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A Comprehensive Interaction Map of the Human Survival of Motor Neuron (SMN) Complex*[S]

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Assembly of the Sm-class of U-rich small nuclear ribonucleoprotein particles (U snRNPs) is a process facilitated by the macromolecular survival of motor neuron (SMN) complex. This entity promotes the binding of a set of factors, termed LSm/Sm proteins, onto snRNA to form the core structure of these particles. Nine factors, including the SMN protein, the product of the spinal muscular atrophy (SMA) disease gene, Gemins 2–8 and unrip have been identified as the major components of the SMN complex. So far, however, only little is known about the architecture of this complex and the contribution of individual components to its function. Here, we present a comprehensive interaction map of all core components of the SMN complex based upon *in vivo* and *in vitro* methods. Our studies reveal a modular composition of the SMN complex with the three proteins SMN, Gemin8, and Gemin7 in its center. Onto this central building block the other components are bound via multiple interactions. Furthermore, by employing a novel assay, we were able to reconstitute the SMN complex from individual components and confirm the interaction map. Interestingly, SMN protein carrying an SMA-causing mutation was severely impaired in formation of the SMN complex. Finally, we show that the peripheral component Gemin5 contributes an essential activity to the SMN complex, most likely the transfer of Sm proteins onto the U snRNA. Collectively, the data presented here provide a basis for the detailed mechanistic and structural analysis of the assembly machinery of U snRNPs.

Several nuclear RNA-protein complexes (RNPs)[3] involved in the processing of mRNAs, such as the snRNPs of the major (U1, U2, U4/6, and U5) and minor (U11, U12, U5, and U4/6atac) spliceosome and the histone-mRNA processing U7 snRNP contain a set of evolutionary conserved proteins of the Sm/LSm class (1, 2). This group of proteins has the propensity to form heptameric rings in the presence of their respective target snRNA. Sm and LSm/Sm rings (also called "cores") can form spontaneously *in vitro* on their target RNAs (3–5). However, assembly *in vivo* occurs in a highly regulated manner and is assisted by *trans*-acting factors. One well characterized entity in this pathway is the SMN complex, whose name-giving component SMN is the product of the spinal muscular atrophy (SMA) disease gene (6, 7). This entity recruits all Sm proteins and promotes their transfer onto the U snRNAs (8–10). Likewise, assembly of the U7 snRNP is facilitated by a specialized SMN complex that is charged with the unique set of Sm and LSm proteins of this particle (11). With a sedimentation coefficient of 25–40 S and an estimated molecular mass exceeding 1 megadalton, the SMN complex represents a macromolecular machine of great complexity. So far, nine major proteins termed SMN, Gemins 2–8, and unrip as well as nine Sm/LSm protein "substrates" (*i.e.* B/B′, D1, D2, D3, E, F, G, LSm10, and LSm11) have been identified as components of this assembly machinery (12, 13).

Our knowledge about the architecture of the core SMN complex (*i.e.* the SMN complex without substrate proteins) is still limited and relies mostly on *in vitro* interaction assays using recombinant proteins. These studies have placed the SMN protein in the center with interactions to Gemins 2, 3, 5, and 7, as well as substrate Sm proteins. In contrast, other factors such as Gemins 2, 4–6, and 8 and unrip appeared to be peripheral and interact only with few other components of the complex (see Refs. 12 and 13 for reviews; Refs. 14–16). The relevance of these interactions in the context of the native SMN complex remained unclear from these studies. To address this issue, we applied a combination of *in vitro* and *in vivo* approaches (*in vitro* binding assays, co-immunoprecipitations and a yeast two-hybrid interaction system) to establish a comprehensive interaction map of the SMN complex. Furthermore, by use of a novel assay we were able to reconstitute the SMN complex in reticulocyte lysate. Interestingly, complex formation is severely compromised by an SMA-causing mutation in the SMN protein. Finally, we have assessed the contribution of the peripheral component Gemin5 to the activity of the SMN complex.

EXPERIMENTAL PROCEDURES

DNA Constructs—Plasmids encoding full-length cDNAs corresponding to the open reading frames of SMN, unrip, and

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3 and Refs. 1–3.

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[3] The abbreviations used are: RNP, ribonucleoprotein; U snRNP, uridine-rich small ribonucleoprotein; snRNA, small nuclear ribonucleic acid; SMN, survival of motor neurons; Gemins 2–8, components of gems number 2–8, respectively; GST, glutathione *S*-transferase; HA, hemagglutinin; siRNA, short interfering RNA; RNAi, RNA interference; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

Gemins 2–7 have been described previously (15). The full-length open reading frame of Gemin8 was purchased from RZPD (Clone IRAUp969F1069D6) and subcloned into the vectors pGEX6P-1 (GE Healthcare), pET28a (Novagen), and pHA (an N-terminal HA-tag containing derivative of pcDNA3.1; Invitrogen).

Recombinant Proteins and in Vitro Protein Binding Assays—Expression and purification of single proteins or protein complexes were performed as described (15). [^{35}S]Methionine-labeled proteins were produced using the TNT-T7 quick coupled transcription/translation system (Promega). *In vitro* co-translations of the entire SMN complex were carried out in one single TNT reaction with [^{35}S]methionine, using a mixture of vectors encoding for the different SMN complex components. In the case of GST binding assays, *in vitro* translated proteins were incubated with ~2 μg of purified GST fusion proteins, immobilized on glutathione-Sepharose (GE Healthcare), and allowed to bind in lysis buffer (50 mM Tris/HCl, pH 7.5, 200 mM NaCl, 0.01% Igepal, 1 mM dithiothreitol, 5 mM EDTA, 5 mM EGTA, 1 μg/ml bovine serum albumin) at 4 °C for 1 h. After washing the resin extensively, bound proteins were eluted by boiling in 2× SDS sample buffer, resolved by SDS-PAGE, and analyzed by Coomassie staining and autoradiography of the dried gel.

Preparation of HeLa Cell Extract, Antibodies, and Immunoprecipitations—HeLa cytosolic extract was prepared as described (17). Antibodies against Gemins 5–8 were raised by injection of full-length human proteins into rabbits. Antibodies were affinity purified on columns with the respective covalently linked antigen. Anti-Gemin4 and anti-SmB/B′ were purchased from Santa Cruz Biotechnology. Immunoprecipitations of the SMN complex were carried out in HeLa cytosolic extract, using a covalently linked 7B10 monoclonal SMN antibody (8). Precipitated SMN complex was washed extensively with buffer (50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 0.01% Igepal) of rising ionic strength (150 mM, 250 mM, 750 mM, 500 mM, 1 M, 1.5 M NaCl). Resulting SMN complexes were eluted by 2× SDS buffer and resolved on 8–20% SDS-PAGE gradient gels. For reconstitution of SMN complexes with SmB/D3, an immobilized complex was washed with 1.5 M NaCl high salt buffer and incubated with recombinant SmB/D3 heterodimer, purified as described (18). After extensive washing, complexes were eluted by 2× SDS buffer and resolved in 8–20% SDS-PAGE.

