Reconstitution of the human U snRNP assembly machinery reveals stepwise Sm protein organization

Nils Neuenkrichen, Clemens Englbrecht, Jürgen Ohmer, Thomas Ziegenhals, Ashwin Chari and Utz Fischer

Corresponding author: Utz Fischer, University of Würzburg

Review timeline:

Submission date: 20 October 2014
Editorial Decision: 01 December 2014
Revision received: 31 March 2015
Editorial Decision: 21 April 2015
Revision received: 28 April 2015
Accepted: 12 May 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 01 December 2014

Thank you for submitting your manuscript for consideration by The EMBO Journal and my apologies for the slightly extended duration of the review process in this case. Your study 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 and acknowledge the high technical quality of the data presented. However, at the same time they do raise a number of questions and comments that will have to be addressed before they can support publication of a revised manuscript.

More specifically - as pointed out by refs #1 and #2 - the manuscript text would benefit from being rewritten to include a more extensive presentation of the existing literature and to make the overall study more accessible for the non-specialist reader. In addition, ref #3 asks you to address the potential discrepancy between the in vitro conditions used in the current system and the crowded nature of protein interactions in the cytoplasm. I would encourage you to address this issue experimentally as far as possible, also integrating the point raised by the same ref concerning cooperativity in the binding of individual factors in the complex (I would be happy to discuss the details of this further, if necessary). Finally, I strongly encourage you to follow the suggestion by ref #1 to include additional known SMA mutations in the vitro analysis.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO
Journal policy to allow only a single round of revision, and 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.

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Referee #1:

In this manuscript, the authors describe the reconstitution of the human PRMT5 and SMN complexes from recombinant sources, and show that these two purified complexes can function to recapitulate important steps in formation of the UsnRNP Sm core particle. This is an important accomplishment. For the most part, the experiments are well performed and the figures are well illustrated. The system has the potential to uncover important mechanistic insights into the assembly of Sm-class snRNPs. However, the manuscript itself needs a fair amount of work, as it falls short both in the introduction (e.g. the motivation for carrying out the experiments) and in the interpretation/discussion of several of the results. Finally, the authors do not take full advantage of their system, as they analyzed only a single SMN point mutation.

General comments:
Title: Does the experiments in the ms. reveal the basis for snRNA proofreading? Not really. They exclude Gemins3-5 from this activity but do not identify the underlying mechanism (see below).
Synopsis: Only one mutation was analyzed. Cannot use plural in last bullet point.

Introduction: The introduction needs to be re-formulated to provide a more accurate description of previous work in the field and better reasoning/motivation for why they performed the experiments.

Specific comments:
1. The first paragraph is fine, but in the second one, they get into trouble. They state that pICln accepts newly-synthesized Sm proteins "at the ribosome itself" citing a paper that is submitted and not provided to the referees. Remove words in quotations above.

2. Also in the second paragraph, the authors go on to talk about Sm protein methylation, stating that "the precise role of this modification in the assembly reaction is controversial." What is the controversy? They cite a ton of papers, but seem to ignore the fact that yeast have UsnRNPs but their Sm proteins completely lack RG dipeptide motifs (e.g. Girard et al. 2004). Moreover, they fail to mention that although Drosophila Sm proteins have RG rich tails, Sm protein methylation is dispensable for snRNP assembly in flies (Gonsalvez et al. 2008, RNA; Gonsalvez et al. 2006, Curr Biol).

If the authors want to limit their discussion to humans (not sure why they would want to do that but...), then they need to explicitly state it. Even then, most of the papers they cite are review articles. The few primary papers they cite don't really address the requirement for the sDMA modification. Pulse chase experiments in HeLa cells showed that Sm protein methylation IS required for proper snRNP assembly, and that two different PRMTs participate in this activity (Gonsalvez et al. 2007).
3. Later on in the Introduction, the authors state that "Likewise, apart from SMN and Gemin2 ..., the functions of other SMN complex subunits (Gemin2-8 and unrip) are less clear (Chari & Fischer, 2010; Kroiss et al, 2008; Palfi et al, 2009)." The authors cite one review, and two primary papers, neither of which address this subject in the human system! Kroiss et al. use Drosophil and Palfi et al. use trypanosomes. The selectivity of the citations paints a disturbing picture here. Which of these subunits (Gemin3-8 and unrip) is required for Sm core assembly? Two papers address this directly in the human system, using RNAi to specifically deplete certain SMN complex subunits and then asking if the extracts from these cells can carry out snRNP assembly (see Shpargel & Matera, 2005; Feng et al. 2005). Curious that these two papers are not mentioned, even among the "multitude of experimental approaches" and primary papers described in the very next sentence to address the functions of Gemins.

4. After describing the ATP requirements (or lack thereof) for Gemin3 in the overall process of snRNP assembly, the authors sum up the paragraph with this sentence: "Therefore, a deep mechanistic analysis of the assisted snRNP assembly process appears unavoidable." I am at a loss for words here. ????

5. The beginning of the next paragraph ("The mechanistic dissection of many...") is good. But the statement they use for motivating their study is untrue: "Since SMN is essential for viability, the effects of disease-causing missense mutations on snRNP assembly cannot be studied in the absence of wild-type protein in vivo." It has yet to be established in mouse knock-in models whether mild SMN point mutations can support viability. However, it has been shown in Drosophila that SMA-causing point mutations recapitulate the full range of viability seen in human patients. Some of the disease-causing point mutants are viable in the absence of any wild-type SMN (Praveen et al. 2012; 2014).

Results
6. The nomenclature is a bit confusing (6S vs. pICln-D3/B). Why use Svedberg numbers for one complex and not the other? Do the two trimeric complexes -D3/B and -D1/D2) have different names? They probably have similar Svedberg numbers, so maybe not.

