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

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Abstract

The assembly of spliceosomal U snRNPs depends on the coordinated action of PRMT5 and SMN complexes \textit{in vivo}. These \textit{trans}-acting factors enable the faithful delivery of seven Sm proteins onto snRNA and the formation of the common core of snRNPs. To gain mechanistic insight into their mode of action, we reconstituted the assembly machinery from recombinant sources. We uncover a stepwise and ordered formation of distinct Sm protein complexes on the PRMT5 complex, which is facilitated by the assembly chaperone pICln. Upon completion, the formed pICln-Sm units are displaced by new pICln-Sm protein substrates and transferred onto the SMN complex. The latter acts as a Brownian machine that couples spontaneous conformational changes driven by thermal energy to prevent mis-assembly and to ensure the transfer of Sm proteins to cognate RNA. Investigation of mutant SMN complexes provided insight into the contribution of individual proteins to these activities. The biochemical reconstitution presented here provides a basis for a detailed molecular dissection of the U snRNP assembly reaction.

Keywords assembly; pICln; PRMT5; SMN; snRNP

Introduction

Macromolecular complexes perform vital activities in virtually all cells. The cellular environment in which these complexes are assembled is highly crowded, and thus not only substantially perturbs diffusion-driven self-assembly, but also increases the likelihood of non-productive interactions. Assembly processes therefore require \textit{trans}-acting factors, which sequester subunits of macromolecular complexes and safeguard them from engaging in unwanted interactions (Ellis, 2006; Chari & Fischer, 2010). Compelling evidence has emerged in recent years that the formation of even comparatively simple structures often requires a plethora of assembly factors (Zemp \textit{et al}, 2009; Chari & Fischer, 2010; Liu \textit{et al}, 2010). An extreme example is the assembly of the common Sm core structure of spliceosomal snRNPs (U1, U2, U4/U6 and U5), which utilizes more assembly factors than parts to be assembled (Fischer \textit{et al}, 2011).

Even though self-recognition of RNA and protein counterparts is sufficient for Sm core assembly \textit{in vitro} (Raker \textit{et al}, 1996), at least 12 \textit{trans}-acting factors, united in PRMT5 and SMN complexes, participate in this process \textit{in vivo} in animals (Meister \textit{et al}, 2002; Paushkin \textit{et al}, 2002). Other eukaryotes, such as \textit{Drosophila melanogaster} and \textit{Trypanosoma brucei}, only contain SMN and Gemin2 genes in their genome, and sometimes also genes for additional SMN complex subunits (Kroiss \textit{et al}, 2008; Palfi \textit{et al}, 2009). However, it remains to be elucidated whether and how these additional SMN complex subunits contribute to snRNP biogenesis (Shpargel \textit{et al}, 2009). The cellular snRNP assembly pathway can be divided into two distinct temporal phases (see Supplementary Fig S1 for a schematic overview of the snRNP assembly pathway). The early phase is dominated by the assembly chaperone pICln (Friesen \textit{et al}, 2001; Meister \textit{et al}, 2001b). PICln accepts newly synthesized Sm proteins and delivers them to the PRMT5 complex, which consists of PRMT5 and WD45 (also referred to as MEPS0) (Friesen \textit{et al}, 2001; Meister \textit{et al}, 2001b). The PRMT5 subunit catalyzes symmetrical dimethylation of arginine residues (sDMA) within the Sm proteins B'-D1 and D3 (Friesen & Dreyfuss, 2000; Brahms \textit{et al}, 2001). Recently, a second methyltransferase was identified in humans and \textit{Drosophila}, termed PRMT7 that is capable of arginine methylation and contributes to the snRNP assembly process (Gonsalvez \textit{et al}, 2007, 2008). Additionally, pulse-chase experiments in HeLa cells have revealed that Sm protein methylation is...
required for proper snRNP biogenesis (Gonsalvez et al., 2007). It is believed from experiments in cell extracts that sDNA modification enhances the affinity of Sm proteins for the SMN complex in vivo (Brahms et al., 2001; Friesen et al., 2001; Meister et al., 2001b; Matera & Wang, 2014). However, in purified, reconstituted systems the effect is less apparent (Chari et al., 2008; Kroiss et al., 2008; Zhang et al., 2011). The early assembly phase eventually segregates into two lines. In one assembly line, a stable hexameric ring intermediate is formed termed the 6S complex, which is composed of pICln and the Sm proteins D1, D2, F, E and G, and pre-organizes these Sm proteins into spatial positions adopted in the assembled Sm core domain (Fisher et al., 1985; Chari et al., 2008; Grimm et al., 2013). The other assembly line consists of pICln-D3/B, which may likely not dissociate from the PRMT5 complex (Chari et al., 2008). The result of both assembly lines is that Sm proteins are kinetically trapped and fail to proceed in the assembly pathway. The late phase of snRNP formation is dominated by the SMN complex, which resolves this kinetic trap by accepting the pre-organized Sm proteins from pICln and catalyzes the subsequent joining of Sm proteins with snRNA (Chari et al., 2008; Zhang et al., 2011; Grimm et al., 2013).

While the basic principles in the cellular formation of snRNPs have been understood in great detail, several mechanistic aspects await their elucidation. For example, in the early assembly phase, the PRMT5 complex appears to contain all seven Sm proteins. Nevertheless, the understanding of how the two lines of assembly entailing 6S- and pICln-D3/B complexes emerge remains entirely elusive. Likewise, apart from SMN and Gemins2, which mediate Sm protein binding and assembly (Fischer et al., 1997; Meister et al., 2001a; Kroiss et al., 2008; Zhang et al., 2011; Grimm et al., 2013), the functions of other SMN complex subunits (Gemins3-8 and unrip) are less clear (Chari & Fischer, 2010). RNAi depletion of Gemins3-8 and unrip has been shown to perturb snRNP assembly in cell culture (Feng et al., 2005; Grimmler et al., 2005; Shpargel & Matera, 2005). However, it remains to be seen whether this requirement for Gemins3-8 and unrip stems from a direct function of these subunits in snRNP assembly or whether this is due to indirect effects such as the destabilization of the SMN complex or other possible roles of these subunits in other steps of snRNP biogenesis. Additionally, several, in part contradictory, functions have been ascribed to each of these subunits in snRNP assembly by a multitude of experimental approaches (Charroux et al., 1999, 2000; Bacon et al., 2002; Gubitz et al., 2002; Pellizzoni et al., 2002a; Carissimi et al., 2005, 2006a,b; Battle et al., 2006, 2007; Otter et al., 2007). As an example, snRNP assembly was shown to be ATP dependent when analyzed in extracts (Meister et al., 2001a) and with purified SMN complexes (Pellizzoni et al., 2002b). By examining the domain structure of proteins in the SMN complex, an ATP requirement has been attributed to the Gemins3 subunit, a DEAD-box helicase. Nevertheless, subsequent studies using purified systems showed that the assembly reaction could occur in the absence of ATP (Chari et al., 2008; Kroiss et al., 2008). Therefore, a deep mechanistic and eventually structure-based analysis of the reaction mechanism of the assisted snRNP assembly process is necessary.

