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Gemin8 Is a Novel Component of the Survival Motor Neuron Complex and Functions in Small Nuclear Ribonucleoprotein Assembly*[S]

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The survival motor neuron (SMN) protein is the product of the spinal muscular atrophy disease gene. SMN and Gemin2–7 proteins form a large macromolecular complex that localizes in the cytoplasm as well as in the nucleoplasm and in nuclear Gems. The SMN complex interacts with several additional proteins and likely functions in multiple cellular pathways. In the cytoplasm, a subset of SMN complexes containing unrip and Sm proteins mediates the assembly of spliceosomal small nuclear ribonucleoproteins (snRNPs). Here, by mass spectrometry analysis of SMN complexes purified from HeLa cells, we identified a novel protein that is evolutionarily conserved in metazoans, and we named it Gemin8. Co-immunoprecipitation and immunolocalization experiments demonstrated that Gemin8 is associated with the SMN complex and is localized in the cytoplasm and in the nucleus, where it is highly concentrated in Gems. Gemin8 interacts directly with the Gemin6-Gemin7 heterodimer and, together with unrip, these proteins form a heteromeric subunit of the SMN complex. Gemin8 is also associated with Sm proteins, and Gemin8-containing SMN complexes are competent to carry out snRNP assembly. Importantly, RNA interference experiments indicate that Gemin8 knock-down impairs snRNP assembly, and Gemin8 expression is down-regulated in cells with low levels of SMN. These results demonstrate that Gemin8 is a novel integral component of the SMN complex and extend the repertoire of cellular proteins involved in the pathway of snRNP biogenesis.

Eukaryotic genes are transcribed as long precursors that need to undergo post-transcriptional processing to produce functional, protein-coding mRNAs. The spliceosome, a large dynamic macromolecular assembly of hundreds of proteins and a few RNAs, is responsible for the proper excision of introns and ligation of exons during pre-mRNA splicing (1). Spliceosomal small nuclear ribonucleoproteins (snRNPs)[2] are a class of abundant cellular particles and the essential components of the spliceosome. Major snRNPs are composed of an snRNA molecule (U1, U2, U4/U6, and U5) and a set of common (Sm proteins) and snRNP-specific proteins (2). The hallmark of snRNPs is the presence of a heptameric ring of Sm proteins, known as the Sm core, around a conserved sequence called the Sm site (3). Although snRNPs function in the nucleus, the biogenesis of spliceosomal snRNPs in higher eukaryotes follows a complex pathway that requires the functions of many cellular proteins and includes nuclear as well as cytoplasmic phases (2).

The survival motor neuron (SMN) protein has emerged in recent years as a key player in the biogenesis of snRNPs (4, 5). Understanding the molecular function(s) of SMN also bears direct relevance to human disease because reduced levels of SMN expression, due to homozygous mutations or deletions of the *SMN1* gene, cause the fatal neurodegenerative disease spinal muscular atrophy (SMA) (6). SMN localizes in the cytoplasm, in the nucleoplasm, and is highly concentrated in Gems, nuclear structures that are often associated with Cajal bodies (7). At least six additional proteins (Gemin2–7) are stably associated with SMN in large macromolecular complexes and display a similar subcellular distribution, including co-localization with SMN in Gems (8–13). Therefore, Gemin2–7 proteins are considered as integral components of the SMN complex. The SMN complex associates transiently with several additional proteins and RNAs and is thought to function in multiple cellular pathways, which are often related to RNA metabolism (4, 14).

The best characterized function of the SMN complex is the assembly of the heptameric core of Sm proteins on spliceosomal snRNAs (4, 5). Newly translated Sm proteins bind first to pICln and the PRMT5 complex, which carries out the symmetrical dimethylation of specific arginine residues of a subset of Sm proteins, and then to the SMN complex (15–17). The SMN complex bound to unrip and the seven Sm proteins is the macromolecular machine poised for snRNP assembly in the cytoplasm (18–22). Through an ordered series of events, this SMN complex interacts directly with newly exported snRNAs, which are transcribed as precursors with short extensions at the 3′-end by RNA polymerase II in the nucleus, and mediates the ATP-dependent assembly of the Sm core (21, 22). Additional cytoplasmic steps of snRNP biogenesis are the hypermethylation of the m7G-cap structure at the 5′-end of snRNAs, trimming of the 3′-end extensions, and binding of nuclear import factors (2). The SMN complex remains associated with snRNPs until their import back into the nucleus and likely plays additional roles in snRNP biogenesis beyond formation of the Sm core (23, 24). Indeed, the association of the SMN complex with the Sm core is important for nuclear import of snRNPs (25). Once in the nucleus and before functioning in pre-mRNA splicing, snRNPs undergo further steps of maturation,

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[S] The on-line version of this article (available at http://www.jbc.org) contains Figs. S1 and S2.