7. The second part of this statement is neither supported by data in the paper nor by citations: "One crucial step in the initial phase of snRNP assembly is the recruitment of all Sm proteins to the PRMT5 complex and the methylation of a subset of them (i.e. B, D1 and D3)." What is the evidence that methylation of Sm proteins is required in vitro? See Gonsalvez et al. 2006, 2007, 2008. Do we know if PRMT5 enzymatic activity is strictly required? What happens to Sm core assembly if you use an MTase dead point mutant PRMT5 in your reconstituted system?

8. The finding that Gemin5 is dispensable for snRNA identification is important. The authors fail to mention that they showed this to be the case in their Kroiss et al. 2008 paper. They showed that human SMN/Gemin2 heterodimers were sufficient for Sm core assembly (see supplemental figures). What they did NOT do in that paper is challenge the assembly reaction with a mixture of RNAs. Evidence for this 'proofreading' activity is an important finding of this study, especially if this activity does not depend on Gemins3-5. Related to this point in the Discussion, the authors state: "Quite unexpectedly, the central SMN complex components are both necessary and sufficient for all aspects of snRNP core formation in vitro." Why is this so unexpected? The authors showed that human SMN/Gemin2 dimers were necessary and sufficient in 2008 (Kroiss et al).

9. The title of the manuscript says that the basis for snRNA proofreading is revealed by these expts. Why is proofreading not specifically discussed in the Discussion?

10. This is a good point: "Surprisingly, neither methylation nor F/E/G binding are sufficient for 6S complex dissociation from PRMT5/WD45. This process appears to be feed-forward driven, where the addition of a new pICln-D1/D2 or pICln-D3/B heterotrimer displaces the 6S complex (Fig. 2)." Is there a way to test this idea (not necessarily in this paper)? But the authors may want to elaborate.

11. In the Discussion: E134K is not a common SMA mutation. None of the point mutations in SMN are 'common'. It is a well-studied mutant but it is by no means "frequently found" in the SMA patient population. Furthermore, why is this the only mutation analyzed? There are a number of
other mutations, analysis of which could really help flesh out this manuscript. T274I, A111G and 
one or two others (not sure off top of my head) should be analyzed to address questions of assembly 
and function of SMN complex.

12. Near the end of the Discussion, this statement should be removed. "The consequences of 
mutations ... cannot be analyzed in these model systems ... in vivo model systems must contain at 
least one copy of wild-type SMN. This statement is patently untrue.

Referee #2:

Neuenkirchen et al report the biochemical reconstitution of Sm complex assembly from purified 
recombinant Sm proteins and the components of the PRMT5 and SMN chaperone complexes. This 
represents a remarkable feat, which paves the way to the detailed analysis of the sequence of events, 
kinetics and modulation (e.g. by SMA-associated mutations) of this key assembly process for 
snRNP biogenesis. Despite the extensive knowledge already available about this process, the initial 
biochemical characterization presented by Fischer and colleagues has already provided relevant 
unexpected insights, including the dispensability of several Gemin proteins and ATP-independence 
for SMN complex function.

The conclusions are supported by very clean, carefully controlled biochemical data. I have only 
three suggestions for the authors' consideration:

1) I fear that the manuscript may be too demanding for non-specialists, some of whom may be put 
down by the complexity of the system, the non-friendly nomenclature and -to some extent- the 
rather dry style of the Results section. I would encourage the authors to improve the latter and also 
to provide a scheme at the beginning of the manuscript schematizing the current knowledge about 
the assembly pathway and the open questions, and display, in their final model, complementary 
information derived from the new results, highlighting how the work has contributed to illuminate 
new features of the pathway.

2) The authors argue that the influence of temperature on assembly efficiency may reveal that the 
SMN complex acts as a Brownian machine by coupling spontaneous conformational changes driven 
by thermal energy to the directed delivery of Sm proteins onto snRNA. It is not entirely clear to me, 
however, what is the mechanism that the authors are proposing. Is thermal energy affecting some 
activity of the SMN complex itself? Or is it enhancing the opportunities of the SMN complex to take 
advantage of spontaneous conformational changes in Sm proteins? Can these -or other scenarios- be 
distinguished?

3) While the result with the SMN(E134K) mutant is of significant interest as a direct demonstration 
that this mutant has intrinsically lower activity to promote Sm complex assembly, the extent to 
which this difference (2-fold increase in Kd) can be rate-limiting for snRNP assembly and function 
in cells, or make a difference between different cell types, and particularly in motor neurons - perhaps the aspect of wider interest- should be discussed further.

Referee #3:

The group of Utz Fischer has been studying the process of U snRNP assembly and cellular 
components involved in this process and has been making important contribution to our 
understanding of U snRNP assembly. In this manuscript the authors attempted to recapitulate the 
assembly process of the core snRNP domain using purified protein components expressed in insect 
cells and E. coli. The results are in general of high quality and largely confirmed what is known 
about this system.

The core snRNP domain assembly takes place in the cytoplasm overcrowded with many proteins 
and RNAs. It is not easy to show why such an elaborate machine is necessary when Sm proteins 
spontaneously assemble with snRNAs when purified components are mixed. Furthermore yeast 
seems to manage without such an elaborate system. It would be more informative if the experiments 
had been done more quantitatively such that we could learn what is critical when these core snRNP
components are present under physiological concentrations in the presence of other competing proteins and nucleic acids.

The results described in this manuscript are important and should be published after the following points have been considered.

(1) Figure 1 could be more informative if size exclusion chromatography was used in conjunction with multi angle light scattering (this is now a standard practice). This will show the homogeneity and molecular mass (hence, subunit stoichiometry) of eluted complexes. The quality of the protein samples shown in Figure 1A and Figure 1C-J does not look the same. For example PRMT5/WD45 looks much cleaner in Figure 4A than in other panels. Some of the input lane has no protein bands and proteins in purified fractions do not look like the sum of input proteins shown in Figure 1A. This is presumably because the protein samples in Figure 1A were stained with Coomassie blue whereas those in Figure 1C-J were silver stained. It would be good if the authors show each input protein preparation used for each experiment on the same gel. For D3/B, D3 looks over-represented even when they are stained with Coomassie blue (Figure 1A lane 4). Does D3 stain better with Coomassie blue or is D3 present in excess?