The mechanistic dissection of many macromolecular machines, foremost the translation machinery and the RNA polymerase II transcription cycle, has greatly benefited over the last decades from the availability of totally reconstituted systems (Rodnina & Wintermeyer, 1995; Myers et al., 1997; Pisarev et al., 2007). To enable such studies for the assisted formation of U snRNPs, we present here the first total in vitro reconstitution of the entire assembly machinery (i.e., PRMT5 and SMN complexes) from recombinant sources. Using this system, we obtained mechanistic insight into the formation of the pICln-Sm assembly intermediates on the PRMT5 complex and their subsequent transfer onto the SMN complex. The investigation of the late phase allowed us to identify subunits of the SMN complex that are important for snRNP assembly and RNA proofreading. Moreover, reduced levels of SMN or expression of mutant versions thereof elicits the neuromuscular disorder spinal muscular atrophy (SMA) (Lunn & Wang, 2008; Burghes & Beattie, 2009; Kolb & Kissel, 2011). 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

Reconstitution of recombinant PRMT5 complex

The cytosolic assembly phase of U snRNPs starts with the sequestration of newly synthesized Sm proteins onto the PRMT5 complex and their subsequent release as distinct pICln-Sm assembly intermediates (i.e., 6S and pICln-D3/B). To analyze in a reconstituted system how the PRMT5 complex generates these units, we initially established protocols for the production of the recombinant human PRMT5, WD45 and pICln proteins (i.e., the components of the PRMT5 complex) and the seven Sm proteins (B, D1, D2, D3, E, F and G). PRMT5 and WD45 were obtained by co-expression in the MultiBac expression system (Berger et al., 2004) and were purified (Fig 1A, lane 1, Fig 1C and Supplementary Fig S2). pICln and the Sm protein heterooligomers D1/D2, F/E/G and D3/B were expressed in bacteria and purified as described previously (Fig 1A, lanes 2–5) (Chari et al., 2008). Subsequently, pICln was incubated with D1/D2 and D3/B, or a mixture of D1/D2 and F/E/G. From these proteins, we reconstituted the pICln-Sm assembly intermediates, pICln-D1/D2, pICln-D3/B and the 6S complex as described (see Fig 1A, lanes 6–8 and Supplementary Fig S3) (Chari et al., 2008). 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) (Brahms et al., 2001; Friesen et al., 2001; Meister et al., 2001b). To test whether the recombinant PRMT5 complex is enzymatically active in our in vitro system, PRMT5/WD45 was incubated with either individual Sm protein heterooligomers or their corresponding pICln complexes. Methylation was measured using an established in vitro methylation assay with [3H]-labeled S-adenosylmethionine (SAM) as a co-factor (Frankel et al., 2002). Recombinant PRMT5/WD45 efficiently symmetrically di-methylated the guanidine group of arginine residues of free and pICln-bound Sm proteins B, D1 and D3 (Fig 1B and Supplementary Fig S2D), as evident by the incorporation of [3H]-labeled methyl groups (see also Supplementary Information and Supplementary Fig S4 for a detailed characterization of the modification). Thus, our recombinant system of the early assembly phase is functional with respect to binding and methylation of individual Sm protein heterooligomers as well as pICln-Sm complexes.
Figure 1.
The availability of the recombinant PRMT5 complex allowed us to WD45 and Sm proteins.

due to their higher affinity toward PRMT5/WD45 and drives the reconstitution of U snRNP assembly machinery

Stepwise formation of the 6S assembly intermediate on the PRMT5 complex

The availability of the recombinant PRMT5 complex allowed us to start a series of experiments addressing the order of events leading to the formation of the 6S complex, that is, the most prominent assembly intermediate arising from the PRMT5 complex. For this, we incubated PRMT5/WD45 with Sm and pICln-Sm heterooligomers and analyzed the resulting complexes by gel filtration chromatography (Fig 1C–J). Consistent with a previous study, PRMT5/WD45 alone migrated as a stoichiometric heterooolomer in gel filtration chromatography (Antonsamy et al., 2012; Fig 1C). We found that pICln, D1/D2 and D3/B alone bound inefficiently to PRMT5/WD45, while F/E/G did not bind at all (Fig 1D–G, note that unbound D1/D2 elutes in a volume not analyzed on the gels, see also Supplementary Fig S3). The weak binding of D1/D2 and D3/B could be inferred from our finding that both are substrates for methylation by PRMT5/WD45 even in the absence of pICln (see Fig 1B, lanes 2 and 4). However, when equimolar amounts of Sm proteins were provided in a pICln-bound form, they were present in higher abundance in the PRMT5 complex (Fig 1H–J). As pICln is able to bind independently to PRMT5/WD45 (Fig 1G), the most likely explanation is that pICln serves as the bridging factor between PRMT5/WD45 and Sm proteins.

In vivo, pICln is part of both 20S and 6S complexes (Chari et al., 2008). The 20S complex consists of PRMT5, WD45, pICln and all Sm heterooligomers. Of note, the relative stoichiometry of the Sm proteins within the 20S complex differs strongly in vivo. Whereas D1/D2 and D3/B are often stoichiometric to each other and to the PRMT5/WD45 dimer, F/E/G is underrepresented and in some cases even absent (Friesen et al., 2001; Chari et al., 2008). In contrast, the 6S complex is a stoichiometric hexamer consisting of pICln-D1/D2/F/E/G. Noteworthy, this unit or parts thereof must have been in contact with the 20S complex as D1 is symmetrically dimethylated. Hence, the 6S complex represents an assembly intermediate bridging the early and late phases of snRNP biogenesis (Chari et al., 2008). These findings, along with the observation that stable incorporation of F/E/G into the 6S complex requires cooperative binding via pICln and D2, suggest a stepwise assembly of 6S on PRMT5/WD45.