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[2] The abbreviations used are: snRNP, small nuclear ribonucleoprotein; SMN, survival motor neuron; SMA, spinal muscular atrophy; RNAi, RNA interference; siRNA, small interfering RNA; PBS, phosphate buffered saline; GST, glutathione *S*-transferase.

including the association with snRNP-specific proteins as well as methylation and pseudouridylation of specific nucleotides (26).

Several individual components of the SMN complex interact with specific subsets of Sm proteins and collectively contribute to the association of Sm proteins with the SMN complex (4, 14). The SMN complex also interacts directly with specific domains of snRNAs, and this interaction is important for the specificity of snRNP assembly (5, 21). This mode of action of the SMN complex applies to the formation of Sm cores on snRNAs of the major (U1, U2, U4, and U5) and of the minor (U11 and U12) spliceosomes as well as on viral small RNAs encoded by the lymphotropic herpesvirus saimiri (21, 27–29). The SMN complex also assembles U7 snRNP, which is required for 3′-end processing of histone pre-mRNAs and whose Sm core contains a unique combination Sm and Lsm proteins (30). Hence, the SMN complex is the general macromolecular assembly machine employed by cells to assemble the Sm core.

As part of our efforts to characterize the protein composition of SMN complexes, we have identified by peptide microsequencing by mass spectrometry a novel protein that we term Gemin8. Here we report the characterization of Gemin8 as a novel integral component of the SMN complex that localizes to nuclear Gems, interacts with the Gemin6-Gemin7 heterodimer, and functions in snRNP biogenesis.

EXPERIMENTAL PROCEDURES

DNA Constructs—The open reading frame of Gemin8 was generated by PCR amplification from the Ultimate open reading frame clone IOH3877 (Invitrogen) with appropriate primers and was cloned into the EcoRI and XhoI sites of pet28, pGEX-5X, and pcDNA3 vectors downstream of the sequences encoding for the GST, His_6, and FLAG epitopes, respectively. For *in vitro* translation experiments, the Gemin8 cDNA was also cloned without any tags into a pcDNA3 vector. A 6xHis-Gemin8 construct was generated by cloning into a pDEST 17 vector using Gateway technology (Invitrogen). All constructs were analyzed by automated DNA sequencing. Plasmids encoding for epitope-tagged Gemin2, Gemin6, Gemin7, and unrip were described previously (12, 13, 18).

Antibodies—For production of monoclonal antibodies, BALB/c females were primed with ImmunEasy adjuvant (Qiagen) and 25 μg of either GST-unrip or 6xHis-Gemin8 purified recombinant proteins. Following two boosts at 2-week intervals, SP2 myeloma cells were fused with mouse splenocytes, and hybridoma supernatants were analyzed onto antigen-coated aminosilane-modified slides using an LS400 scanner (Tecan) and the GenePix Pro 4.1 software as described previously (31). The antibodies used are as follows: anti-Gemin8 1F8 (this work); anti-unrip 3G6 (this work); anti-SMN clone 8 (BD Transduction Laboratories); anti-SMN 7F3 (18); anti-Gemin2 2E17 (8); anti-Gemin3 12H12 (9); anti-Gemin4 17D10 (10); anti-Gemin5 10G11 (11); anti-Gemin6 rG6 (27); anti-hnRNP A1 4B10 (32); anti-Sm Y12 (Lab Vision Corp.); anti-pICln clone 32 (BD Transduction Laboratories); anti-coilin clone 56 (BD Transduction Laboratories); anti-H1 and core histones (Chemicon); anti-tubulin (Sigma); anti-FLAG M2 (Sigma); anti-T7 tag (Novagen); and purified mouse IgG immunoglobulins (Sigma).

Protein Sequencing by Mass Spectrometry—Bands were excised from silver-stained polyacrylamide gels and in-gel digested with trypsin. Tryptic peptides were analyzed by nano-electrospray tandem mass spectrometry as described previously (33).

Cell Culture—HeLa and 293T cells were cultured in Dulbecco's modified Eagle's medium with high glucose (BioWhittaker) containing 10% fetal bovine serum and penicillin/streptomycin. 293T cells were transfected using the calcium phosphate method and manufacturer's instructions (Clontech). Following overnight incubation with DNA, medium was replaced, and cells were harvested after 1 additional day in culture.

Immunofluorescence Analysis—HeLa PV cells grown on 35-mm tissue culture plates were washed briefly with phosphate-buffered saline, fixed in 50% methanol, 50% acetone for 5 min at −20 °C, and air-dried for 30 min. Cells were then blocked with 3% bovine serum albumin in phosphate-buffered saline for 1 h at room temperature. Single- or double-label immunofluorescence experiments were carried out by separate sequential incubations of each primary antibody followed by the specific fluorescently labeled secondary antibody as described previously (12). Indirect epifluorescence microscopy was performed with an Olympus AX70 microscope equipped with a SPOT digital camera (Diagnostic Instruments).