(2) It is possible that these proteins tend to aggregate, when present in high concentration in isolation, and these experiments may have to be done under particular conditions. The authors should specify buffer conditions for analytical gel-filtration. Ideally binding assays should be quantitative and Kd for each interaction should be described although this may be difficult. D1/D2 binds much more strongly to PRMT5/WD45 in the presence of pICln. Does D1/D2 bind more strongly to pICln when PRMT5/WD45 is present? Does B/D3 bind to PRMT5/WD45 more strongly in the presence of pICln?

(3) On page 12 the authors refer to Figure 4D but there is no Figure 4D. The authors must have meant Figure 4C. No experimental conditions are described for this experiment. In low salt tRNA may bind to Sm proteins and precipitate with Y12. Figure 3 and Raker et al. (1996) show that binding of Sm proteins is specific to the nucleotide sequence within snRNAs. Is the binding of tRNA to Sm proteins non-specific?

The manuscript may be published if these points are satisfactorily addressed.

We have responded to the helpful comments of the reviewers in the following way:

Specific points raised by the reviewers:
Referee #1:
In this manuscript, the authors describe the reconstitution of the human PRMT5 and SMN complexes from recombinant sources, and show that these two purified complexes can function to recapitulate important steps in formation of the UsnRNP Sm core particle. This is an important accomplishment. For the most part, the experiments are well performed and the figures are well illustrated. The system has the potential to uncover important mechanistic insights into the assembly of Sm-class snRNPs. However, the manuscript itself needs a fair amount of work, as it falls short both in the introduction (e.g. the motivation for carrying out the experiments) and in the interpretation/discussion of several of the results.

We have now extensively re-written parts of the introduction to acknowledge the literature and to discuss in more depth what is known about the individual factors of the SMN complex and the contribution of methyltransferases PRMT5 and 7 in U snRNP biogenesis. These changes are highlighted in the revised manuscript.

Finally, the authors do not take full advantage of their system, as they analyzed only a single SMN point mutation.

We have now included the generation and functional investigation of three additional mutations (one missense mutation and two deletions of SMN) in our study. The results of these studies have been summarized in a new Fig. 6. See also remarks to the other reviewers.
Title: Does the experiments in the ms. reveal the basis for snRNA proofreading? Not really. They exclude Gemins3-5 from this activity but do not identify the underlying mechanism (see below).

We agree with the reviewer and have now re-phrased the title. It now reads:
Reconstitution of the human U snRNP assembly machinery reveals stepwise Sm protein organization and the mechanistic basis of defects in Spinal Muscular Atrophy

Synopsis: Only one mutation was analyzed. Cannot use plural in last bullet point.

We have now included several additional mutations in our analysis and hence can now use plural in the synopsis.

Introduction: The introduction needs to be re-formulated to provide a more accurate description of previous work in the field and better reasoning/motivation for why they performed the experiments.

We have revised extensively our introduction. We hope that the reviewer will now find this manner of presentation more appealing and approve of this.

1. The first paragraph is fine, but in the second one, they get into trouble. They state that pICln accepts newly-synthesized Sm proteins "at the ribosome itself" citing a paper that is submitted and not provided to the referees. Remove words in quotations above.

As this paper is still under consideration, we have re-phrased the sentence and left out the respective reference.

2. Also in the second paragraph, the authors go on to talk about Sm protein methylation, stating that "the precise role of this modification in the assembly reaction is controversial." What is the controversy?

We have extensively re-written this paragraph, the changes are highlighted in red in the revised manuscript.

They cite a ton of papers, but seem to ignore the fact that yeast have UsnRNP's but their Sm proteins completely lack RG dipeptide motifs (e.g. Girard et al. 2004). Moreover, they fail to mention that although Drosophila Sm proteins have RG rich tails, Sm protein methylation is dispensable for snRNP assembly in flies (Gonsalvez et al. 2008, RNA; Gonsalvez et al. 2006, Curr Biol). If the authors want to limit their discussion to humans (not sure why they would want to do that but...), then they need to explicitly state it. Even then, most of the papers they cite are review articles. The few primary papers they cite don’t really address the requirement for the sDMA modification. Pulse chase experiments in HeLa cells showed that Sm protein methylation IS required for proper snRNP assembly, and that two different PRMTs participate in this activity (Gonsalvez et al. 2007). The reviewer is correct in that we focus on higher eukaryotes in our paper. In our revised introduction, we now also mention that “simpler” SMN complexes exist in other (mostly lower) eukaryotes. However, yeast (S. cerevisiae) does not contain an SMN complex and hence it is likely that the snRNP biogenesis follows an entirely different route. Therefore, the situation in yeast, albeit of considerable interest, is not important for our work and is hence not mentioned in detail.

3. Later on in the Introduction, the authors state that "Likewise, apart from SMN and Gemin2 ..., the functions of other SMN complex subunits (Gemins2-8 and unrip) are less clear (Chari & Fischer, 2010; Kroiss et al, 2008; Palfi et al, 2009)." The authors cite one review, and two primary papers, neither of which address this subject in the human system! Kroiss et al. use Drosophil and Palfi et al. use trypanosomes. The selectivity of the citations paints a disturbing picture here. Which of these subunits (Gemins3-8 and unrip) is required for Sm core assembly? Two papers address this directly in the human system, using RNAi to specifically deplete certain SMN complex subunits and then asking if the extracts from these cells can carry out snRNP assembly (see Shpargel & Matera, 2005; Feng et al. 2005). Curious that these two papers are not mentioned, even among the
"multitude of experimental approaches" and primary papers described in the very next sentence to address the functions of Gemins.

We apologize for having ignored and mis-cited important literature. In the revised version, this has now been corrected and additional publications are cited that address the role of individual SMN complex components in the assembly pathway.