RNA Interference (RNAi)—Gemin2 levels were reduced by transfection of HeLa cells with two double-stranded 21-nucleotide-long siRNAs (sequences: RNAi-1, 5′-GGAAGCAAAGUGUGAAUAUTT-3′ and RNAi-2, 5′-GCAGCUCAAUGUCCAGAUGTT-3′). siRNAs were purchased from IBA Nucleic Acids Synthesis (Göttingen, Germany) and transfected with Oligofectamine™ (Invitrogen), following the protocol of the manufacturer. Silencing of Gemin2 was assessed 52 h after transfection by Western blotting of total cell extract, using a monospecific Gemin2 antibody. Immunoprecipitations from these extracts were performed as described above, using extracts normalized for SMN by Western blotting with specific antiserum.

Purification of Baculoviral Expressed Gemin5 and in Vitro Assembly of U snRNPs—His-tagged Gemin5 protein was expressed using the MultiBac system (19). Cells were harvested 5 days post-infection by centrifugation and lysed with detergent (20 mM NaHEPES, 200 mM NaCl, 0.25% Triton X-100, pH 7.5). The clarified lysate was subjected to nickel-nitrilotriacetic acid purification, washed, and eluted with imidazole. 60–200 mM imidazole elution fractions were pooled and dialyzed against 1× phosphate-buffered saline, 0.01% Igepal, and 1 mM dithiothreitol before further use.

To analyze the role of Gemin5 in U snRNP assembly, an immunoprecipitated SMN complex in the presence and absence of recombinant SmB/D3 was incubated with 0.75 μg of recombinant Gemin5 in an assembly assay using ^{32}P-labeled U1 snRNA (15). Sm core formation was assessed by native gel electrophoresis after addition of buffer containing 4 M urea, 12.5 mg/ml heparin, and 16% glycerol as described (8).

Glycerol Gradient Centrifugation of the in Vitro Reconstituted SMN Complex—*In vitro* reconstituted, ^{35}S-labeled SMN complexes were diluted with 1× phosphate-buffered saline to a final volume of 250 μl, layered on a 10–30% glycerol gradient, and centrifuged for 16 h in an SW60Ti rotor (4 °C, 24,000 rpm, Beckman Coulter Optima L-80 XP). Gradients were manually harvested in 22 fractions from top to bottom.

Yeast Two-hybrid Interaction Trap—All yeast manipulations and the yeast two-hybrid interaction trap assay were carried out as described (20, 21). In brief, to analyze all possible protein-protein interactions within the SMN complex, the complex components (*i.e.* SMN, Gemins 2–8, and unrip) were subcloned into pEG202 vector (Clontech), which allows constitutive expression of LexA fusion proteins (DNA-binding domain, bait) and into the galactose inducible vector pJG4-5 to express B42-HA-fusion proteins (activation domain, prey). Plasmids were co-transformed in *Saccharomyces cerevisiae* strain EGY48 harboring the reporter plasmid pSH18–34 (Invitrogen). Positive clones were selected by blue staining of colonies on X-gal containing galactose/URA$^-$HIS$^-$TRP$^-$ dropout plates. Yeast clones were grown on glucose-containing dropout plates as a control.

RESULTS

Interactions of Recombinant SMN Complex Components in Vitro—In a first series of experiments we used GST-tagged recombinant proteins to analyze the interactions with *in vitro* translated ^{35}S-labeled components of the SMN complex, produced in reticulocyte lysate (Fig. 1*A*). In our hands, only SMN and Gemins 2 and 6–8 could be produced in *Escherichia coli* as soluble, full-length proteins, whereas Gemins 3–5 and unrip were degraded and/or misfolded. Robust binding of SMN, Gemins 2, 3, and 8 to immobilized GST-SMN was detected (*lane 3*), but no significant association of any translated protein was observed to GST alone (*lane 2*). An identical pattern could be observed when a complex composed of GST-SMN and Gemin2 was used as bait (*lane 4*), suggesting that Gemin2 binds predominantly to SMN. Consistently, GST-Gemin2 bound SMN only but no other protein of the SMN complex in this assay (*lane 5*). Next, we analyzed GST-Gemins 6 and 7, which have convincingly been shown to form a heterodimer (13). In