Protein Production and in Vitro Binding Experiments—*In vitro* translated proteins were produced in the presence of [^{35}S]methionine (Amersham Biosciences) using a coupled transcription-translation system according to the manufacturer's protocol (Promega). All His_6- or GST-tagged recombinant proteins were expressed in *Escherichia coli* BL21(DE)pLysE cells (Invitrogen) and purified by affinity chromatography on nickel-chelated agarose (Pierce) or glutathione-Sepharose (Amersham Biosciences), respectively. GST-TEV-Gemin6 and 6xHis-Gemin7 (or GST-TEV-Gemin7 and 6xHis-Gemin6) proteins were co-expressed and purified as described previously (13, 34). Gemin6-Gemin7 heterodimers were purified by a tandem affinity purification strategy. Briefly, following cleavage of GST-TEV-Gemin6/6xHis-Gemin7 complexes bound to glutathione-Sepharose beads with recombinant AcTEV protease (Invitrogen) according to the manufacturer's instructions, Gemin6/6xHis-Gemin7 heterodimers were further purified by affinity chromatography on nickel-chelated agarose (Pierce). For *in vitro* binding experiments, 2 μg of GST or GST-tagged proteins bound to glutathione-Sepharose beads were incubated either with *in vitro* translated [^{35}S]methionine-labeled proteins or with 1 μg of purified recombinant proteins in binding buffer (200 mM NaCl, 50 mM Tris-HCl, pH 7.4, 2 mM EDTA) containing 0.1% Nonidet P-40 and EDTA-free protease inhibitor mixture (Roche Applied Science) for 2 h at 4 °C. Following extensive washing with the same buffer, bound proteins were eluted by boiling in SDS-PAGE sample buffer and were analyzed by SDS-PAGE on 12% polyacrylamide gels.

Extract Preparation, Sucrose Gradient Centrifugation, and Immunoprecipitation Experiments—Mouse tissues from C57BL/6 embryos at stage 18 were collected by manual dissection, quickly frozen in liquid nitrogen, and stored at −80 °C until further processing. Whole tissue extracts for Western blot analysis were prepared by homogenization of tissues in SDS-PAGE sample buffer, followed by sonication, boiling, and centrifugation for 15 min at 10,000 rpm at room temperature. For total cell extract preparation, HeLa or 293T cells were resuspended in RSB-100 buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 2.5 mM $MgCl_2$) containing 0.1% Nonidet P-40, EDTA-free protease inhibitor mixture (Roche Applied Science), and phosphatase inhibitors (20 mM NaF, 0.2 mM Na_3VO_4), briefly sonicated three times on ice, and centrifuged for 15 min at 10,000 rpm at 4 °C. For sucrose gradient centrifugation experiments, HeLa total cell extracts were fractionated on 10 ml of 10–30% sucrose gradients in RSB-100 buffer by centrifugation for 4 h at 38,000 rpm in an SW 41 rotor at 4 °C. Immunoprecipitation experiments were carried out from HeLa or 293T total cell extracts using either antibodies bound to protein G-Sepharose (Sigma) or FLAG M2-agarose (Sigma) in RSB-100 buffer containing 0.1% Nonidet P-40 for 2 h at 4 °C. Following five washes with the same buffer, bound proteins were eluted by boiling in SDS-PAGE sample buffer or analyzed by snRNP assembly. Immunoprecipitations of snRNP assembly reactions with anti-Sm (Y12) anti-

bodies bound to protein G-Sepharose were carried out for 2 h at 4 °C in RSB-500 buffer (500 mM NaCl, 10 mM Tris-HCl, pH 7.4, 2.5 mM $MgCl_2$) containing 0.1% Nonidet P-40 and protease inhibitors. Following five washes with the same buffer, bound RNAs were recovered from immunoprecipitates by proteinase K treatment, phenol/chloroform extraction, and ethanol precipitation. RNAs were then analyzed by electrophoresis on denaturing polyacrylamide gels and autoradiography.

Purification of SMN Complexes—Parental HeLa Tet-On cells (Clontech) constitutively expressing a tetracycline-regulated transcriptional transactivator and the HeLa Tet-On stable cell line containing FLAG-Gemin2 under the control of a tetracycline-inducible promoter were grown in the presence of doxycycline (5 μg/ml) to induce FLAG-Gemin2 expression (12). Native SMN complexes were affinity-purified using the high salt-wash procedure established previously and described below (12). Cells were resuspended in ice-cold RSB-100 buffer containing 0.1% Nonidet P-40 and protease and phosphatase inhibitors, passed five times through a 27-gauge needle, and sonicated briefly. After centrifugation for 15 min at 10,000 rpm at 4 °C, extracts were passed through a 0.2-μm filter and incubated with anti-FLAG M2-agarose beads (Sigma) for 2 h at 4 °C. Beads were first extensively washed with RSB-100 containing 0.1% Nonidet P-40 and then three times with 10 bead volumes of RSB-500 containing 0.02% Nonidet P-40 for 15 min at 4 °C. Native SMN complexes were eluted under mild conditions by competitive displacement of FLAG-Gemin2 with 0.5 mg/ml of 3X-FLAG peptide (Sigma) in RSB-100 buffer containing 0.02% Nonidet P-40 for 1 h at 4 °C.