To investigate this possibility, we initially incubated recombinant PRMT5/WD45 with the pICln-D1/D2 trimer, which led to the formation of a defined complex (Fig 2A and B). Fractions containing the PRMT5/WD45/pICln-D1/D2 complex (Fig 2B) were then pooled, incubated with F/E/G and re-analyzed by gel filtration chromatography. This resulted in the stable recruitment of the F/E/G trimer to the PRMT5 complex (Fig 2C). As the F/E/G trimer does not interact with any of the PRMT5 complexes independently (Fig 1E), this recruitment is likely to reflect the formation of the 6S complex. To proceed in the assembly reaction, the 6S complex must leave the PRMT5/WD45 unit (Chari et al., 2008). In order to elucidate the basis for this reaction, we asked whether the release is diffusion driven, dependent on D1 methylation or caused by another protein factor involved in the snRNP assembly pathway. Neither the prolonged incubation of the PRMT5 complex nor methylation of D1 resulted in 6S complex release, as evident by gel filtration analysis (Fig 2E, lanes 1–4). However, addition of pICln, pICln-D1/D2 or pICln-D3/B quantitatively expelled the 6S complex (Fig 2E, lanes 5–10). To evaluate whether pICln-mediated dissociation of the 6S complex is a consequence of its reduced affinity to PRMT5/WD45, we reconstituted a PRMT5/WD45/pICln-D1/D2 complex (Fig 2F and G). The reconstituted complex was stable over time and was also not dissociated by the addition of 6S, indicating a higher affinity of pICln-D1/D2 toward PRMT5/WD45 and proving directionality in the assembly and release of the 6S complex (Fig 2H). These data suggest a sequence of events, in which initially pICln-D1/D2 binds to PRMT5/WD45 and the methylation reaction on D1 can occur. The complex then accepts F/E/G, which upon joining pICln-D1/D2 forms 6S that remains associated with the PRMT5 complex. pICln alone or containing either D1/D2 or D3/B expels the 6S complex due to their higher affinity toward PRMT5/WD45 and drives the assembly reaction in a forward direction.

Reconstitution of recombinant SMN complex

The results above illustrate how Sm building blocks are generated on the PRMT5 complex and are made available for the SMN complex-dominated late phase of assembly. The core machinery of the SMN complex consists of eight core proteins (SMN and Gemins2-8) and the seven Sm protein “substrates” that are transferred onto snRNA. The SMN-mediated assembly process can be subdivided into: (i) the binding of Sm proteins to the SMN complex, (ii) the concomitant release of the assembly chaperone pICln, and finally (iii) the assembly of the Sm proteins and snRNA to mature snRNP cores. To establish an assay that allowed us to recapitulate these events and to gain insight into its mechanism, we generated all components of the human SMN complex in a recombinant form. Based on a recently published interaction map of the SMN complex (Otter et al., 2007), the components SMN, Gemin2, Gemin6, Gemin7 and Gemin8 form a central protein network, which allowed their co-expression in bacteria and purification as a stable unit as reported (termed SMNAGemin3-5, Fig 3A, lane 1) (Chari et al., 2008). The three remaining and peripheral components Gemin3, Gemin4 and Gemin5 were produced in insect cells, as their size prevented...
Figure 2.

A) PRMT5/WD45

B) Gel filtration fractions

C) Gel filtration fractions

D) PRMT5/WD45/6S

E) 1 5

F) PRMT5/WD45

G) Gel filtration fractions

H) 1 5

(i): PRMT5 complex

(ii): pICln-Sm protein complex

Concentration of peak fractions

SDS-PAGE

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Reconstitution of U snRNP assembly machinery
Gemin5 was shown to bind specifically to precursor and mature snRNAs and thus identifies RNA targets for the assembly reaction (Yong et al., 2010). Our recombinant system allowed us to directly test whether these proteins indeed perform the above-mentioned activities in the assembly reaction.

To analyze the energy requirement of the assembly reaction, we initially incubated Sm protein heterooligomers with U1 snRNA in the absence of SMN complex (Fig 3D, lane 1). In accordance with previous reports (Raker et al., 1996; Meister et al., 2001a; Pellizzoni et al., 2002b; Wan et al., 2005), assembly occurred in a spontaneous reaction as core formation was observed not only at 37°C but also at 4°C (Fig 3D, lanes 1 and 6). In striking contrast, snRNP core formation was entirely blocked at 4°C when Sm proteins were loaded onto the recombinant SMN complex prior to incubation with U1 snRNA (Fig 3D, lanes 9 and 10). When the reaction was carried out at 37°C, however, the assembly was efficient (Fig 3D, lanes 4 and 5). These results, in conjunction with our observation that ATP has no measurable effect on the assembly reaction (Fig 3E), suggest that the SMN complex could be considered a Brownian machine that couples spontaneous conformational changes driven by thermal energy to the directed delivery of Sm proteins onto snRNA.

**Gemin5 is dispensable for snRNA identification and snRNP assembly in vitro**

Next, we investigated whether Gemin5 was required for snRNA identification in our in vitro system as reported previously (Battle et al., 2006). For this, we generated [32P]-labeled U1 snRNA and pre-U1 snRNA as target RNAs. The 3'-extension of the latter has been shown to form a stem-loop, which constitutes the snRNP code recognized by Gemin5 (Yong et al., 2010). Both snRNAs were subsequently incubated with either the complete recombinant SMN complex or SMNAGemin3-5, and the assembly reaction was analyzed over time by native gel electrophoresis (Fig 4A). Surprisingly, both SMN complexes were active in snRNP assembly and also displayed very similar assembly kinetics for wild-type U1 snRNA (Fig 4A, upper panel and Fig 4B) and its precursor (Fig 4A, lower panel and Fig 4B; note that pre-U1 snRNA was assembled faster than the wild-type RNA as reported earlier (Yong et al., 2010)). These data indicate that Gemin3, 4 and 5, while being an integral part of the SMN complex, are not essential for the identification of target RNA and their faithful assembly into the Sm core domain in vitro.

The SMN complex also prevents mis-assembly of Sm proteins onto non-target snRNAs (Pellizzoni et al., 2002b). We hence asked

expression in E. coli, and were purified to homogeneity (Fig 3A, lane 2). As determined by gel filtration analysis, Gemin3/Gemin4 formed a heterohexamer, whereas Gemin5 formed a tetramer. Upon incubation of these Gemin with SMNAGemin3-5, the complete reconstitution of the SMN complex was accomplished (Fig 3B, lane 2). The recombinant SMN complex was first characterized by its ability to bind Sm proteins and to release pICln (Fig 3B). For this, we incubated immobilized SMN complex with pICln-D3/B and 6S, that is, those pICln-Sm complexes released from the PRMT5 complex. Analysis by gel electrophoresis revealed Sm protein transfer from both pICln-Sm intermediates (Fig 3B, lanes 3 and 4, see also Supplementary Fig S5B). Of note, even though the Sm proteins bound efficiently to the SMN complex, pICln was absent as determined by Western blotting (Fig 3B, lanes 3 and 4 lower panel; lanes 5 and 6 show input 6S and pICln-D3/B complexes. See also Supplementary Fig S5B, lower panel). Thus, similar to its endogenous counterpart (Meister et al., 2001a; Pellizzoni et al., 2002b; Chari et al., 2008), the recombinant SMN complex accepts pre-organized Sm proteins and simultaneously expels pICln.