4. After describing the ATP requirements (or lack thereof) for Gemin3 in the overall process of snRNP assembly, the authors sum up the paragraph with this sentence: "Therefore, a deep mechanistic analysis of the assisted snRNP assembly process appears unavoidable." I am at a loss for words here. ????

We have toned down this specific statement, which now reads: “Therefore a deep mechanistic and eventually structural-based analysis of the reaction mechanism of the assisted snRNP assembly process appears unavoidable.”

5. The beginning of the next paragraph ("The mechanistic dissection of many...") is good. But the statement they use for motivating their study is untrue: "Since SMN is essential for viability, the effects of disease-causing missense mutations on snRNP assembly cannot be studied in the absence of wild-type protein in vivo." It has yet to be established in mouse knock-in models whether mild SMN point mutations can support viability. However, it has been shown in Drosophila that SMA-causing point mutations recapitulate the full range of viability seen in human patients. Some of the disease-causing point mutants are viable in the absence of any wild-type SMN (Praveen et al. 2012; 2014).

We have re-phrased this final paragraph. It now reads:
“The reconstituted system presented here enables us to dissect the contribution of disease-causing mutations and deletions, and undoubtedly will be a valuable tool for future mechanistic and eventually structure-based studies of the snRNP assembly reaction mechanism.”

Results 6. The nomenclature is a bit confusing (6S vs. pICln-D3/B). Why use Svedberg numbers for one complex and not the other? Do the two trimeric complexes -D3/B and -D1/D2) have different names? They probably have similar Svedberg numbers, so maybe not.

We certainly agree with the reviewer. However, due to studies over the past years, these (sometimes) strange names have been established in the literature. To avoid even more confusion by re-naming some of the complexes, we would prefer to use the same nomenclature that was used in the past by several labs including ours (for example Fisher et al., 1985; Chari et al., 2008).

7. The second part of this statement is neither supported by data in the paper nor by citations: "One crucial step in the initial phase of snRNP assembly is the recruitment of all Sm proteins to the PRMT5 complex and the methylation of a subset of them (i.e. B, D1 and D3)." What is the evidence that methylation of Sm proteins is required in vitro? See Gonsalvez et al. 2006, 2007, 2008. Do we know if PRMT5 enzymatic activity is strictly required? What happens to Sm core assembly if you use an MTase dead point mutant PRMT5 in your reconstituted system?

We have included in the revised version citations that support this scenario. As now also stated in the introduction, methylation is not required for the assembly reaction per se in vitro. The suggested experiment with MTase dead point mutant PRMT5 in our view is unnecessary as the entire early (PRMT5-dependent) assembly phase is methylation independent in vitro as we have described in Fig. 2. The reason why the in vitro situation obviously differs from the in vivo situation is currently unclear to us. We consider it likely that some “fine-tuning” of the reaction is necessary in vivo, which we override in our in vitro systems. Additional experiments that go beyond the scope of our manuscript and taking the “crowded” environment of the cell into account are necessary to further elaborate on this aspect.

8. The finding that Gemin5 is dispensable for snRNA identification is important. The authors fail to mention that they showed this to be the case in their Kroiss et al. 2008 paper. They showed that human SMN/Gemin2 heterodimers were sufficient for Sm core assembly (see supplemental figures).
What they did NOT do in that paper is challenge the assembly reaction with a mixture of RNAs. Evidence for this 'proofreading' activity is an important finding of this study, especially if this activity does not depend on Gemins3-5. Related to this point in the Discussion, the authors state: "Quite unexpectedly, the central SMN complex components are both necessary and sufficient for all aspects of snRNP core formation in vitro." Why is this so unexpected? The authors showed that human SMN/Gemin2 dimers were necessary and sufficient in 2008 (Kroiss et al).

The reviewer is correct in that we previously reconstituted the assembly reaction with SMN/Gemin2 dimers. Nevertheless, we were quite surprised that we did not detect any contribution of the other Gemins to the assembly reaction, be it stimulatory, inhibitory or as specificity factors.

9. The title of the manuscript says that the basis for snRNA proofreading is revealed by these expts. Why is proofreading not specifically discussed in the Discussion?

As suggested, we have now included the following modified paragraph in the discussion (marked in red in the text):

"Accordingly, as previously reported by us (Kroiss et al, 2008), SMN and Gemin2 were alone sufficient to proof-read snRNAs. The view that emerges from the experiments presented here in Fig. 4C and Kroiss et al (2008) is that SMN and Gemin2 subunits provide a platform, where Sm proteins themselves are able to proof-read the presence of cognate snRNAs. Additionally, in our reconstituted system we find no dependence of SMN complex-mediated snRNP assembly on ATP. We thus put forward the hypothesis that the SMN complex is a Brownian machine driven by thermal energy (Fig. 7B). Interestingly, several labs including ours have shown that assembly depends on metabolic energy in cellular extracts (Meister et al, 2001a; Pellizzoni et al, 2002b). This raises the possibility that steps in the biogenesis pathway not covered in our in vitro system are driven by the hydrolysis of ATP."

10. This is a good point: "Surprisingly, neither methylation nor F/E/G binding are sufficient for 6S complex dissociation from PRMT5/WD45. This process appears to be feed-forward driven, where the addition of a new pICln-D1/D2 or pICln-D3/B heterotrimer displaces the 6S complex (Fig. 2)." Is there a way to test this idea (not necessarily in this paper)? But the authors may want to elaborate.