RNA Interference—For silencing experiments, HeLa cells were transfected with 21-nucleotide-long small interfering RNA duplexes (siRNAs) using Lipofectamine 2000™ (Invitrogen) according to the manufacturer's instructions and harvested 72 h after transfection. Preliminary time course analysis showed that the greatest reduction in the levels of Gemin8 is observed at 72 h after transfection, and this time point was consequently used in the experiments (data not shown). Furthermore, indirect immunofluorescence experiments indicated that over 90% of cells were transfected with siRNAs and displayed reduced expression of the target proteins (data not shown). The following siRNAs (sense strand), selected using the BLOCK-iT™ RNAi Designer, were synthesized, purified, and annealed with the corresponding antisense RNA oligonucleotides by Invitrogen: Gemin8a (5′-GCAAGAUACUGGCAACAUUdTdT-3′) and Gemin8b (5′-GCAAUGGCUUGGAUGCAAAdTdT-3′) against Gemin8; (5′-GAAGAAUACUGCAGCUUCCdTdT-3′) against SMN (35); and (5′-GCUGGAGAGCAACUGCAUAdTdT-3′) against firefly luciferase as a control.

In Vitro snRNP Assembly—U1 and U1ΔSm snRNAs were transcribed *in vitro* from linearized template DNAs in the presence of [α-^{32}P]UTP (3000 Ci/mmol) and purified from denaturing polyacrylamide gels according to standard procedures. Extracts for snRNP assembly were prepared as described previously with minor modifications (21). HeLa cells were resuspended in ice-cold reconstitution buffer (20 mM Hepes-KOH, pH 7.9, 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 5% glycerol) containing 0.01% Nonidet P-40, passed five times through a 25-gauge needle, and then centrifuged at 10,000 rpm for 15 min at 4 °C. Supernatants were collected and either used directly or stored in frozen aliquots at −80 °C. For snRNP assembly experiments, beads-bound protein complexes were immunopurified from 293T total cell extracts using FLAG M2-agarose (Sigma) as described above and washed for five additional times with ice-cold reconstitution buffer containing 0.01% Nonidet P-40. snRNP assembly reactions with HeLa cell extracts or beads-bound protein complexes were carried out for 1 h at 30 °C in a volume of 20 μl of reconstitution buffer containing 0.01% Nonidet P-40, 10,000 cpm of *in vitro* transcribed [α-^{32}P]UTP-labeled U1 snRNA, 2.5 mM ATP, and 10 μM *E. coli* tRNA. Reactions were then analyzed by immunoprecipitation experiments or by electrophoresis on 6% polyacrylamide native gels at 4 °C and autoradiography as described previously (18).

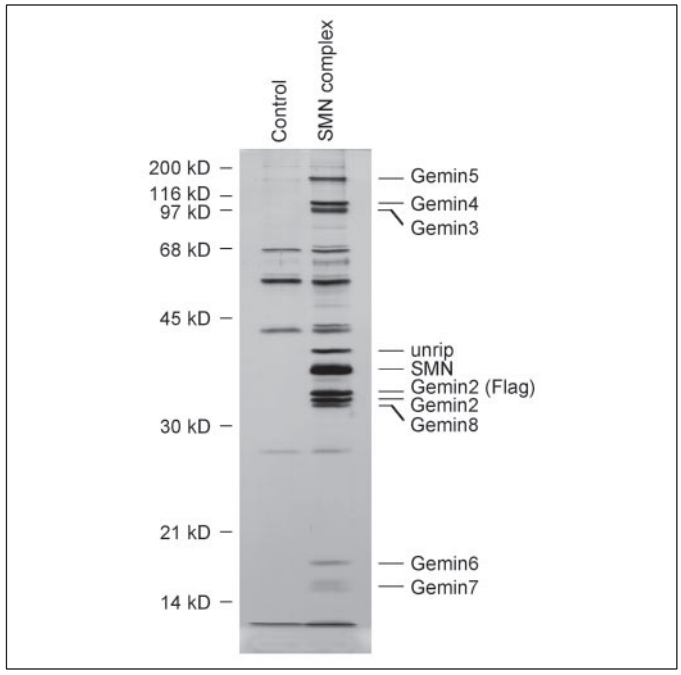

FIGURE 1. **Protein composition of native SMN complexes.** SDS-PAGE and silver staining analysis of SMN complexes purified with anti-FLAG antibodies from HeLa Tet-On cells that stably express FLAG-tagged Gemin2 (SMN complex) and of nonspecific proteins from parental HeLa Tet-On cells (Control). Molecular weight standards and the known protein components of the SMN complex are indicated.