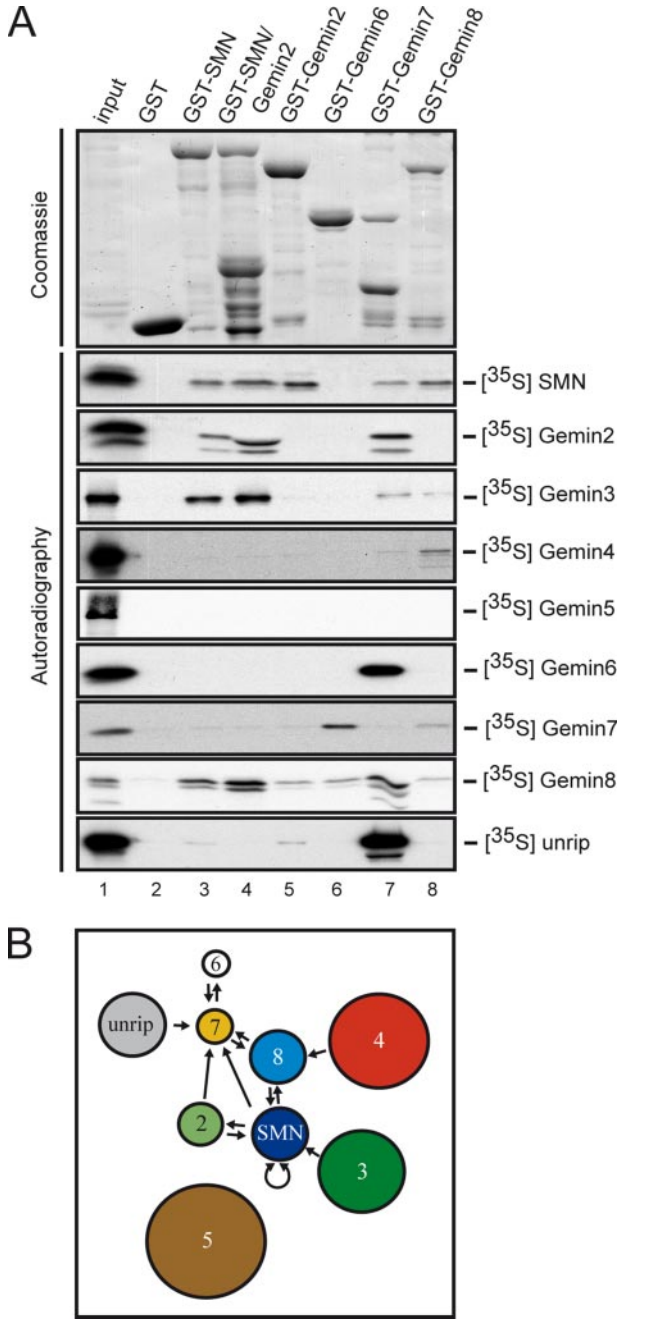

FIGURE 1. **GST pulldown assay with SMN complex components.** *A*, GST-fusion proteins (*i. e.* SMN, Gemin2, GST-SMN/His_6-Gemin2, and Gemins 6–8) were immobilized on glutathione-Sepharose and incubated with *in vitro* translated, ^{35}S-labeled components of the SMN complex. Bound proteins were eluted by boiling in loading buffer, resolved by SDS-PAGE, visualized by Coomassie staining (*upper panel*, *lanes 3–8*), and by autoradiography of the dried gel (*lower panels*, *lanes 3–8*). 10% input of the ^{35}S-labeled proteins is shown in *lane 1*. GST was used as a control (*lane 2*). *B*, schematic of all resulting interactions shown in *A*.

agreement with earlier findings, we observed binding of translated Gemin7 to GST-Gemin6 and vice versa (*lanes 6* and *7*). Whereas Gemin6 appears to bind to no other component, strong binding of Gemin7 to Gemins 2, 6, and 8 and unrip, and weak binding to SMN could be detected (*lane 7*). In the same assay, GST-Gemin8 bound to SMN as well as Gemin4 (*lane 8*). In conclusion, this first experimental strategy suggests that Gemin8 links the proteins Gemins 6 and 7 and unrip with the remainder of the SMN complex (see also supplemental Fig. 1 for additional binding assays that support this view). The data obtained from this set of experiments are depicted in Fig. 1*B*.

A Co-immunoprecipitation Strategy Reveals Interactions of Gemins 3, 4, and 5—Three proteins of the SMN complex, namely Gemins 3, 4, and 5 could not be produced in *E. coli* as full-length proteins in a properly folded manner and in sufficient amounts to perform the experiments described above. Hence, the interactions of these proteins were analyzed by co-immunoprecipitation of co-translated and ^{35}S-labeled components. In a first set of experiments, the interactions of Gemin3 were studied in detail (Fig. 2*A*). For this, Gemin3 was co-translated with single proteins of the SMN complex, tagged with either an HA-epitope (SMN, Gemins 2, 4, and 6–8 and unrip) or a V5 epitope (Gemin5). The co-translations were then precipitated via the respective tag and analyzed by autoradiography (see *lanes 4–11* for inputs). Using this assay, we observed reproducible association of Gemin3 with SMN, Gemins 2, 4, and 5 (*lanes 12–15*). No other component of the SMN complex was co-precipitated in this experiment, and neither antibody precipitated Gemin3 alone (Fig. 2*A*, *lanes 2*, *3*, and *16–19*), implying specific interactions.

Next, binding of Gemin4 with co-translated proteins of the SMN complex was determined by the same strategy (see Fig. 2*B*, for inputs see *lanes 4–11*). Only Gemin3 and to a lesser extent Gemin5 could be co-precipitated with Gemin4 (compare *lanes 14* and *15* with *lanes 12*, *13*, and *16–19*). Finally, V5-tagged Gemin5 was analyzed (Fig. 2*C*). Weak but reproducible binding was observed to Gemin2 and Gemin4 (*lanes 12* and *14*), whereas the other components failed to interact in the same assay (*lanes 11*, *13*, and *15–18*). To further validate the unexpected interaction between Gemin2 and Gemin5, expression levels of Gemin2 were reduced by RNAi in HeLa cells. As shown in Fig. 2*E*, transfection of two different 21-nucleotide-long siRNA duplices complementary to the Gemin2 mRNA but not a control siRNA reduced Gemin2 protein levels. SMN and Gemin7 levels were unaffected, whereas Gemin5 expression was reduced marginally (Fig. 2*E*, *left panel*, compare *lane 1* with *lanes 2* and *3*). Extracts prepared from these cells were then immunoprecipitated with an anti-SMN monoclonal antibody and co-precipitated components detected by Western blotting. Whereas Gemins 2, 5, and 7 could be co-precipitated with SMN from extracts derived from control cells, the association of SMN with Gemin2 and Gemin5 was markedly reduced in the Gemin2 knockdown extracts (by at least 80%), while Gemin7 association was unaltered (Fig. 2*E*, *right panel*, compare *lane 1* with *lanes 2* and *3*). These data illustrate that Gemin5 is tethered to the SMN complex predominantly via an interaction with Gemin2 *in vivo*. This conclusion was further supported by additional co-immunoprecipitation experiments and bacterial co-expression studies with truncation mutants of Gemin5 (supplemental Fig. 2). These confirmed the results obtained so far and further revealed the C-terminal half of Gemin5 (amino acids 721–1508) as the binding site for Gemin2 (supplemental Fig. 2).

Analysis of Protein Contacts within the SMN Complex by a Yeast Two-hybrid System—The experiments described above revealed specific interactions of individual SMN complex com-