Next, we tested whether the recombinant SMN complex also promotes the assembly of bound Sm proteins onto snRNA. For this purpose, the SMN complex was initially incubated with either the 6S complex or a mixture of 6S and pICln-D3/B, resulting in loading with Sm proteins of the subcore assembly intermediate and the mature core, respectively (Chari et al., 2008). Hereafter, both complexes were incubated with either [32P]-labeled wild-type U1 snRNA or a mutant thereof lacking a functional Sm site (U1ΔSm snRNA) and RNP formation was visualized by native gel electrophoresis (Fig 3C). Whereas both complexes failed to assemble Sm proteins onto U1ΔSm snRNA (Fig 3C, lanes 7 and 8), efficient formation of the subcore and core snRNP on the wild-type U1 snRNA could be observed (Fig 3C, lanes 3 and 4). We conclude that the recombinant SMN complex is active in snRNP core assembly onto RNAs containing a functional Sm site and, regarding this activity, is indistinguishable from the endogenous complex.

**The SMN complex acts as a Brownian machine in U snRNP assembly**

The availability of functional recombinant SMN complex enabled us to investigate individual components in the assembly reaction. We initially focused on the function of the peripheral SMN complex components Gemin3, 4 and 5. The Gemin3-4 heterodimer has been implied to hydrolyze ATP in the course of snRNP assembly, while
whether the recombinant SMN complex also fulfills this unique proofreading activity. Additionally, encouraged by the results above, we asked whether this proposed proofreading activity is dependent on Gemins3-5. For this purpose, we incubated a mixture of different cellular RNAs (3'-end-labeled with [32P]) with Sm proteins that were either bound to SMNΔGemins3-5 or present as free heterooligomers under physiological conditions. The reaction mixture was subsequently immunoprecipitated with antibodies against the Sm proteins, and the co-precipitated RNAs were detected by electrophoresis. Under these conditions, the SMN

\[ \text{SMN(WT) complex} \]

\[ \text{Sm subcore} \]

\[ \text{Sm core} \]

\[ \text{core RNP} \]

\[ \text{subcore RNP} \]

\[ \text{RNA} \]

\[ \text{6S} \]

\[ \text{pICln-D3/B} \]

\[ \text{Gemin3/4} \]

\[ \text{Gemin5} \]

\[ \text{Gemin6} \]

\[ \text{Gemin7} \]

\[ \text{Gemin8} \]

\[ \text{SMN} \]

\[ \text{D1/Gemin2} \]

\[ \text{D2/Gemin7} \]

\[ \text{D3/Gemin3/4} \]

\[ \text{D3/B/Gemin7} \]

\[ \text{D2/Gemin7} \]

\[ \text{Gemin5} \]

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complex assembled Sm cores only onto snRNAs, whereas other RNAs of the mixture (i.e., tRNAs) were no substrates (Fig 4C, lane 3).

In striking contrast, incubation of free Sm protein heterooligomers leads to their non-specific association with any RNA present in the mixture (Fig 4C, lane 2).

We conclude that the recombinant SMN complex assembles Sm core domains in an ATP-independent manner and proofreads the assembly onto cognate snRNA. However, neither the putative ATPase/helicase Gemin3 nor the snRNA binding protein Gemin5 is required for this reaction in vitro.

SMA-causing missense mutations or deletions in SMN affect either the activity of the SMN complex or its assembly

Finally, we investigated the function of the SMN protein in U snRNP assembly. Structural, biochemical and bioinformatic studies have revealed that SMN together with Gemin2 forms the core of the SMN complex that mediates the binding of Sm proteins and their assembly onto U snRNA. SMN is essential for the formation of the SMN complex and hence for the assembly reaction per se. Mutations within the SMN gene cause the devastating neuromuscular disorder

Figure 4. Gemin3, 4 and 5 are dispensable for snRNA identification and snRNP assembly in vitro.

A Assembly activity for SMN(WT)ΔGemin3-5 (left panel) and the entire SMN complex (right panel) over time with human U1 snRNA (upper panel) and pre-snRNA (lower panel). Lanes 1 and 9 show RNA only.

B Densitometric quantification of the experiment shown in (A). Measurements were performed with the software Image Lab (Bio-Rad) and fitted to saturation kinetics by Solver in Microsoft Excel (n = 2). Data points indicate the average value; bars represent the data range of all measurements.

C Autoradiography of the specificity assay. Sm core proteins alone or pre-bound to SMN(WT)ΔGemin3-5 were incubated with [α-32P]-labeled total cell RNA. The mixture was subsequently immunoprecipitated with an antibody against Sm proteins (Y12), and bound fractions were resolved on a urea gel after phenol extraction. Lane 1 shows the input.

Source data are available online for this figure.
spinal muscular atrophy (SMA). How SMN acts mechanistically, however, is still unclear. While it is impossible to assemble an SMN complex without the SMN protein itself, pathogenic missense mutations have been described in SMA patients that allow their functional characterization in our reconstituted system. We initially focused our studies on a missense mutation in SMN that is located in

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\text{SMN(WT)} \Delta \text{Gemin3-5} + \text{Sm Core proteins}
\]

\[
\text{SMN(E134K)} \Delta \text{Gemin3-5} + \text{Sm Core proteins}
\]

**Figure 5. Recombinant SMN(E134K) complex shows defects in snRNP assembly.**

A Transfer of Sm proteins from pICln-Sm complexes to SMN complexes containing either wild-type or mutant SMN protein. Immobilized wild-type and mutant complexes were incubated with the 6S complex (lanes 2 and 6), 6S and pICln-D3/B (lanes 3 and 7), or treated with buffer only (lanes 1 and 5). Analysis of retained proteins was achieved by a SDS–PAGE.

B Immobilized wild-type and mutant complexes shown in (A) were subsequently used for in vitro assembly reactions with \[^{32}\text{P}\]-U1 snRNA. snRNP formation was monitored by native gel electrophoresis (lanes 1–3 and 5–7, respectively). Lane 4 shows RNA only.

C In vitro snRNP assembly reactions were performed as described above with increasing concentrations of the SMN(WT)ΔGemin3-5 or SMN(E134K)ΔGemin3-5 complexes, respectively.

D Quantification of the experiments shown in (C) with Image Lab (Bio-Rad). The data were fitted to the Boltzmann equation by Solver in Microsoft Excel (\(n = 2\)). Rep1 and Rep2 indicate the datasets of independent experiments.