RESULTS AND DISCUSSION

Identification of Gemin8 by Mass Spectrometry—We purified native SMN complexes from a stable HeLa cell line that expresses FLAG-tagged Gemin2 using affinity chromatography on anti-FLAG-agarose beads and the high salt-wash procedure established previously (12). A parallel purification was carried out from the parental HeLa cell line as a control. Bound proteins were eluted from beads by competition with a molar excess of FLAG peptides and analyzed by SDS-PAGE and silver staining (Fig. 1). In addition to the known integral components of the SMN complex, peptide microsequencing by mass spectrometry revealed three distinct peptides whose amino acid sequences perfectly match several expressed sequences in the data base and belong to a novel 32-kDa protein, which migrates close to Gemin2 and which we term Gemin8 (Fig. 1 and supplemental Fig. S1). Gemin8 cDNA contains both start and stop codons as well as an in-frame stop codon upstream of the initial methionine, indicating that the open reading frame is complete (supplemental Fig. S1). Gemin8 cDNA encodes for a protein of 242 amino acids with a predicted molecular mass of 28.6 kDa and an isoelectric point of 6.8. Protein analyses *in silico* using various web-based search engines indicated that Gemin8 neither contains identifiable protein motifs nor shares significant homology with other proteins that may hint to a possible function. We then performed BLAST search of the data base and identified several putative orthologues of human Gemin8 in diverse organisms. Fig. 2 shows an alignment of the amino acid sequences of Gemin8 from *Homo sapiens*, *Canis familiaris* (73.2% identity, 79.7% similarity), *Rattus norvegicus* (66.5% identity, 74.2% similarity), *Mus musculus* (66% identity, 72.9% similarity), *Bos taurus* (58.9% identity, 62.1% similarity), *Xenopus laevis* (50% identity, 54.5%

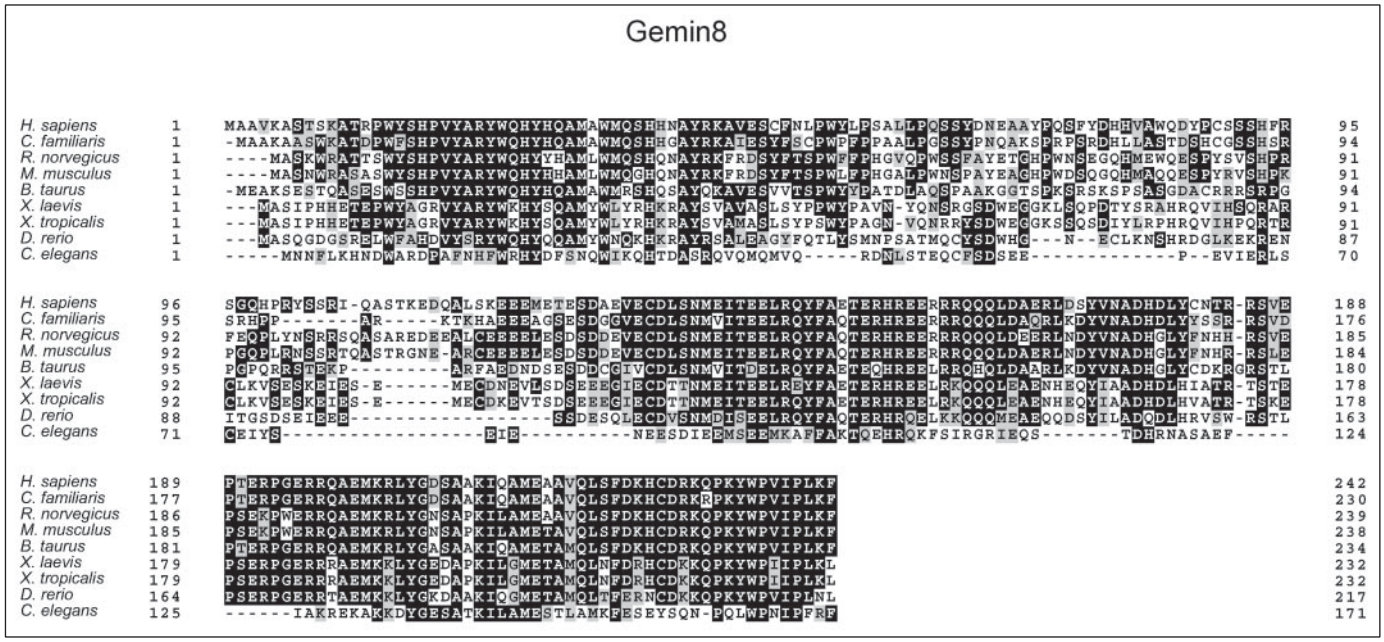

FIGURE 2. **Alignment of Gemin8 protein sequences.** The amino acid sequences of human Gemin8 (GenBank™ accession number DQ224033) and putative orthologues of *C. familiaris* (GenBank™ accession number XM_537960), *R. norvegicus* (GenBank™ accession number BC079160), *M. musculus* (GenBank™ accession number NM_146238), *B. taurus* (GenBank™ accession number XM_594084), *X. laevis* (GenBank™ accession number BC056116), *X. tropicalis* (GenBank™ accession number CR762163), *D. rerio* (GenBank™ accession number BC085647), and *C. elegans* (GenBank™ accession number AAR25647.1) were aligned using ClustalW and BOXSHADE 3.21. *Black boxes* highlight identical amino acids; *gray boxes* highlight similar amino acids.