In order to study feed-forward mechanism suggested by us that expels the 6S complex from the PRMT5 complex, a stable isotope labelling by amino acids in cell culture (SILAC) experiment could be performed. Conceptually, the experiment we suggest is analogous to a pulse-chase experiment. In the SILAC approach, cells are adapted to two kinds of media. While both contain 19 naturally occurring amino acids, one amino acid (commonly arginine or lysine) is either present as it natural form (light) or containing heavy isotopes of carbon or nitrogen (heavy). Consequently, the 'light' culture will incorporate the naturally occurring amino acid, while the 'heavy' culture will only contain the isotope-labelled amino acid with an increased molecular weight. After combining both 'light' and 'heavy' cell samples and controlling the identical protein concentration, LC-MS/MS can be performed to identify the ratio of individual proteins. Upon treatment of the 'heavy' cell line with for example growth factors or inhibitors, the amount of differential expression can be determined.

To study the suggested feed-forward mechanism, we envision to adapt a human cell line, such as HEK293 cells, to SILAC media. In the suggested approach, it would be essential that the cell line constitute a stable one where pICln, SmD1 and SmD2 (or pICln, SmD3 and SmB) are under the control of a Tetracycline inducible promoter. Once the cells are adapted, we could either use the Tet-On system to induce pICln-D1/D2 (or pICln-D3/B) overexpression by doxycycline and/or additionally add a PRMT5 inhibitor to prevent Sm protein methylation. Subsequently, we would immunoprecipitate the PRMT5 complex and quantify the ratio of Sm proteins within the 20S complex (PRMT5 complex). The PRMT5 protein would serve as the internal control to ensure equal starting concentrations of immunoprecipitated proteins. If the methylation of Sm proteins is required for 6S release, we would expect to see an enrichment of Sm proteins F/E/G on the PRMT5 complex compared to the sample that was not treated with the inhibitor. For the case that large quantities of pICln-D1/D2 are sufficient for 6S release, the amount of F/E/G should decrease as all 6S is replaced by the overexpressed pICln-D1/D2.
The SILAC approach comprises six major steps: 1) Preparation of the SILAC labelling medium 2) Adaptation of cells from cell-specific growth medium to SILAC labelling medium (5-6 doublings for full labelling) 3) Differential treatment applied to the SILAC cells (such as inhibitor-treatment) 4) Mixing of cells, cell lysis and protein estimation (Bradford, etc.) 5) Trypsin digestion and peptide isolation 6) MS analysis and determination of the fold abundance of specific proteins of interest.

The fold abundance can be determined by either (1) calculating the ratio of intensities of each peptide from individual MS spectra (light and heavy) or (2) the ion chromatograms of light and heavy peptides eluted from a reversed-phase column to subsequently determine the ratio of the areas under the curves.

Needless to say that the proposed experiment would require a substantial amount of experimentation to setup the required cell line. Additionally, the adaptation to the SILAC media is expected to be enormously time consuming. Therefore, we maintain that the actual in vivo experiment to verify our unequivocal biochemical experimental evidence that a feed-forward mechanism drives the progression to the 6S-complex, is expected to far exceed the scope of the present manuscript.

References


11. In the Discussion: E134K is not a common SMA mutation. None of the point mutations in SMN are 'common'. It is a well-studied mutant but it is by no means "frequently found" in the SMA patient population. Furthermore, why is this the only mutation analyzed? There are a number of other mutations, analysis of which could really help flesh out this manuscript. T274I, A111G and one or two others (not sure off top of my head) should be analyzed to address questions of assembly and function of SMN complex.

As suggested by this (and other reviewers/the editor), we have now included the reconstitution and analysis of additional SMN complexes containing mutant SMN (see new Fig. 6 and the corresponding text). We have chosen three different SMN mutants, one pathogenic missense mutation in the N-terminus (D44V), a C-terminal truncation (SMNDExon7), which represents the major product of the SMN2 gene copy and an artificial deletion of the YG box, in which by far the most pathogenic missense-mutations occur.

The generation of these mutant complexes turned out to be technically quite challenging and new protocols were needed to purify them in sufficient quantities for functional studies, which explains the delay of our revision. Nevertheless, the new data show that these mutations differentially affect both the formation of the SMN complex and also its activity.

12. Near the end of the Discussion, this statement should be removed. "The consequences of mutations ... cannot be analyzed in these model systems ... in vivo model systems must contain at least one copy of wild-type SMN. This statement is patently untrue.

We apologize for this overstatement. We have now deleted this sentence. It now reads:

“The reconstitution system we describe here enables mechanistic analyses of snRNP assembly and the significance of disease-causing mutations in detail. Our in vitro reconstitution scheme will therefore be essential for the understanding of the molecular etiology of the devastating disease spinal muscular atrophy.”

Referee #2:

Neuenkirchen et al report the biochemical reconstitution of Sm complex assembly from purified recombinant Sm proteins and the components of the PRMT5 and SMN chaperone complexes. This represents a remarkable feat, which paves the way to the detailed analysis of the sequence of events, kinetics and modulation (e.g. by SMA-associated mutations) of this key assembly process for snRNP biogenesis. Despite the extensive knowledge already available about this process, the initial biochemical characterization presented by Fischer and colleagues has already provided relevant unexpected insights, including the dispensability of several Gemin proteins and ATP-independence.
for SMN complex function. The conclusions are supported by very clean, carefully controlled biochemical data.

I have only three suggestions for the authors’ consideration:

1) I fear that the manuscript may be too demanding for non-specialists, some of whom may be put down by the complexity of the system, the non-friendly nomenclature and -to some extent- the rather dry style of the Results section. I would encourage the authors to improve the latter and also to provide a scheme at the beginning of the manuscript schematizing the current knowledge about the assembly pathway and the open questions, and display, in their final model, complementary information derived from the new results, highlighting how the work has contributed to illuminate new features of the pathway.

To simplify the reading of the manuscript, we now have included a supplementary figure, where we schematically summarize the current knowledge about the pathway and highlight the steps addressed in this study (see new Supplementary Fig. 1).

The system is indeed very complex and the nomenclature confusing but we hope that our revised introduction is now understandable for a broader readership. Regarding the style of the results section, we would prefer to describe the experiments in an unbiased observatory manner and later discuss the results in a “less dry” manner.