Source data are available online for this figure.
Figure 6. SMN complex formation and assembly activity with mutant SMN proteins.

A Recombinant wild-type SMN protein was used to assemble the SMN(Gemin3-5) complex. After incubation with 6S complex, the mixture was separated by gradient centrifugation and the peak fraction (dashed box) was used for snRNP assembly assays (right panel) with radiolabeled U1 snRNA.

B–D The reconstitution and assembly activity of SMN complexes containing either SMN(D44V), SMN(ΔExon7) or SMN(ΔYG) is shown in (B), (C) and (D), respectively.
its central Tudor domain. The mutation alters a glutamate to a lysine in position 134 [termed SMN(E134K)] and has been shown to disrupt an aromatic cage designed to bind dimethylarginines of Sm proteins (Tripsianes et al., 2011). To functionally characterize this mutant in the context of the SMN complex, we co-expressed and purified the SMN(E134K)ΔGemins-3-5 complex as described above for its wild-type counterpart (see Fig 5A and Materials and Methods for details). A stable mutant complex was obtained whose biochemical composition was indistinguishable from the wild-type complex. This illustrated that the mutant protein had folded properly and did not induce an assembly defect on the SMN complex itself (Fig 5A, compare lanes 1 and 5). Furthermore, the mutant complex bound Sm proteins and expelled plCln as described for its wild-type counterpart (Fig 5A, lanes 2–3 and 6–7, Supplementary Fig S5C, lanes 15–16). To test whether the SMN(E134K) mutation caused a defect in U snRNP assembly, wild-type and mutant complexes were loaded with Sm proteins of the subcore and the core proteins, respectively, and were subsequently incubated with [32P]-labeled wild-type U1 snRNA. U snRNP assembly was then analyzed by native gel electrophoresis as described above (Fig 5B). Strikingly, the mutant complex, although not entirely inactive, displayed a markedly reduced assembly activity (Fig 5B, lanes 6 and 7). A very similar picture was observed when wild-type and mutant SMN complexes also containing Gemins-3-5 were analyzed (Supplementary Fig S5). To gain insight into the molecular basis of this defect, the dissociation constant (Kd) of the assisted assembly process was determined. Whereas the assembly reaction mediated by the wild-type complex had a Kd of approximately 126 nM, this value doubled with the mutant complex (Fig 5C and D). These data suggest that the Sm proteins are less efficiently transferred onto the U snRNA because they are either more tightly bound or mis-arranged on the mutant complex.

To extend our studies further, we analyzed three additional mutant SMNAGemins-3-5 complexes containing SMN with either a pathogenic missense mutation (SMN(D44V)), a C-terminal deletion (SMN(ΔExon7)) that is the major product of the second human SMN gene (SMN2), or an artificial deletion of the highly conserved C-terminal YG box (SMN(AYG box)). The complexes were generated as described for SMN(E134K)ΔGemins-3-5 and analyzed by gradient centrifugation and in vitro assembly assays. As shown in Fig 6B, SMN(D44V) readily formed the SMNAGemins-3-5 complex and also displayed assembly activity comparable to the wild-type complex (compare Fig 6A with B). We note, however, that the SMN(D44V)ΔGemins-3-5 complex appeared to sediment in a lighter molecular weight range than the wild-type complex on density gradients, indicating that the stoichiometry of the complex had changed. Likewise, SMN(ΔExon7) formed SMN complexes, which sedimented in a similar molecular weight range as the SMN(D44V)ΔGemins-3-5 complex. The snRNP assembly activity of the SMN(ΔExon7)ΔGemins-5 complex, however, was entirely abrogated (Fig 6C, right panel), even though no gross defects in the SMNAGemins-3-5 complex were appreciable and Sm proteins were bound (Fig 6C, left panel). On the other hand, the artificial SMN(AYG box)ΔGemins-3-5 complex had a severe propensity to aggregate in our hands (Fig 6D, left panel). Concomitantly, this mutant SMNAGemins-3-5 complex was inactive in snRNP assembly (Fig 6D, right panel). Therefore, we conclude that SMN mutations/deletions affect the SMN complex differentially. Whereas some may affect the assembly activity directly (SMN (E134K)), others may affect the integrity/stoichiometry of the complex (i.e., SMN(ΔExon7) and SMN(AYG box)) or even processes yet to be identified (SMN(D44V)). The detailed structural, biochemical and functional analysis of these and potential other mutant complexes will hence enable the dissection of the assembly pathway and provide insight into the etiology of SMA.

Discussion

The detailed mechanistic analysis of the mode of action of cellular macromolecular complexes greatly benefits from the availability of reconstituted systems. Keeping with this notion, a series of in vivo experiments, extract systems and partial purification from cell lysates have defined the basic machinery and principles of cellular snRNP assembly (Friesen & Dreyfuss, 2000; Meister et al., 2001a;b; Pellizzoni et al., 2002b; Battle et al., 2006; Yong et al., 2010). Moreover, most recently, partial reconstitution (Chari et al., 2008) and structural characterization of key intermediates (Zhang et al., 2011; Sarachan et al., 2012; Grimm et al., 2013) have provided mechanistic insight at near atomic resolution. Despite these achievements, several aspects remain unanswered and a matter of debate. To establish experimental systems that may help to resolve these issues, we report here the total reconstitution of the human snRNP assembly machinery from recombinant proteins.

Employing this system, we show that the 6S complex, a key assembly intermediate, which constitutes a kinetic trap in snRNP assembly (Chari et al., 2008), is formed in a stepwise manner on the PRMT5 complex (Fig 7A). The heterotramer plCln-D1/D2 binds to the PRMT5/WD45 heterodimer, which we confirm to form a quaternary structure, composed of 4 PRMT5/WD45 heterodimers (Antonykamy et al., 2012). Note that this quaternary structure enables the simultaneous binding of plCln-D1/D2 and plCln-D3/B to the PRMT5/WD45 heteroctamer. In this complex, mono- and symmetrical dimethylation of B, D1 and D3 can occur. In vivo, the PRMT5 complex contains only substoichiometric amounts of F/E/G compared to D1/D2 and D3/B (Chari et al., 2008). Using an in vitro competition assay, we are able to provide a basis for this finding and show that the 6S complex is formed in a sequential manner on the PRMT5 complex with plCln-D1/D2 binding initially and F/E/G joining subsequently (Fig 2). Surprisingly, neither methylation nor F/E/G binding is sufficient for 6S complex dissociation from PRMT5/WD45. This process appears to be feed-forward driven, where the addition of a new plCln-D1/D2 or plCln-D3/B heterotrimer displaces the 6S complex (Fig 2). Therefore, it might be a combination of two effects that cause the newly formed 6S complex from interacting with the PRMT5 complex and support it to pass on to the SMN complex. First, plCln-D1/D2 or plCln-D3/B expels 6S from the PRMT5 complex. Second, the methylated D1 protein in 6S might make it more susceptible to bind to the SMN complex.

