex dimerisation. We can understand that the initially submitted Fig 1E can lead to confusion. We have therefore changed it and used schematic drawings of the main-view B complex without using a mirrored sketch.
The B complex is probably asymmetric. Negative-stain EM images are two-dimensional projections of three-dimensional images. It is possible that some B complexes adsorb to the carbon film with a certain part of their surface (orientation 1) and other B complexes adsorb to the carbon film with a surface on almost the opposite site of the particle (orientation 2). This would lead to the two-dimensional projections of orientation 1 and orientation 2 having mirror symmetry in the x-y plane. Indeed Fabrizio *et al* 2009 (Fig 4B panels 1 and 3) and this work (frames 1A and 7B in Fig7) observed class averages and raw images, respectively that are very close to being mirrored versions of the B complex main-view. In those pseudo-mirrored views the arm domain protrudes from the main axis towards the opposite side when compared with the B complex main-view. The existence of two large surfaces on opposing sides of the B complex that preferably interact with the carbon film during EM specimen preparation indicates that the B complex is a rather “flat” particle, comparable to the yeast tri-snRNP (Nguyen *et al* 2015).

(4) What is in fractions 11 and 12 of the 'spliceosomal' peak in Figure EV2A?

As can be seen in Fig EV2A there is a peak in the radioactivity profile of the gradient in fractions 11-15. Fractions 13-15 contain the pre-mRNA and all snRNAs involved in splicing (albeit with a weak band for the U1 snRNA; Fig EV2B). The conclusion is that these fractions contain the B complex. Fractions 11 and 12 contain the pre-mRNA, U1 snRNA and the U2 snRNA but almost no U4, U5L, U5S or U6 snRNA (Fig EV2B). Thus these fractions mainly contain spliceosomal A complex which has a lower sedimentation coefficient than the B complex.

(5) Is the TAP-tagged spliceosome functional? Might the complex isolated in some cases be not B but a complex whose assembly has been stalled because of interference by the tag?

This point addresses the potential effects of the TAP tag and the anti-TAP antibody addition on the stability of the B complex. These questions have also been raised by referee #1 (points (2) and (3)). To address these questions we included growth tests of the yeast strains expressing TAP-tagged proteins (Appendix Fig S1) and splicing tests of whole-cell extracts from these yeast strains (Appendix Fig S2) into the manuscript. Further we performed splicing tests in the presence of the

antibody (Response Fig R1). Our conclusions regarding the stability and identity of the purified spliceosomes are discussed in detail in the answers to points (2) and (3) of referee #1.

Accepted 19 October 2015

Thank you for submitting a revised version of your manuscript to The EMBO Journal. It has now been seen by one of the original referees whose comments are shown below.

As you will see, the referee finds that all major criticisms have been sufficiently addressed and therefore recommends the manuscript for publication. However, the referee does still suggest a change to figure 8 to better overlay your findings with work published elsewhere during the revision of this study. In addition, I would encourage you to also include a reference to the recent structure work from Yan and colleagues (PMID: 26292707) in the manuscript. This slightly amended final version can be sent to me directly by email and we will then upload it in house before transferring the files for production.

If you have any questions, please feel free to contact me. Thank you for your contribution to The EMBO Journal and congratulations on this nicely executed work.

REFEREE REPORT

Referee #1:

The authors have addressed all the points raised by the reviewers and the manuscript can be published with only minor changes (optional)

Page 22
"During revision of the manuscript the 3D structure of the S. cerevisiae tri-snRNP, determined by cryo-EM at a resolution of 5.9 Å, was reported (Nguyen et al, 2015). On the basis of our fitting of the tri-snRNP into the B complex, our protein locations are consistent with the protein positions in the 3D structure."

Figure 8D may be replaced with a figure based on Nguyen et al., 2015 and more precise location of the C-termini of tri-snRNP proteins could be indicated.