2) The authors argue that the influence of temperature on assembly efficiency may reveal that the SMN complex acts as a Brownian machine by coupling spontaneous conformational changes driven by thermal energy to the directed delivery of Sm proteins onto snRNA. It is not entirely clear to me, however, what is the mechanism that the authors are proposing. Is thermal energy affecting some activity of the SMN complex itself? Or is it enhancing the opportunities of the SMN complex to take advantage of spontaneous conformational changes in Sm proteins? Can these-or other scenarios-be distinguished?

The postulate of a Brownian machine has several mechanistic implications. The first is that the substrate (snRNA) is not recognized by an induced fit mechanism but by a conformational selection. Speaking in favour of a conformational selection, our previous results (Chari et al., 2008) indicate that the SMN complex is able to disassemble a fully formed snRNP as long as it is provided in excess even though it is a thermodynamically unfavourable reaction. Additionally, the conformational selection would offer an immediate and plausible explanation for the proofreading activity of snRNA by the SMN complex. In this scenario, as we describe in the present manuscript, no subunit of the SMN complex would contribute to snRNA proofreading but instead provide a passive platform, where the Sm proteins themselves would proofread the presence of a cognate snRNA. Ultimately, the question of a Brownian machine can only be resolved by the structural determination of several intermediate stages in the reaction pathway and the consequent determination that the conversion from one stage to another occurs in an iso-energetic manner. The evidence we provide here, that not energy in the form of ATP hydrolysis is required for the assembly reaction but heat, would strongly favour this scenario. We therefore postulate this in the discussion section, however, if the reviewer thinks that this postulate is too premature, we would be prepared to remove it from the text.

3) While the result with the SMN(E134K) mutant is of significant interest as a direct demonstration that this mutant has intrinsically lower activity to promote Sm complex assembly, the extent to which this difference (2-fold increase in Kd) can be rate-limiting for snRNP assembly and function in cells, or make a difference between different cell types, and particularly in motor neurons-perhaps the aspect of wider interest-should be discussed further.

We have included the characterization of three additional mutants and this now described in the text and illustrated in a new Fig. 6. However, we believe it to be premature to hypothesize how this partial loss in activity would affect the observed neuropathy in Spinal Muscular Atrophy. In our manuscript, we have solely analysed snRNP assembly, it is likely that Spinal Muscular Atrophy causing mutations affect other steps in snRNP biogenesis as well, not recapitulated in the present manuscript. Until the consequence of SMA-causing mutations are assessed in all aspects of snRNP biogenesis, the verdict on their effect on neuropathy remains open and wholly speculative.
Referee #3:

The group of Utz Fischer has been studying the process of U snRNP assembly and cellular components involved in this process and has been making important contribution to our understanding of U snRNP assembly. In this manuscript the authors attempted to recapitulate the assembly process of the core snRNP domain using purified protein components expressed in insect cells and E. coli. The results are in general of high quality and largely confirmed what is known about this system. The core snRNP domain assembly takes place in the cytoplasm overcrowded with many proteins and RNAs. It is not easy to show why such an elaborate machine is necessary when Sm proteins spontaneously assemble with snRNAs when purified components are mixed. Furthermore yeast seems to manage without such an elaborate system. It would be more informative if the experiments had been done more quantitatively such that we could learn what is critical when these core snRNP components are present under physiological concentrations in the presence of other competing proteins and nucleic acids. The results described in this manuscript are important and should be published after the following points have been considered.

We thank the reviewer for his/her encouraging comments. The system we describe here can be considered a starting point for a complete and fully quantitative description of the entire assembly pathway. While it would be very interesting to study this process in more complex environments (i.e. molecular crowding conditions etc.) as suggested by the reviewer, we would not have been able to understand the effects described in this manuscript without working in a fully purified system. Based on the results we present in this manuscript, we believe we will be in the position to address the question raised by this reviewer in depth in the near future.

(1) Figure 1 could be more informative if size exclusion chromatography was used in conjunction with multi angle light scattering (this is now a standard practice). This will show the homogeneity and molecular mass (hence, subunit stoichiometry) of eluted complexes. The quality of the protein samples shown in Figure 1A and Figure 1C-J does not look the same. For example PRMT5/WD45 looks much cleaner in Figure 4A than in other panels. Some of the input lane has no protein bands and proteins in purified fractions do not look like the sum of input proteins shown in Figure 1A. This is presumably because the protein samples in Figure 1A were stained with Coomassie blue whereas those in Figure 1C-J were silver stained. It would be good if the authors show each input protein preparation used for each experiment on the same gel. For D3/B, D3 looks over-represented even when they are stained with Coomassie blue (Figure 1A lane 4). Does D3 stain better with Coomassie blue or is D3 present in excess?

We have attempted to perform MALS experiments in conjunction with size exclusion chromatography to obtain more precise molecular masses. However, due the high sample amount and the high concentrations required for these measurements in a quantitative manner, these experiments turned out to be technically very difficult. We would be prepared to provide the raw data of these studies but think that this would not substantially contribute to the conclusion of the manuscript.

Several experiments were re-done and a new Fig. 1 was assembled where the gels in panels of Fig.1 C, E, F, G and J were replaced.

Regarding the comments on apparent different protein staining in Fig. 1A and Fig. 1C-J: Fig. 1A indeed is a Coomassie-stained representation of the different proteins used in this study stemming from the peak fraction of the final protein purification step (size exclusion chromatography). In subsequent experiments the protein amounts were not sufficient for Coomassie-staining and silver staining was performed (Fig. 1C-J), in our hands D3 indeed stains better in Coomassie.