In the late phase of snRNP biogenesis, the SMN complex sequesters the Sm proteins from the assembly intermediates 6S and plCln-D3/B and expels plCln. Subsequently, the snRNA interacts with the SMN complex-bound Sm proteins to form snRNPs. In order to address specific steps of the late phase, we initially verified whether the SMN complex, reconstituted from recombinant proteins, indeed had the same activities as its cellular counterpart.
Quite unexpectedly, the central SMN complex components are both necessary and sufficient for all aspects of snRNP core formation in vitro. This finding is in contrast to former reports, which have emphasized the function of Gemin5 in binding to snRNAs and pre-snRNAs in a sequence-specific manner (Battle et al., 2006; Yong et al., 2010). While we were able to recapitulate that pre-snRNAs enhanced snRNP biogenesis, we found Gemin3-5 to be dispensable for assembly and proofreading of snRNAs (Figs 3 and 4). However, this result confirms our former findings that a minimal subcomplex of SMN/Gemin2 is sufficient to carry out the assembly reaction in vitro (Chari et al., 2008). Notably, Drosophila and Trypanosome SMN complexes consist of only these two subunits in vivo, enforcing this notion (Kroiss et al., 2008; Palfi et al., 2009). Accordingly, as previously reported by us (Kroiss et al., 2008), SMN and Gemin2 were alone sufficient to proofread 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 proofread 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 laboratories 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.

Finally, we focused on the SMN protein itself. Since decreased levels of functional SMN protein lead to spinal muscular atrophy, we hypothesized that distinct patient mutations in the SMN protein give rise to defects in snRNP assembly. Consequently, we generated a recombinant SMN complex with a common patient mutation (E134K). We demonstrate that this mutation decreases the rate of snRNP formation, while it does not affect the binding of Sm proteins of assembly intermediates (Fig 5). This suggests a crucial role of the SMN protein in the transfer of Sm proteins from the SMN complex onto snRNA. It is interesting to note that the E134K mutation inhibits SMN's ability to interact with sDMA-methylated Sm proteins when analyzed in the absence of other Geminis (Bühler et al., 1999; Tripsianes et al., 2011). This finding may hint to an interaction of SMN's Tudor domain with Sm proteins at stages of the assembly pathway that are not covered in our *in vitro* assay. Furthermore, we assessed the SMN(D44V), SMN(ΔExon7) and SMN(ΔVG box) mutations in the SMN gene for their capacity to form complexes of similar quaternary structure as the wild-type SMN gene and interrogated the snRNP assembly activity of these mutant complexes (Fig 6). The SMN(D44V) complex appears to form complexes that are somewhat smaller than the wild type and is not impaired in snRNP assembly. It might well be true that this SMN mutation exhibits a defect in snRNP biogenesis not recapitulated in our *in vitro* assay. The SMN(ΔExon7) complex appeared to have a similar quaternary structure as the SMN(D44V) complex. However, this complex was entirely abrogated in its snRNP assembly activity. Note, the SMN(ΔExon7) protein is the predominant product expressed from the SMN2 gene and has also been shown to be unstable in human cells (Cho & Dreyfuss, 2010). Our data indicate that the lack of exon 7 not only interferes with the stability of SMN but also with its function in the assembly reaction. In striking contrast to the aforementioned two mutant SMN proteins, we found in the SMN(ΔVG box) protein to impart a strong aggregation behavior for the SMN complex. This finding was surprising, considering that the YG box is believed to induce oligomerization of the SMN complex (Martin et al., 2012; Praveen et al., 2014) and is a common source of patient mutations (Wirth, 2000). In keeping with the aggregation propensity of this mutant SMN complex, there was no snRNP assembly activity.

Cellular and organismic approaches have thus far been utilized to study the effects of disease-causing mutations and/or deletions of the SMN gene (Lefebvre et al., 1995; Wang & Dreyfuss, 2001; Shpargel & Matera, 2005; Praveen et al., 2014). 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.

**Materials and Methods**

**Expression and purification of PRMT5/WD45**

The genes encoding human His$_6$-tagged PRMT5 isoform 1, WD45 and enhanced green fluorescent protein (EGFP) were cloned between the EcoRI and Xhol sites (PRMT5, WD45) and Ndel and Stul sites (EGFP) of a modified pFBDM vector (pFBDM4) of the MultiBac system (Berger et al., 2004 and Supplementary Methods). This plasmid was transformed into *E. coli* DH10MultiBac cells, and positive colonies were identified by blue/white screening. Twelve micrograms of isolated bacmid DNA was transfected into 3 × 10$^6$ S2/1 insect cells using Cellfectin II (Invitrogen) and incubated for 10 days at 27°C in 10 ml EX-CELL TiterHighem medium (Sigma-Aldrich). The P1 virus titer of this was applied to 250 ml of S2/1 insect cell suspension culture (2 × 10$^6$ cells/ml) at 100 rpm and 27°C for 72–79 h. Baculovirus titer concentrations were determined by end-point dilution identifying EGFP-expressing cells (O’Reilly et al., 1993). S2/1 insect cells (2 × 10$^6$ cells/ml) were infected with recombinant baculovirus titer at 3 MOI (PRMT5, WD45, EGFP) and incubated for 72–79 h. The overexpressed protein was purified using Ni-NTA chromatography (Qiagen) with a binding buffer of 20 mM HEPES-NaOH (pH 7.5), 1 M NaCl, 10% (v/v) glycerol, 10 mM imidazole, 1 mM PMSF, 20 mg/l aprotinin, 20 mg/l leupeptin/pepsstatin, 0.2 mM AEBSF and 5 mM β-mercaptoethanol, and an elution buffer lacking protease inhibitors but containing 250 mM imidazole. Elution fractions were pooled, dialyzed twice against 20 mM HEPES-NaOH (pH 7.5), 90 mM NaCl and 5 mM DTT at 4°C for 3 h and applied to anion exchange chromatography (HiTrapQ 1 ml, GE Healthcare). Proteins were eluted by increasing the NaCl concentration. Finally, recombinant proteins were separated by gel filtration chromatography (Superose 6 10/300 GL, GE Healthcare) in 20 mM HEPES-NaOH (pH 7.5), 200 mM NaCl and 5 mM DTT.