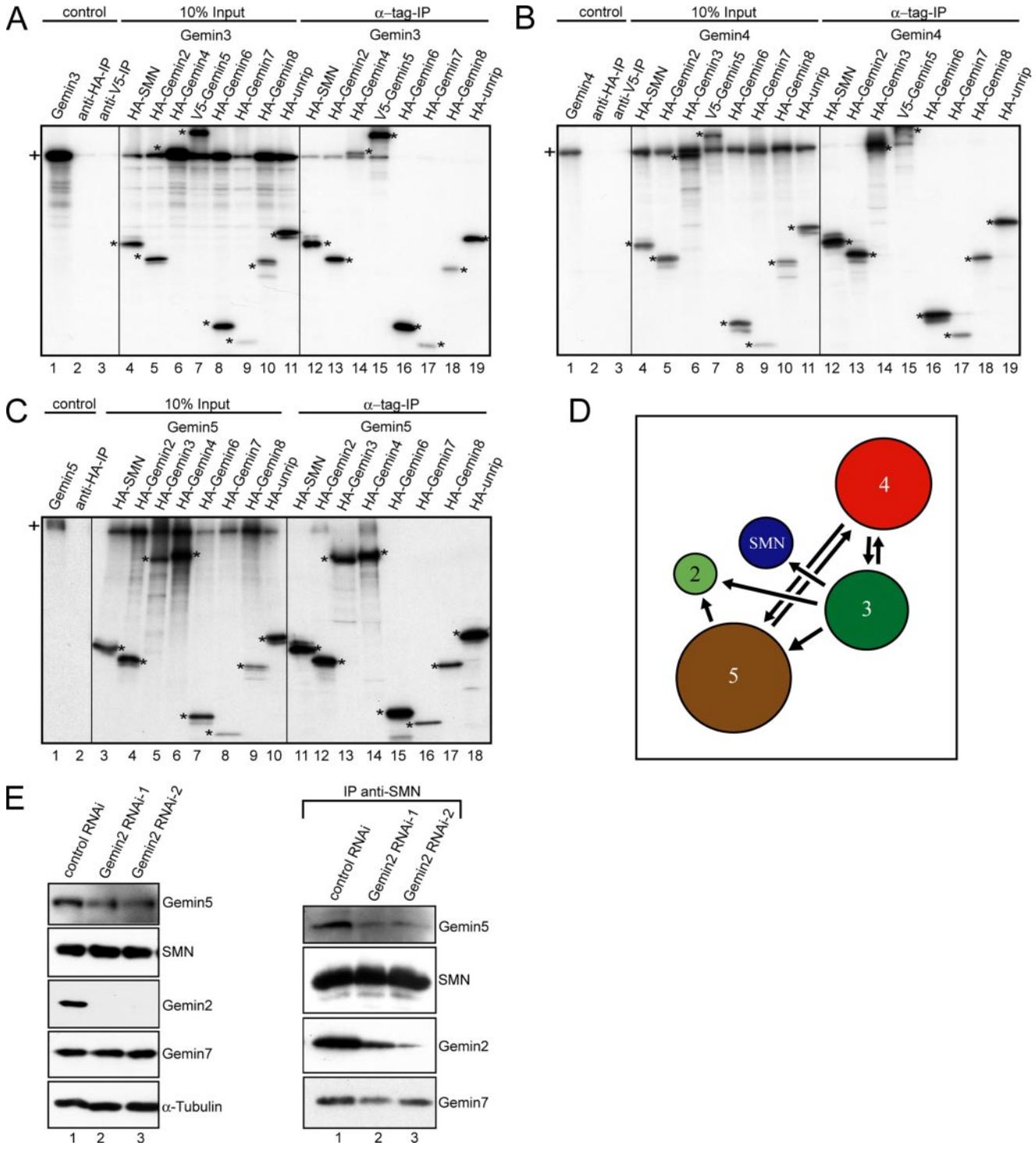

FIGURE 2. **Co-immunoprecipitation assay with Gemins 3–5.** HA-tagged versions of all SMN complex components were co-translated *in vitro* with His_6-tagged Gemin3 (*A*), Gemin4 (*B*), or Gemin5 (*C*) and labeled with [^{35}S]methionine, respectively. Proteins were co-immunoprecipitated with either an α-HA (SMN, Gemins 2–4, Gemins 6–8, and unrip) or an α-V5 antibody (Gemin5) and resolved by SDS-PAGE. Labeled proteins were visualized by autoradiography. Immunoprecipitated bait proteins are marked by *asterisks* and prey proteins by *plus signs*. *A*, co-immunoprecipitations of Gemin3 with SMN complex components (*lanes 12–19*). As a control ^{35}S-labeled His_6-Gemin3 was immunoprecipitated by α-HA (*lane 2*) and α-V5 antibody (*lane 3*). 10% input is shown (*lanes 1* and *4–11*). *B*, co-immunoprecipitations of Gemin4 with SMN complex components (*lanes 12–19*). As a control ^{35}S-labeled His_6-Gemin4 was immunoprecipitated by α-HA (*lane 2*) and α-V5 antibody (*lane 3*). 10% input is shown (*lanes 1* and *4–11*). *C*, co-immunoprecipitations of Gemin5 with SMN complex components (*lanes 11–18*). As a control ^{35}S-labeled V5-Gemin5 was immunoprecipitated by α-HA antibody (*lane 2*). 10% input is shown (*lanes 1* and *3–10*). *D*, schematic of all resulting interactions shown in *A–C*. *E*, *left panel*: Gemin5 is incorporated into the SMN complex predominantly via Gemin2. HeLa cells were transfected with Gemin2 siRNAs (RNAi-1, *lane 2* and RNAi-2, *lane 3*). Silencing efficiency was assessed 52 h post-transfection by Western blotting with the indicated antibodies. An unrelated siRNA was used as control (*lane 1*). *E*, *right panel*: SMN complex was immunoprecipitated from extract (*E*, *left panel*) with 7B10 anti-SMN antibody, resolved on an 8–20% gradient gel, and analyzed by Western blotting with specific antibodies.