(2) It is possible that these proteins tend to aggregate, when present in high concentration in isolation, and these experiments may have to be done under particular conditions. The authors should specify buffer conditions for analytical gel-filtration. Ideally binding assays should be quantitative and Kd for each interaction should be described although this may be difficult. D1/D2 binds much more strongly to PRMT5/WD45 in the presence of pICln. Does D1/D2 bind more strongly to pICln when PRMT5/WD45 is present? Does B/D3 bind to PRMT5/WD45 more strongly in the presence of pICln?
We can conclusively disprove an aggregation of these complexes since these purified proteins do not elute in the void volume of the column. Even at a concentration up to 5mg/ml (data not shown), we were not able to observe such an elution behaviour. We agree with the reviewer that obtaining Kd values would be highly interesting at this point, however, the only experimental way we can think of addressing this in a clean way would be to perform ITC experiments, which would require amounts which we are unable to purify.

All biochemical data indicate that pICln has the strongest affinity for individual Sm heterooligomers in the assembly line. Any other component has a lesser affinity, therefore it is unlikely that D1/D2 bind more strongly to pICln when PRMT5/WD45 is present. This is also supported by the experiments in Figures 1 and 2, which strongly indicate that Sm proteins bind only stably to the PRMT5 complex when pICln is present. The stronger affinity of the Sm protein heterodimers to PRMT5/WD45 would therefore stem from an interaction of Sm protein heterodimers to pICln and an increased avidity of Sm proteins to PRMT5/WD45 aided by the interaction of pICln with PRMT5/WD45.

(3) On page 12 the authors refer to Figure 4D but there is no Figure 4D. The authors must have meant Figure 4C. No experimental conditions are described for this experiment. In low salt tRNA may bind to Sm proteins and precipitate with Y12. Figure 3 and Raker et al. (1996) show that binding of Sm proteins is specific to the nucleotide sequence within snRNAs. Is the binding of tRNA to Sm proteins non-specific?

We have corrected this error and now also provide information regarding the buffer conditions for this experiment (physiological conditions in PBS buffer). The binding of isolated Sm proteins to tRNA under these conditions is indeed non-specific. However, once bound to the SMN complex, Sm proteins are specifically directed towards snRNAs due to the proofreading activity of the SMN complex (see also comment 2 of reviewer 2).

We thank all reviewers for their comments and helpful suggestions to the present manuscript. We do now hope that all points are addressed satisfactorily, such that they will find the manuscript now suitable for publication.

2nd Editorial Decision

21 April 2015

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

As you will see they both find that all experimental criticisms have been sufficiently addressed and recommend the manuscript for publication, pending minor textual revision. I would therefore ask you to incorporate the response to these few remaining referee comments in a final revision of you manuscript.

Regarding the title of your study I do agree with referee #1 that it would (for clarity) be better to keep the original title 'Reconstitution of the human U snRNP assembly machinery reveals stepwise Sm protein organization'. For the last point raised by this referee; if you have data available for additional mutant analysis at this point we would encourage you to include it but this will not be a requirement for publication.

We now generally encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.
Thank you again for giving us the chance to consider your manuscript for The EMBO Journal and please feel free to contact me with any questions. I look forward to receiving the final revision of your study.

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Referee #1:

In this revised manuscript, the authors describe the reconstitution of the human PRMT5 and SMN complexes from recombinant sources, and show that these two purified complexes can function to recapitulate important steps in formation of the UsnRNP Sm core particle. For the most part, the experiments are well performed and the figures are well illustrated. The system has the potential to uncover important mechanistic insights into the assembly of Sm-class snRNPs.

Specific concerns that should be addressed:

1. The title is still a bit problematic. There is no actual link between the reconstitution of the assembly machinery and any mechanistic defects in SMA. Suggest the authors truncate the current title after the word "organization." Or revise to remove inference that Sm core assembly is directly connected to the neuromuscular phenotype.

2. The following revisions are unsatisfactory.
   
   Even though self-recognition of RNA and protein counterparts is sufficient for Sm core assembly in vitro (Raker et al, 1996), at least 12 trans-acting factors, united in PRMT5 and SMN complexes, participate in this process in vivo in higher eukaryotes (Meister et al, 2002; Paushkin et al, 2002). Lower eukaryotes also contain SMN and Gemin2 genes in their genome, and sometimes also genes for additional SMN complex subunits (Kroiss et al, 2008; Palfi et al, 2009).

Reply - I hate to remind the authors about points of basic phylogeny, but it seems important to do so here. The problem with the above paragraph is that yeast (and plants) are both considered to be HIGHER eukaryotes, as are all animals such as fruit flies or worms. Animals are definitely NOT lower eukaryotes.

3. After describing the ATP requirements (or lack thereof) for Gemin3 in the overall process of snRNP assembly, the authors sum up the paragraph with this sentence: "Therefore, a deep mechanistic analysis of the assisted snRNP assembly process appears unavoidable." I am at a loss for words here. ????__

We have toned down this specific statement, which now reads: "Therefore a deep mechanistic and eventually structural-based analysis of the reaction mechanism of the assisted snRNP assembly process appears unavoidable."

Reply - Apologies for the miscommunication. There is no need to tone things down here. The problem was with the word "unavoidable." That is, I was at a loss for words as to why the authors
would want to avoid gaining a deep mechanistic understanding of the snRNP assembly process. I still am unclear on this after reading the revised sentence. Why is it unavoidable? Why would you want to avoid it?

Additional non-essential suggestions for improving the study:

Finally, the authors do not take full advantage of their system, as they analyzed only a single SMN point mutation... We have now included the generation and functional investigation of three additional mutations (one missense mutation and two deletions of SMN) in our study. The results of these studies have been summarized in a new Fig. 6. See also remarks to the other reviewers.

It is a pity that the authors did not use more informative YG box point mutants in the revision. The only point mutant besides E134K from the first submission was D44V. For example, T274I and Y272C have both been tested extensively and should have opposite phenotypes. I suppose if the authors plan a follow up study with many different mutations, then they can explore this issue in greater depth.

Referee #3:

The authors addressed all the points raised by the referees and the revision is satisfactory.

page 6 the end of the first paragraph.