similarity), *Xenopus tropicalis* (47.6% identity, 51.7% similarity), *Danio rerio* (45.5% identity, 54.1% similarity), and *Caenorhabditis elegans* (26.6% identity, 30% similarity). The high degree of evolutionary conservation suggests that Gemin8 may perform important cellular functions. However, we did not identify Gemin8 orthologues in organisms such as *Drosophila melanogaster* or *Schizosaccharomyces pombe* that contain SMN and whose genomes have been entirely sequenced. This situation is also observed for several other Gemin proteins and may indicate either lack of Gemin8 in these organisms or, as is the case for Gemin2 (36), a low degree of amino acid sequence conservation because of significant evolutionary divergence. Most interestingly, the Gemin8 gene is located on human chromosome X at p22.2, and sporadic cases of several neurological disorders including X-linked recessive Charcot-Marie-Tooth neuropathy and X-linked mental retardations have been associated with this region (37–39). It will be interesting to investigate the potential involvement of Gemin8 dysfunctions in the pathophysiology of these diseases.

Gemin8 Is a Component of the SMN Complex—The presence of Gemin8 in SMN complexes purified under highly stringent conditions suggests that it may represent a novel integral component of the SMN complex. To address this possibility directly, we transiently transfected 293T cells either with a vector expressing FLAG-tagged Gemin8 or with an empty vector as a control. Total extracts from these cells were then analyzed by immunoprecipitation experiments with anti-FLAG antibodies and Western blot. FLAG-Gemin8 is associated specifically with SMN, Gemin2–6, and unrip proteins but not with the abundant hnRNP A1 protein, and none of the SMN complex components are present in the control immunoprecipitates from extracts that do not express FLAG-Gemin8 (Fig. 3*A*). These results identify Gemin8 as a *bona fide* component of the SMN complex.

To demonstrate the association of endogenous Gemin8 with the SMN complex, we generated a monoclonal antibody against Gemin8. Supplemental Fig. S2A shows that the monoclonal antibody 1F8 recognizes recombinant Gemin8 and a protein of the expected molecular weight in HeLa total cell extracts by Western blot. We then carried out immunoprecipitation experiments from HeLa cell extracts with antibodies against Gemin8, SMN, Sm proteins, and pICln as well as mouse immunoglobulins as a control. Western blot analysis of the immunoprecipitates demonstrates the specific association of Gemin8 with SMN, unrip, and Sm proteins but not with pICln (Fig. 3*B*). Although Gemin8 is associated with Sm proteins *in vivo*, we did not find evidence that Gemin8 interacts directly with Sm proteins *in vitro* (data not shown), suggesting that their association may be mediated by the other components of the SMN complex.

We further investigated the association of Gemin8 and SMN by sucrose gradient centrifugation of HeLa cell extracts and co-immunoprecipitation experiments. Gemin8 has a distribution very similar to that of SMN and is part of large heterodisperse macromolecular complexes ranging from 20 S to 80 S (Fig. 3*C*). In contrast, most unrip is found at the top of the gradient, and only a small proportion co-fractionates with SMN and Gemin8 (Fig. 3*C*) (18). Consistent with its presence in the PRMT5 complex (15, 17), pICln is found in complexes up to 20 S. More importantly, immunoprecipitation from the sucrose gradient fractions with anti-SMN antibodies demonstrates that Gemin8 is associated with the entire spectrum of SMN complexes (Fig. 3*D*). Collectively, these results demonstrate that Gemin8 is a novel integral component of cellular SMN complexes.

Gemin8 and Unrip Proteins Are Ubiquitously Expressed—We sought to investigate the expression of Gemin8 and unrip in different tissues of mouse embryos. To do so, we carried out Western blot analysis of whole tissue extracts from spinal cord, brain, kidney, skeletal muscle, and heart of embryos at stage 18 and found that both Gemin8 and unrip proteins are expressed in all tissues (Fig. 4). When total protein extracts from these embryonic tissues are normalized per cell number as indicated by similar amounts of core histone proteins, SMN and unrip expression levels are approximately equal, whereas slightly reduced levels of Gemin8 are found in skeletal muscle and heart. These results indicate