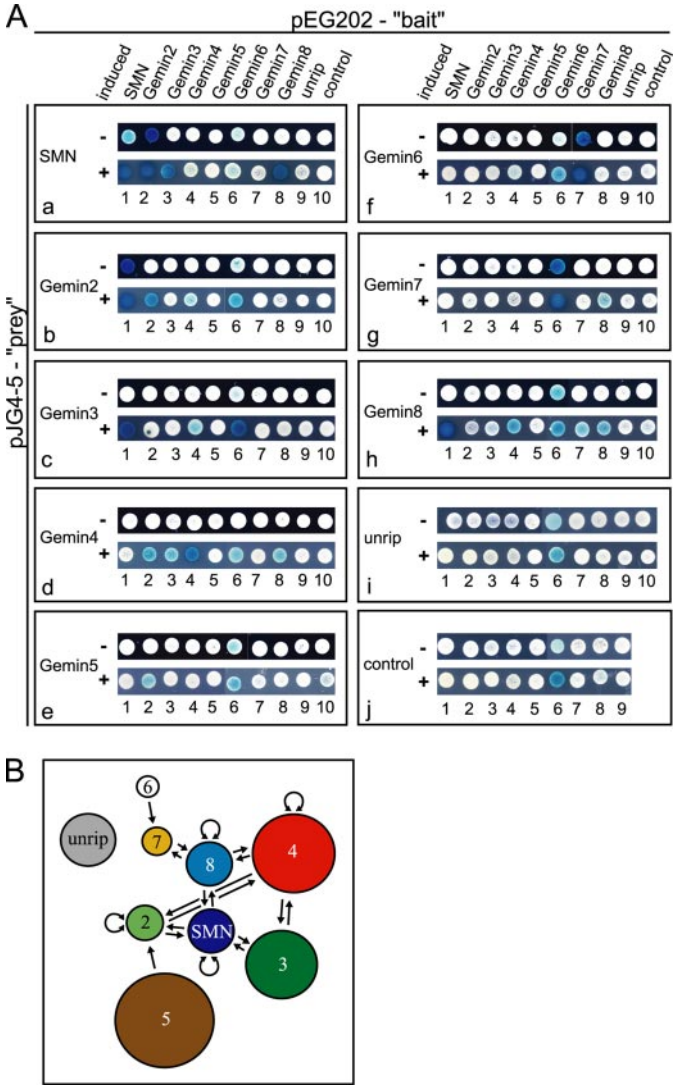

FIGURE 3. **Yeast two-hybrid interaction trap assay with SMN complex components.** *A*, all components of the SMN complex (*i. e.* SMN, Gemins 2–8, unrip) were transformed into *S. cerevisiae* as galactose-inducible prey plasmids pJG4-5 (*panels a–j*). A single tested clone of each transformation was co-transformed with bait plasmids pEG202, encoding all SMN complex components (*lanes 1–9* in each panel), respectively. As a control, yeast clones with complex components subcloned in pJG4-5 were co-transformed with the empty vector pEG202 (*lane 10*). Positive clones were selected by blue staining on galactose/URA$^-$HIS$^-$TRP$^-$ X-gal dropout plates (*panels a–j*, *lower row*). As a control, clones were tested on glucose containing dropout plates (*upper row*). *B*, schematic of all resulting interactions shown in *A*.

ponents to each other *in vitro*. We next set forth to analyze interactions within the SMN complex *in vivo* using a yeast two-hybrid assay (20, 21). For this purpose, a panel of "bait"-vector constructs were created, encoding for fusions of the DNA-binding domain of the transcription factor LexA and either SMN, Gemins 2–8, or unrip (Fig. 3*A*, *lanes 1–9*, *lane 10* shows the LexA control without a fusion partner). These plasmids were co-transformed with "prey"-vectors encoding for fusions of the activation domain B42 and the same proteins as in the bait vectors (*panels a–i*, *panel j* shows the B42 control). Binding of prey to bait fusion proteins was then assessed in a β-galactosidase enzyme assay upon induction of prey fusion expression.

Interactions of SMN expressed from the prey-vector are shown in Fig. 3*A*, *panel a*. In agreement with earlier data and those shown in Figs. 1 and 2, SMN interacts with itself, Gemin2, Gemin3, and Gemin8 (*panel a*, *lanes 1–3* and *8*). Importantly, these interactions can also be observed when SMN is expressed as bait protein (see *panels a–c* and *h*, *lane 1*). The weak β-galactosidase activity observed in yeast co-transformed with SMN and Gemin6 most likely does not reflect a true interaction, as the latter fusion protein is auto-activating (*panels a–j*, *lane 6* and data not shown). In the same system, we also observed binding of Gemin3 with Gemin4 and Gemin7 with Gemins 6 and 8. Furthermore, we could detect novel protein contacts of Gemins 4–8, and Gemin 2–4 as well as Gemin5 (compare *panels a–i*, *lanes 1–9*). Note that certain interactions such as SMN and Gemin2 (*panel a*, *lane 2*; *panel b*, *lane 1*), Gemins 6 and 7 (*panel f*, *lane 7*), and SMN oligomerization (*panel a*, *lane 1*) can be detected prior to induction, implying very strong binding. Taken together, this strategy supports most data obtained *in vitro* (summarized in Fig. 3*B*). However, binding partners of unrip that could readily be detected *in vitro* were not identified in the yeast system possibly due to misfolding in this host.

A Novel Reconstitution System Reveals the Framework of the SMN Complex—The studies described thus far focused on interactions of single, isolated components. We next developed an experimental strategy to address whether the observed interactions also occur in the context of the SMN complex and how they contribute to its overall architecture. To this end, all proteins of the complex, including HA-tagged SMN were simultaneously translated and radiolabeled in rabbit reticulocyte lysate. Following immunoprecipitation by anti-HA antibodies, the proteins were subjected to SDS-PAGE and autoradiography. Strikingly, this led to an efficient co-precipitation of Gemins 2–4 and 6–8, whereas no proteins were precipitated from a mixture lacking tagged SMN (Fig. 4*A*, *lanes 8* and *9*, the inputs are depicted in *lanes 1* and *2*). Only Gemin5 and unrip are incorporated less efficiently into the complex and therefore co-precipitate weakly. Thus, a significant proportion of the SMN complex forms when all core components are co-translated. This observation is further supported by gradient centrifugation studies shown in Fig. 5. Co-translated SMN complex components sediment very similar to the endogenous complex (Fig. 5*A*, *lanes 12–22* and data not shown), indicating the formation of a stable entity. In contrast, when SMN is omitted from the co-translation reaction the sedimentation behavior of all Gemins is in a lower molecular weight range (compare Fig. 5, *A* and *B*, *lanes 12–22*; note that lower migrating subcomplexes or single translated proteins can be observed in both translations, Fig. 5, *A* and *B*, *lanes 2–11*). Next, co-translation experiments were performed in the absence of selected single components. Consistent with the *in vitro* binding assays shown above all other complex components could still be co-immunoprecipitated with HA-SMN when Gemin2 was omitted from the co-translation (Fig. 4*A*, *lane 10*). Gemin2 is therefore a peripheral protein that is recruited to the complex predominantly via SMN. In contrast, co-translation in the absence of Gemin3 led to the formation of a complex that also lacked Gemin4 (*lane 11*), whereas omission of Gemin4 had no effect on the incorporation of the other proteins into the complex (*lane 12*). These data are consistent with the notion that Gemin3 and 4 interact and associate with the SMN complex predominantly via