"Therefore, a deep mechanistic and eventually structure-based analysis of the reaction mechanism of the assisted snRNP assembly process appears unavoidable" sounds very strange. Do the authors really mean "unavoidable"? "desirable", "necessary" or "indispensable" sounds more appropriate here. The author may wish to rephrase this sentence at the proof stage.

Referee #1:

In this revised manuscript, the authors describe the reconstitution of the human PRMT5 and SMN complexes from recombinant sources, and show that these two purified complexes can function to recapitulate important steps in formation of the U1snRNP Sm core particle. For the most part, the experiments are well performed and the figures are well illustrated. The system has the potential to uncover important mechanistic insights into the assembly of Sm-class snRNPs.

Specific concerns that should be addressed:
1. The title is still a bit problematic. There is no actual link between the reconstitution of the assembly machinery and any mechanistic defects in SMA. Suggest the authors truncate the current title after the word "organization." Or revise to remove inference that Sm core assembly is directly connected to the neuromuscular phenotype.

Reply - The title has been adjusted as suggested and now reads:
Reconstitution of the human U snRNP assembly machinery reveals stepwise Sm protein organization

2. The following revisions are unsatisfactory.
Even though self-recognition of RNA and protein counterparts is sufficient for Sm core assembly in vitro (Raker et al, 1996), at least 12 trans-acting factors, united in PRMT5 and SMN complexes, participate in this process in vivo in higher eukaryotes (Meister et al, 2002; Paushkin et al, 2002). Lower eukaryotes also contain SMN and Gemin2 genes in their genome, and sometimes also genes for additional SMN complex subunits (Kroiss et al, 2008; Palfi et al, 2009).

Reply – Our intention was to point out the differences of the composition of SMN complex is various types of eukaryotes as it is known today. We appreciate the reviewer’s comment on the word choice and agree that the use of the terms “higher” and “lower” eukaryotes is inappropriate and have rephrased the sentence in the manuscript as follows:

Even though self-recognition of RNA and protein counterparts is sufficient for Sm core assembly in vitro (Raker et al, 1996), at least 12 trans-acting factors, united in PRMT5 and SMN complexes, participate in this process in vivo in animals (Meister et al, 2002; Paushkin et al, 2002). Other eukaryotes, such as Drosophila melanogaster and Trypanosoma brucei, only contain SMN and Gemin2 genes in their genome, and sometimes also genes for additional SMN complex subunits (Kroiss et al, 2008; Palfi et al, 2009).

It is disappointing to see that the authors revert to this sort of ‘bail-out’ explanation that "perhaps invertebrate snRNPs are just assembled differently." Studies in model organisms have shown time and time again that processes like spliceosome assembly are conserved.

Reply – We agree with the reviewer that many aspects related to the spliceosome, including snRNP composition and function have been conserved in evolution. However, this seems not (or only partially) true for the assembly event. This is most evident by the fact that S. cerevisiae does neither contain a confirmed gene for pICln nor for any of the Gemins. To our knowledge, there also no indication that the assembly pathway follows a segmented pathway that involves nucleocytoplasmic transport events as has been reported for vertebrates. We would therefore very much prefer to leave the text as is.

3. After describing the ATP requirements (or lack thereof) for Gemin3 in the overall process of snRNP assembly, the authors sum up the paragraph with this sentence: "Therefore, a deep mechanistic analysis of the assisted snRNP assembly process appears unavoidable." I am at a loss for words here. ?????

We have toned down this specific statement, which now reads: "Therefore a deep mechanistic and eventually structural-based analysis of the reaction mechanism of the assisted snRNP assembly process appears unavoidable." Reply - Apologies for the miscommunication. There is no need to tone things down here. The problem was with the word "unavoidable." That is, I was at a loss for words as to why the authors would want to avoid gaining a deep mechanistic understanding of the snRNP assembly process. I still am unclear on this after reading the revised sentence. Why is it unavoidable? Why would you want to avoid it?

Reply – The word “unavoidable” was chosen to emphasize the necessity of further investigation to better understand the mechanism of the snRNP assembly process rather than stating that we want to avoid additional analyses. We changed the word “unavoidable” with the word “necessary” as suggested by the second reviewer (see below) to clarify this point. The sentence now reads:

Therefore, a deep mechanistic and eventually structure-based analysis of the reaction mechanism of the assisted snRNP assembly process is necessary.

Additional non-essential suggestions for improving the study: Finally, the authors do not take full advantage of their system, as they analyzed only a single SMN point mutation... We have now included the generation and functional investigation of three additional mutations (one missense mutation and two deletions of SMN) in our study. The results of these studies have been summarized in a new Fig. 6. See also remarks to the other reviewers. It is a pity that the authors did not use more informative YG box point mutants in the revision. The only point mutant besides E134K from the first submission was D44V. For example, T274I and...
Y272C have both been tested extensively and should have opposite phenotypes. I suppose if the authors plan a follow up study with many different mutations, then they can explore this issue in greater depth.

Reply – We are aware that many SMN mutations have been identified which can be tested for their effect on snRNP assembly using the presented reconstitution system. Due to time constraints during the revision process, we decided to focus on a small subset of these (D44V, DYG box and Dexon7). Future studies will enable us to characterize a larger number of known mutations in the SMN gene as well as other SMN complex components.

Referee #3:

The authors addressed all the points raised by the referees and the revision is satisfactory. Page 6 the end of the first paragraph.

"Therefore, a deep mechanistic and eventually structure-based analysis of the reaction mechanism of the assisted snRNP assembly process appears unavoidable" sounds very strange. Do the authors really mean "unavoidable"? "desirable", "necessary" or "indispensable" sounds more appropriate here. The author may wish to rephrase this sentence at the proof stage.

Reply – This concern was equally raised by both reviewers and has been addressed above (see replies to reviewer 1).

We hope that our revised manuscript is now acceptable for publication in the EMBO Journal. We would like to thank the reviewer for their constructive criticism and you for your time and consideration. Please let me know if you need any additional information from our side.