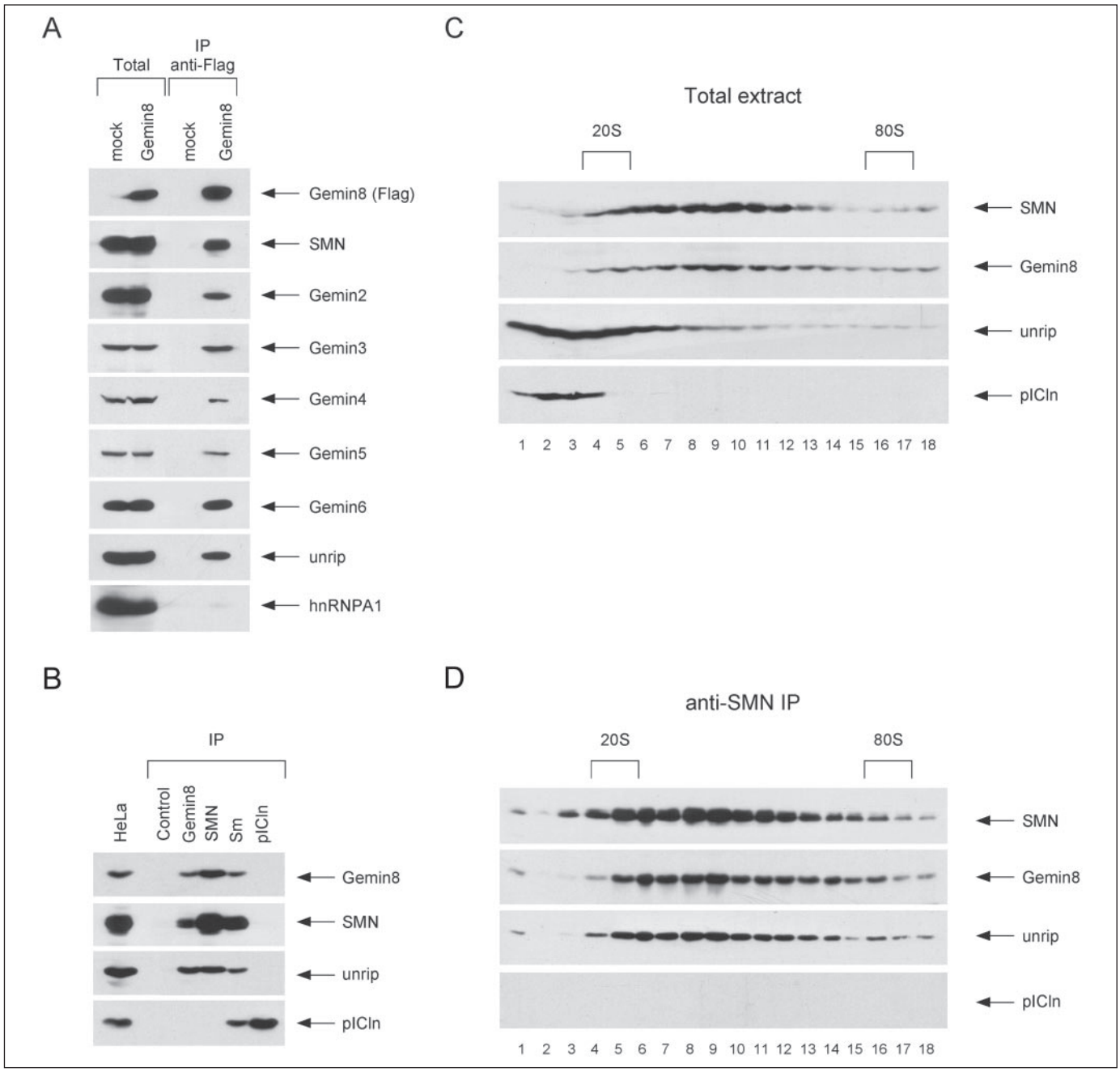

FIGURE 3. **Gemin8 is a component of the SMN complex.** *A*, total cell extracts from 293T cells transiently transfected with either a pcDNA3 vector encoding FLAG-Gemin8 (*Gemin8*) or an empty pcDNA3 vector (*mock*) were immunoprecipitated with anti-FLAG antibodies. 5% of the input (*Total*) and the immunoprecipitates (*IP anti-FLAG*) were analyzed by SDS-PAGE and Western blot. *B*, total cell extracts from HeLa cells were immunoprecipitated with anti-Gemin8 (1F8), anti-SMN (7F3), anti-Sm (Y12), and anti-pICln antibodies or mouse IgG immunoglobulins (*Control*). 5% of the input (*HeLa*) and the immunoprecipitates (*IP*) were analyzed by SDS-PAGE and Western blot. *C*, total cell extracts from HeLa cells were analyzed by centrifugation on a 10–30% sucrose gradient and Western blot. *D*, fractions in *C* were analyzed by immunoprecipitation with anti-SMN (7F3) antibodies and Western blot. Sedimentation (*S*) values are indicated. Western blot analyses were carried out with antibodies against the proteins indicated on the *right*.

that, similar to SMN, Gemin8 and unrip proteins are expressed ubiquitously.