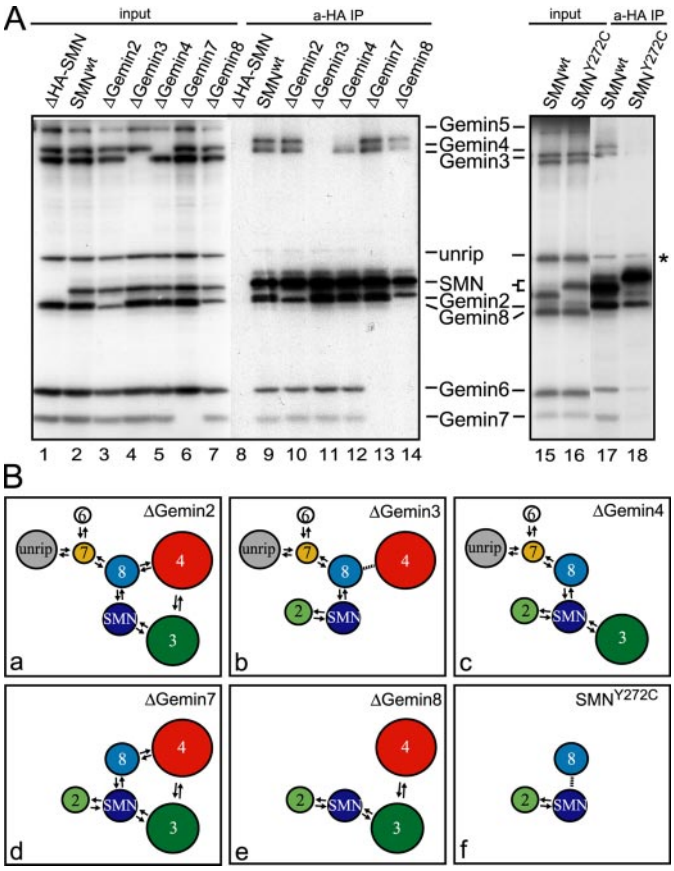

FIGURE 4. ***In vitro* reconstitution of the entire SMN complex in reticulocyte lysate.** *A*, *left panel*: all SMN complex components were ^{35}S-labeled and co-translated as His_6 fusion proteins together with HA-tagged SMN in one TNT reticulocyte lysate reaction as indicated. The resulting SMN complexes were co-precipitated by an α-HA antibody (*lanes 9–14*). As a control all SMN complex components were co-translated without HA-SMN and immunoprecipitated as above (*lane 8*). 10% input is shown (*lanes 1–7*). *A*, *right panel*: all SMN complex components were ^{35}S-labeled and co-translated as His_6 fusion proteins together with HA-tagged SMN^{wt} or SMN^{Y272C} and co-immunoprecipitated (*lanes 17* and *18*). 10% input is shown (*lanes 15* and *16*). An unspecific band is indicated by an *asterisk*. *B*, schematic of all precipitated partial SMN complexes. *Panels a–e*, co-precipitations of reconstituted SMN complexes lacking the indicated components. *Panel f*, co-precipitated reconstituted SMN^{Y272C} complex.

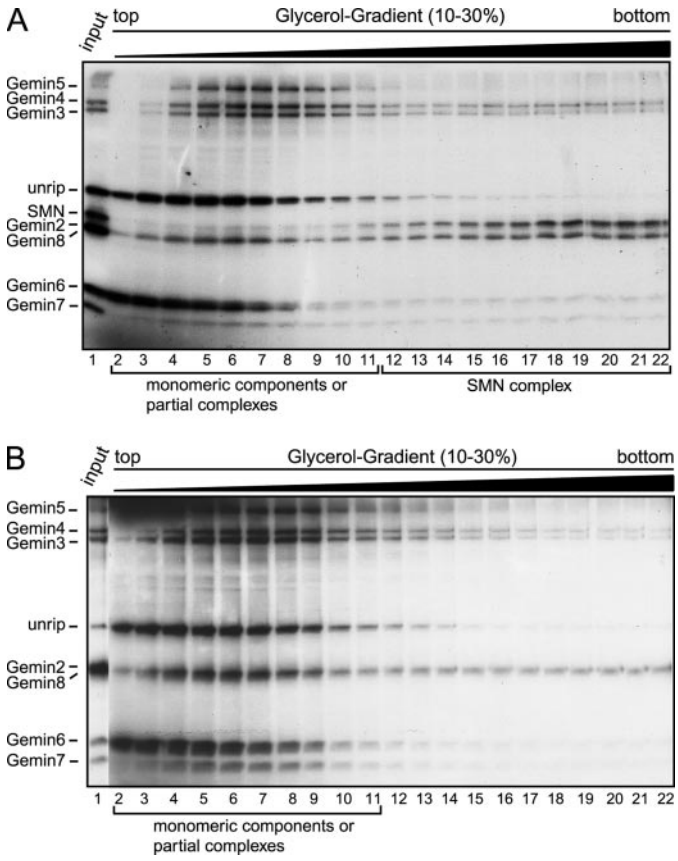

FIGURE 5. **Analysis of the *in vitro* translated SMN complexes by gradient centrifugation.** All SMN complex components were ^{35}S-labeled and co-translated as His_6 fusion proteins together with (*A*) or without (*B*) HA-tagged SMN in the TNT reticulocyte lysate system. The reactions were then layered on 10–30% glycerol gradients. Gradients were harvested manually from top to bottom in 22 fractions. A sample of each fraction was analyzed by 12% SDS-PAGE and autoradiography of the dried gel (*lanes 2–22*). 10% input is shown (*lane 1*).

Gemin3 (22, 23). We note, however, a weak but reproducible binding of Gemin4 to the SMN complex in the absence of Gemin3 (*lane 11* and data not shown). This may indicate that Gemin4 also associates weakly with another protein, presumably Gemin8 as has been observed in the *in vitro* binding experiments and in the yeast interaction assay (Figs. 1 and 3). Co-translation in the absence of Gemin7 strongly affected binding of Gemin6 and unrip (Fig. 4*A*, *lane 13*), and omission of Gemin8 led to the specific loss of Gemins 6 and 7 and unrip association to the complex (*lane 14*). These data in conjunction with those in the preceding sections indicate that Gemin8 constitutes the bridge between the Gemin6/Gemin7/unrip trimer and the rest of the SMN complex. These findings are summarized in Fig. 4*B* (*panels a–e*).

The neuromuscular disorder SMA is caused by reduced levels of or production of mutant SMN protein. It was therefore interesting to analyze whether pathogenic mutations in SMN would interfere with its incorporation into the complex. To address this, we chose the well characterized C-terminal mutation Y272C that has previously been reported to abolish SMN-oligomerization and Sm protein binding (7, 24, 25). Strikingly, when the Y272C mutant was co-translated with all SMN complex components only Gemin2 could efficiently be co-precipitated, whereas binding to all other components was severely impaired (Fig. 4*A*, *lane 18*). Reduced or aberrant formation of the SMN complex may hence be a biochemical defect in patients carrying this and possibly other mutations.