**Expression and purification of Gemin3, Gemin4 and Gemin5**

Genes encoding human His$_6$- and GST-tagged Gemin3 and Gemin5 were introduced into MCS2 of individual pFBDM4 transfer vectors using the Ncol and NotI sites and EGFP into MCS1 applying the Ndel and Stul sites of the same constructs. His$_6$-tagged Gemin4 was inserted into MCS1 of pFBDM4 using the EcoRI and Xhol sites, whereas EGFP was introduced into MCS2 via the Ncol and NotI sites. All coding sequences of protein affinity tags were followed by a tobacco etch virus (TEV) cleavage site. Resulting transfer vectors were transfected into S2/1 insect cells as described above and used to generate two baculovirus titers. S2/1 insect cells (2 × 10$^6$ cells/ml) were infected at 3 MOI with combinations of recombinant baculoviruses (GST-Gemin3/EGFP + His$_6$-Gemin4/EGFP, His$_6$-Gemin3/EGFP + His$_6$-Gemin4/EGFP + GST-Gemin5/EGFP or His$_6$-Gemin5 alone) and incubated for 72–79 h at 27°C.

Cells were harvested by centrifugation, resuspended in buffer C [50 mM sodium phosphate (pH 7.5), 500 mM NaCl, 10 mM EDTA, 1 mM spermidine, 1 mM TCEP] containing protease inhibitors and broken by sonication. A cleared lysate was prepared by ultracentrifugation in a 45Ti rotor (Beckman) for 1 h at 185,500 g and 4°C. The cleared lysate was incubated with Glutathione Sepharose® 4B (GE Healthcare) in batch for 2 h at 4°C. After extensive washing with
buffer C, the protein complex was eluted with buffer C containing 40 mM glutathione. Fractions were identified by SDS–PAGE and stored at 4°C until further use.

Expression and purification of the SMN\Gemin3-5 complex

For the expression and purification of the SMN\Gemin3-5 complex, E. coli BL21 Star™ (DE3) cells (Novagen) were co-transformed with both Gemin7-Gemin6:pET21a and Gemin2-Gemin8- His\_6-GST-TEV-SMN:pET28b* plasmids (for cloning strategy see Chari et al, 2008). Additionally, the plasmid pRARE (Novagen) was co-transformed. Cells were cultured in Super Broth medium containing 2% glucose, 500 mM sorbitol, 1 mM betaine, 100 µg/ml ampicillin, 25 µg/ml kanamycin and 34 µg/ml chloramphenicol. Cultures were incubated at 37°C to an OD\_600 of 0.2 and cooled to 15°C, and protein expression was induced with 0.5 mM IPTG at an OD\_600 of 0.4 for additional 20 h. Cells were harvested by centrifugation, resuspended in buffer A [50 mM imidazole (pH 6.8), 300 mM Na\_2SO\_4, 10 mM EDTA, 10% (w/v) galactose, 1 mM spermidine and 1 mM TCEP] containing protease inhibitors and broken by sonication. A cleared lysate was prepared by ultracentrifugation in a 45Ti rotor (Beckman) for 1 h at 72,400 g and 4°C. This was then incubated with Glutathione Sepharose™ 4B (GE Healthcare) in batch for 2 h at 4°C. After extensive washing with buffer A and buffer B [50 mM imidazole (pH 6.8), 150 mM Na\_2SO\_4, 10% (w/v) galactose, 1 mM spermidine and 1 mM TCEP], the Sepharose beads were supplemented with a 1:50 ratio (protease:protein) of TEV protease and incubated for 14 h at 4°C. The supernatant and four wash fractions containing the cleaved SMN\Gemin3-5 complex were identified by SDS–PAGE and stored at 4°C until further use.

Expression and purification of pICln and Sm protein heterooligomers D1/D2, D3/B and F/E/G

pICln, the heterodimers D1/D2 and D3/B as well as the heterotrimer F/E/G were expressed in E. coli and purified as described (Kambach et al, 1999; Chari et al, 2008).

Reconstitution of pICln-Sm protein complexes and PRMT5-Sm protein complexes

pICln-Sm protein complexes were reconstituted as described previously (Chari et al, 2008). To reconstitute PRMT5 complexes in vitro, recombinant PRMT5/WD45 was incubated with a 2- to 5-fold excess of Sm protein heterooligomers (D1/D2, F/E/G or D3/B), pICln or pICln-Sm protein complexes (pICln-D1/D2, 6S or pICln-D3/B) in 20 mM HEPES-NaOH (pH 7.5), 1 M NaCl, 10% (v/v) glycerol and 5 mM β-mercaptoethanol. These were then dialyzed overnight at 4°C against the same buffer containing 200 mM NaCl. Finally, protein complexes were resolved by gel filtration chromatography (Superose 6 10/300 GL, GE Healthcare). The formation of protein complexes was verified by SDS–PAGE and subsequent silver staining. Protein standards of known molecular weight (dextran blue: 2,000 kDa, thyroglobulin: 669 kDa, ferritin: 440 kDa and BSA: 67 kDa) were applied separately to the same gel filtration column. The resulting elution profiles were plotted against the elution volume and are indicated above the SDS gels.

Sequential formation of 6S on PRMT5/WD45 and subsequent release

Recombinant PRMT5/WD45 was incubated with a 2-fold excess of pICln-D1/D2 in 20 mM HEPES-NaOH (pH 7.5), 1 M NaCl and 5 mM DTT and dialyzed against 20 mM HEPES-NaOH (pH 7.5), 200 mM NaCl and 5 mM DTT overnight at 4°C. Complex formation was monitored by gel filtration chromatography using identical buffer conditions on a Superose 6 10/300 GL column (GE Healthcare) and subsequent SDS–PAGE. Elution fractions containing the protein complex of PRMT5/WD45/pICln-D1/D2 were pooled and incubated with a 5-fold excess of F/E/G, and reconstitution was analyzed as before. Finally, fractions containing PRMT5/WD45/6S were treated with a 3.5-fold excess of pICln-D1/D2 and assayed for complex formation.

In vitro methylation of Sm proteins by recombinant PRMT5/WD45

One picomole of recombinant His\_6-PRMT5/WD45 was incubated with a hundredfold molar excess of D1/D2, pICln-D1/D2, 6S, D3/B or pICln-D3/B and 219 pmol radioactively labeled co-factor S-adenosylmethionine [SAM; mixture of 50% [\(^3\)H]-SAM (Perkin Elmer: 10 Ci/mmol) and 50% SAM (Sigma-Aldrich)] and a reaction buffer containing 100 mM HEPES-NaOH (pH 8.2), 200 mM NaCl and 5 mM DTT in a total volume of 20 µl at 37°C for 60 min. Reactions were stopped by the addition of 6× SDS–PAGE loading buffer and subsequent incubation at 95°C for 5 min. Proteins were separated on a 13% SDS gel and fixed with 30% (v/v) methanol and 10% (v/v) acetic acid. The radioactive signal was amplified by incubation with NAMP-100 amplifying reagent (GE Healthcare) for 45 min. Subsequently, gels were dried and exposed to Amersham Hyperfilm™ MP (GE Healthcare) at −80°C for 18 h.