An Essential Role for Gemin5 in the Assembly Reaction—The assembly map established above places Gemin5 in the periphery of the complex with an interaction to Gemin2 (and possibly Gemin4). As Gemin5 appears to be unimportant for the integrity of the remaining SMN complex, we reasoned that it might have a functional role in the U snRNP assembly reaction. To explore this possibility, we devised a strategy to generate SMN complexes lacking Gemin5. This was achieved by treatment of immobilized purified human SMN complex with increasing concentrations of NaCl. Although most components of the complex remain stably associated up to 1.5 M salt, SmB/B′, D3, and Gemin5 dissociate almost quantitatively at 500 mM and 1.5 M NaCl, respectively (see Fig. 6*A*, *left panel* for a silver-stained gel of the purified complex, *right panel* for Western blots of the same samples with various antibodies). Native and Gemin5/

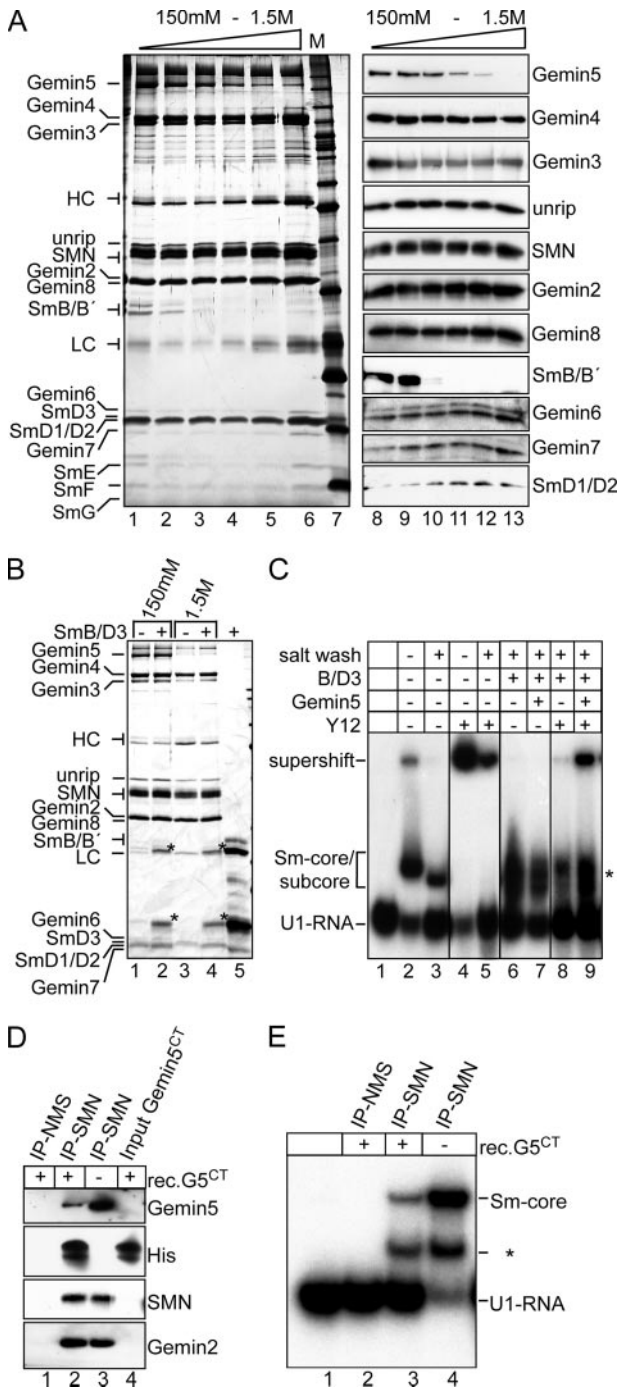

FIGURE 6. **Gemin5 is a peripheral component of the SMN complex but essential for U1 snRNP biogenesis.** *A*, *left panel*: SMN complex was immunoprecipitated from HeLa cytosol. Immobilized complex was washed with buffer containing rising salt concentrations (150 mM, 250 mM, 500 mM, 750 mM, 1 M, and 1.5 M NaCl). Complex was eluted from protein G-Sepharose by SDS-sample buffer, resolved on an 8–20% SDS-polyacrylamide gradient gel, and visualized by silver staining (*lanes 1–6*). *A*, *right panel*: immunoprecipitated complexes from *A* were resolved on an 8–20% gradient gel and analyzed by Western blotting with specific antibodies as indicated. *B*, SMN complex was immunoprecipitated from HeLa cytosol and washed with buffer containing 150 mM NaCl (*lanes 1* and *2*) or 1.5 M NaCl (*lane 3* and *4*). *Lanes 2* and *4* indicate complexes that were reconstituted with purified recombinant B/D3. *Asterisks* indicate bound recombinant SmB/D3. *Lane 5* shows total input of used recombinant B/D3 heterodimer. *C*, Gemin5 is an essential factor in U snRNP biogenesis. Complexes used in *B* were incubated with ^{32}P-labeled U1 snRNA in an assembly assay and analyzed by native gel electrophoresis for Sm core formation (*lanes 2*, *3*, and *6*). *Lane 7* shows the assembly reaction upon addition of 0.75 μg of purified Gemin5. Specificity of Sm core formation was assessed by incubation with Sm-specific antibody Y12 to the assembly reaction (*lanes 4*, *5*, *8*, and *9*). *Lane 1* shows U1 snRNA in the absence of SMN complex. *D*, immunopurified SMN complex was incubated with recombinant His-tagged Gemin5^{CT} (purified as described in supplemental Fig. 2). Mock-treated SMN complex and a normal mouse serum (*NMS*) immunoprecipitation form HeLa cytosolic extract incubated with Gemin5^{CT} served as a control. Western blotting confirmed the incorporation of His-tagged Gemin5^{CT} at the expense of endogenous full-length Gemin5 (compare *lanes 2* and *3*). *Lane 4* shows 10% of histidine-tagged, recombinant Gemin5^{CT}. Indicated proteins were detected with monospecific antibodies after SDS-PAGE and blotting. *E*, complexes used in *D* were incubated with ^{32}P-labeled U1 snRNA in an assembly assay and analyzed by native gel electrophoresis for Sm core formation (*lanes 2–4*). *Lane 1* shows U1 snRNA in the absence of SMN complex.

SmB/D3-depleted SMN complexes were then tested for their ability to promote formation of the Sm core domain on radiolabeled U1 snRNA. Core formation was assessed by native gel electrophoresis. The native SMN complex efficiently promoted the formation of the Sm core domain, as evident by the slower migration of the U1 snRNA in the native gel and further retardation upon addition of the anti-Sm antibody Y12 (Fig. 6*C*, *lanes 2* and *4*). Interestingly, the salt-washed complex lacking Gemin5, B/B′, and D3 also promoted the formation of an RNP that could be super-shifted with antibody Y12 (*lanes 3* and *5*). This Sm-containing RNP most likely corresponds to the subcore particle that has been shown previously to form *in vitro* when isolated Sm proteins D1, D2, E, F, and G (but not B/B′ and D3) are incubated with U1 snRNA (3). Next, we asked whether Sm core domain formation could be restored upon re-addition of the missing Sm proteins B/B′ and D3. Surprisingly, although a recombinant B/D3 heterooligomer was efficiently bound by the salt washed complex (Fig. 6*B*, *lane 4*, indicated by *asterisks*), no Sm core formation could be observed. Note that the retardation of U1 snRNA in the native gel could not be further increased upon addition of Y12 and hence was not caused by Sm proteins (Fig. 6*C*, *lanes 6* and *8*, indicated by an *asterisk*). Thus, an SMN complex containing the complete set of Sm proteins but lacking Gemin5 can neither promote subcore formation nor Sm core assembly. To address the question whether the functionality of this defective SMN complex could be restored upon re-addition of Gemin5, this protein was expressed in insect cells and purified to homogeneity (supplemental figure 3). Strikingly, addition of recombinant Gemin5 to the assembly mixture partially restored the assembly reaction, as evident by the formation of a complex that could be super-shifted with Y12-antibody (Fig. 6*C*, *lanes 7* and *9*). In contrast, upon incorporation of a truncated version consisting of the C-terminal part of the Gemin5 protein, the SMN complex was inactive (Fig. 6 D and E, asterisk indicates unspecific RNA shift). In sum, these data are consistent with the notion that Gemin5 mediates an essential step in the assembly of U snRNPs, most likely in the transfer of Sm proteins onto the U snRNA.

DISCUSSION

The function of the multisubunit SMN complex relies on the ordered interplay of the nine components SMN, Gemins 2–8, and unrip. Previous studies have revealed a great number of interactions among these proteins using various *in vitro* and *in vivo* assays (12–16). However, as most interaction assays are error prone, it is not surprising that conflicting data regarding

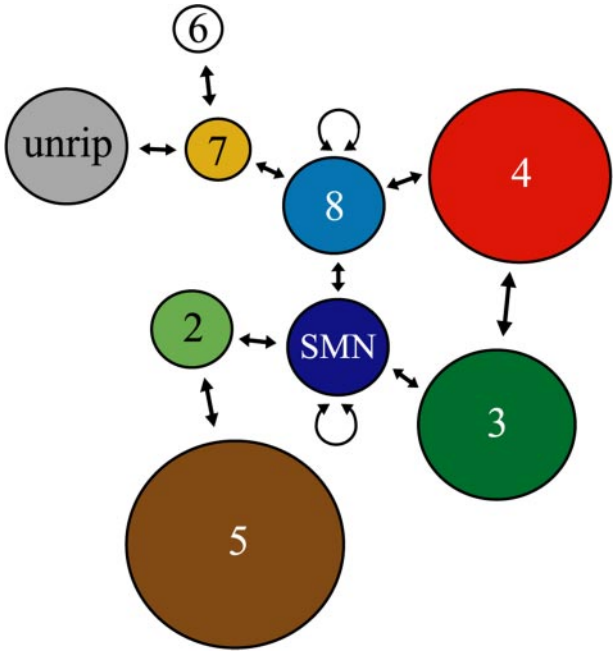

FIGURE 7. **Consensus interaction map of the human SMN complex.** Schematic of all interactions within the SMN complex obtained by the methods described in this work. Only protein-protein interactions that were observed in at least two independent experimental systems were considered.

the interactions within the core SMN complex have been obtained. To circumvent this problem, we have delineated an interaction map that relies on several different and independent interaction assays. Only protein contacts that could be observed in at least two independent experimental systems were considered in the construction of an interaction network. Our studies have revealed a framework of the SMN complex with SMN, Gemin7, and Gemin8 as its backbone. These three proteins provide a binding platform for the other components of the complex via multiple interactions: SMN binds to Gemin2 (26), Gemin3 (27, 28), and Gemin8. Gemin8, in turn, interacts with Gemins 4 and 7. Finally, Gemin7 recruits unrip and Gemin6 via direct interactions (14, 15, 29). Fig. 7 shows a consensus interaction map derived from published data (summarized in Refs. 12 and 13) and from results obtained in this report. Whereas most components of the SMN complex form a very stable structure, the peripheral protein Gemin5 can be dissociated upon treatment with high salt. This finding suggests its incorporation into the complex via weak interactions mediated by Gemin2 and possibly Gemin4. Based on data presented here and in previous reports, we are now confident that we understand the architecture of the SMN complex. Nevertheless, we cannot exclude the possibility that some protein-protein contacts might have escaped our detection or were erroneously excluded based on the stringent criteria described above. In addition, with the availability of the experimental strategies detailed above, we are now in a position to map the binding sites of other peripheral components of the SMN complex. These include substrate Sm/LSm proteins (12, 13), the nuclear import factors importin β and snurportin (30, 31), and coilin, a binding partner of SMN in nuclear coiled bodies (32). These studies will provide additional mechanistic insights into the U snRNP biogenesis cycle.

Most cases of SMA are caused by the reduced production of SMN protein. However, some cases of disease are also known, where patients express mutant versions of the protein. The majority of pathogenic mutations (deletions and missense mutations) cluster in the C-terminal domain responsible for SMN oligomerization and/or interaction with Sm proteins (7, 24, 25). Here, we provide evidence that the pathogenic SMN mutation Y272C is incorporated inefficiently into the SMN complex. This effect might be due to the inability of SMN to form oligomers and/or the disability to bind other proteins such as Gemin8, which contacts amino acids 242–294 of SMN, where the missense mutation Y272C is located (see supplemental Fig. 1*B*, *lanes 3* and *6*). Future studies based on the novel reconstitution assay reported here will be required to resolve the issue whether other pathogenic SMN mutations result in the reduced formation of functional SMN complexes and if this is the underlying cause for the development of the disease SMA in patients.

Conflicting data regarding the function of Gemin5 have recently been reported (33–35). In two reports RNA interference was used to show that Gemin5 is dispensable for the assembly reaction (33, 34). In contrast, evidence for an essential role of Gemin5 in the formation of U snRNPs has been provided in another recent report (35). In this latter study, Gemin5 was shown to bind snRNAs in a sequence specific manner, suggesting its role in the recruitment of these to the SMN complex. Our finding that an SMN complex lacking Gemin5 fails to promote U snRNP assembly is consistent with this notion. However, we do not rule out additional functions of Gemin5 in the assembly reaction such as transfer of Sm proteins onto the U snRNA. The availability of Gemin5 expressed in insect cells will allow us to address these questions.

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