In vitro reconstitution of recombinant SMN complexes

In order to reconstitute the entire human SMN complex from recombinant sources, 40 pmol of bacterially expressed SMN\Gemin3-5 (containing either the wild-type SMN protein or the mutants E134K, D44V, ΔExon7 or ΔYG box) was combined with: (i) buffer only, (ii) 40 pmol of insect cell-expressed Gemin3/Gemin4, (iii) Gemin5, or (iv) Gemin3/Gemin4 + Gemin5. Each of these reactions was then supplemented with: (i) buffer only, (ii) 200 pmol of 6S, (iii) 200 pmol pICln-D3/B, or (iv) 200 pmol of 6S and pICln-D3/B. Finally, BSA was added to a final concentration of 1 mg/ml to prevent protein precipitation. Reaction samples were dialyzed overnight against 20 mM HEPES-NaOH (pH 7.5), 200 mM NaCl and 5 mM DTT at 4°C followed by a dialysis against the same buffer lacking DTT for 3 h at 4°C. Samples were centrifuged at 13,000 g for 30 min at 4°C, and the supernatant was incubated with 40 µl Protein G-Sepharose™ beads (GE Healthcare) coupled with 2.5 mg/ml 7B10 antibody (anti-SMN) at 600 rpm and 4°C for 90 min. Beads were washed twice with 1.2 ml 1× PBS, 300 mM NaCl and 0.01% NP-40, and twice with 1× PBS and 300 mM NaCl. Finally, the beads were resuspended in 20 µl 1× PBS and 300 mM NaCl, supplemented with 5 µl 6× SDS–PAGE loading buffer and analyzed by 12.5% SDS–PAGE and Coomassie staining. For subsequent band shifts, larger protein amounts were deployed in complex reconstitutions.
Native gel electrophoresis of RNA–protein complexes

Band shift assays were performed essentially as described (Meister et al., 2001a; Chari et al., 2008). In brief, 5 pmol proteins were added to 0.025 pmol radiolabeled RNA in 16 µl reactions with 0.4 U/µl RNasin and 0.1 µg/µl RNA and BSA. The mixtures were incubated for 60 min at 37°C (or respective time). After incubation, the mixtures were briefly centrifuged, supplemented with heparin to a final concentration of 0.5 µg/µl and separated on 6% native polyacrylamide gels (acrylamide/bisacrylamide ratio 80:1) containing 4% (v/v) glycerol and 1× TBE buffer. Gels were pre-run for 1 h at 4°C at 100 V in 1× TBE and run with samples for 2 h at 4°C at 300 V. Gels were exposed wet to Amersham Hyperfilm® MP (GE Healthcare) at −80°C. Densitometric measurements were quantified with the Image Lab software integrated into the Gel Doc™ XR+ system (Bio-Rad). Free RNA was taken as a reference (100%). Values were fitted to saturation kinetics via the Solver add-in of Microsoft Excel and plotted. All values shown are averages of two independent experiments.

In vitro transcription of U snRNAs

[32P]-labeled X. laevis U1 snRNA and U1ΔSm snRNA (Hamm et al., 1987) were obtained by an in vitro run-off transcription. pUC9 vectors containing the coding sequences were linearized with BamHI and purified by phenol–chloroform extraction. The transcription was carried out at 37°C for 4 h. Transcripts were separated by electrophoresis on a 5% polyacrylamide gel under denaturing conditions. Respective bands were cut out, purified via ethanol precipitation, resuspended in water and stored at −20°C until further use.

Cloning of the human U1 snRNA and pre-snRNA

Primers hU1-for–EcoRI-T7 and Pre-hU1-rev-BamHI were used to amplify human U1 pre-snRNA from a genomic DNA preparation. After EcoRI and BamHI cleavage, the fragment was ligated into an analogously cleaved pUC19 vector. An altered Sm site was introduced by site-specific mutations with primers hU1-ΔSm-upr and hU1-ΔSm-lwr.

In vitro transcription was carried out with hU1-for-EcoRI-T7 and hU1-rev (human U1 snRNA) and hU1-for–EcoRI-T7 and pre-hU1-rev (human U1 pre-snRNA).

hU1-DSm-upr: 5′-GGAAACTCGACTGCATACGGACTCGTAGTGGGG GACTG-3′
hU1-DSm-lwr: 5′-CAGTCCCCCATACGACTCGTAGGACTCGAGT TTTCC-3′
hU1-for–EcoRI-T7: 5′-GGAAATCTCCTAATACGACTCACTATAGGATCT TACCTGGCAGGGGAGATAC-3′
Pre-hU1-rev-BamHI: 5′-CGGGATCCGAAAAATATGACCATTTGGC GTACAGTCTG-3′
prehU1-rev: 5′-AAAGATATGACCTTGGCCGTACAGTCTG-3′

The RNA concentration was measured via a spectrophotometer at 260 nm.

Assembly reactions in vitro

RNA was prepared from HeLa nuclear cell extract via TRIzol® (Life Tech) treatment according to the manufacturer’s specification. Additionally, snRNAs were isolated from snRNPs (Sumpter et al., 1992) and mixed with nuclear RNA (10 mg each). The RNA mixture was 3′-end-labeled with 3P overnight and subsequently phenolized. A total of 80 pmol of Sm core proteins loaded onto SMNAGemin3-5, Sm core proteins alone or no protein was incubated with 500 ng of labeled RNA at 37°C or 4°C for 1 h in the presence of 1 mg/ml BSA in 1× PBS. Notably, this reaction was performed in the absence of non-specific competitors such as heparin. Sm proteins and bound RNA were immunoprecipitated via Y12 antibody coupled to protein G-Sepharose (1.5 h, 4°C on a head-over-tail rotor). The beads were washed three times with 1× PBS and phenolized. The resulting RNA pellet was resuspended in 20 µl ddH2O. Samples were separated on an 8% denaturing urea gel (30 W constantly).

Supplementary information for this article is available online:
http://emboj.embopress.org

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Author contributions

NN and CE contributed equally to this work. NN, CE, AC and UF designed the experiments and analyzed the results. NN, CE, AC, JO and TZ performed the experiments. NN, CE, AC and UF wrote the manuscript. UF and AC supervised the research.

Conflict of interest

The authors declare that they have no conflict of interest.

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