Gemin8 Co-localizes with SMN and Is a Component of Nuclear Gems—Next, we analyzed the subcellular localization of Gemin8 in HeLa cells by indirect immunofluorescence experiments using anti-Gemin8 antibodies. Gemin8 localizes both in the cytoplasm and in the nucleus, where it is highly concentrated in distinct nuclear foci (Fig. 5, *A–C*). A similar subcellular localization was also observed by immunofluorescence analysis of epitope-tagged Gemin8 transiently transfected in HeLa cells (data not shown). In addition to their stable association, the hallmark of the integral components of the SMN complex is their localization in nuclear structures known as Gems (7–13). Gems were initially discovered as twin nuclear structures of Cajal bodies that are very prominent in HeLa PV cells and can be found either separate or more often co-localized with Cajal bodies (7). Cajal bodies are subnuclear domains containing high local concentrations of several components of the transcription and RNA-processing machineries, including small nuclear and nucleolar ribonucleoproteins, and may have a role in some aspects of their assembly or maturation (40). The presence of Gems and the degree of their co-localization with Cajal bodies vary in different cells as well as during development and, at least in part, are linked to the meth-

ylation state of coilin (41–43). To determine whether Gemin8 foci correspond to Gems or Cajal bodies, we carried out co-localization studies by double-label immunofluorescence experiments with antibodies against Gemin8 and either SMN or coilin, the respective markers of Gems and Cajal bodies. Fig. 5, *D–F*, shows that the nuclear foci containing Gemin8 and SMN overlap completely in HeLa PV cells. In contrast, Fig. 5, *G–I*, indicates that Gemin8 nuclear foci can be found either separate from, adjacent to, or coincident with Cajal bodies, which are identified by coilin staining. The localization of Gemin8 in nuclear foci that contain SMN but are distinct from Cajal bodies is a feature also shared by Gemin2–7 proteins and identifies Gemin8 as a novel protein component of nuclear Gems.

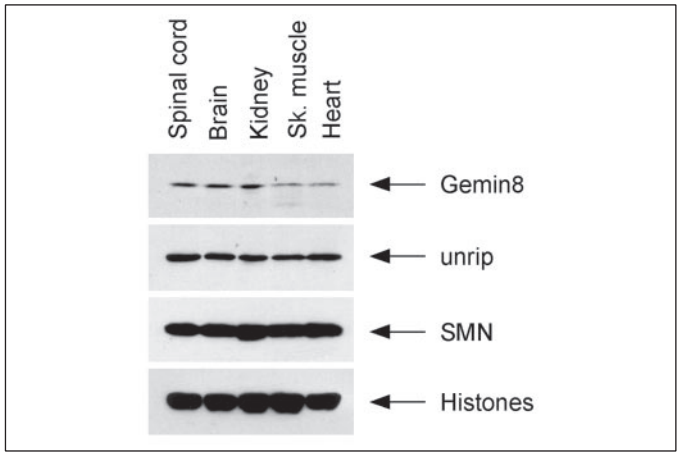

FIGURE 4. **Expression of Gemin8 and unrip proteins in mouse embryonic tissues.** Total proteins from spinal cord, brain, kidney, skeletal muscle, and heart tissues isolated from mouse embryos at stage 18 were analyzed by Western blot with anti-Gemin8, anti-unrip, anti-SMN, and anti-histones antibodies.

Gemin8 Interacts with the Gemin6-Gemin7 Heterodimer—We sought to identify the interaction(s) responsible for Gemin8 association with the SMN complex using *in vitro* binding experiments. To do so, *in vitro* translated [^{35}S]methionine-labeled Gemin8 was incubated with several GST-tagged components of the SMN complex as well as with GST as a control. Gemin6-Gemin7 complexes were co-expressed and purified as described previously (13, 34). Fig. 6*A* shows that Gemin8 interacts very weakly with Gemin7 and efficiently with the Gemin6-Gemin7 complex. We did not detect significant binding of Gemin8 to the other components of the SMN complex (data not shown). More importantly, Gemin8 binds to the Gemin6-Gemin7 complex with high affinity regardless of the presence of the GST on either protein, indicating that the GST tag does not likely interfere with the interactions of Gemin6 or Gemin7 alone. To exclude the possibility that Gemin8 interaction was mediated by components present in the reticulocyte lysate, we carried out direct binding experiments using purified recombinant proteins and Western blot analysis. Gemin8 binds directly and efficiently to the Gemin6-Gemin7 complex, whereas little, if any, binding to the individual proteins or to GST was observed (Fig. 6*B*). Moreover, recombinant Gemin6-Gemin7 heterodimers, isolated using a tandem purification strategy similar to that described previously (34), bind strongly to GST-tagged Gemin8 immobilized on beads but not GST (Fig. 6*C*). Altogether these results indicate that Gemin8 binds directly and with very high affinity to the Gemin6-Gemin7 heterodimer.

In contrast to Gemin8, the unrip protein binds very strongly to both Gemin7 and the Gemin6-Gemin7 complex and weakly to Gemin6 (Fig. 6*B*) (18, 19). We then investigated whether the interactions of Gemin8 and unrip proteins with the Gemin6-Gemin7 heterodimer were mutually exclusive or whether they can form heteromeric complexes. To do so, GST and GST-Gemin8 were incubated in the presence of excess of purified recombinant Gemin6-Gemin7 heterodimers or buffer alone.

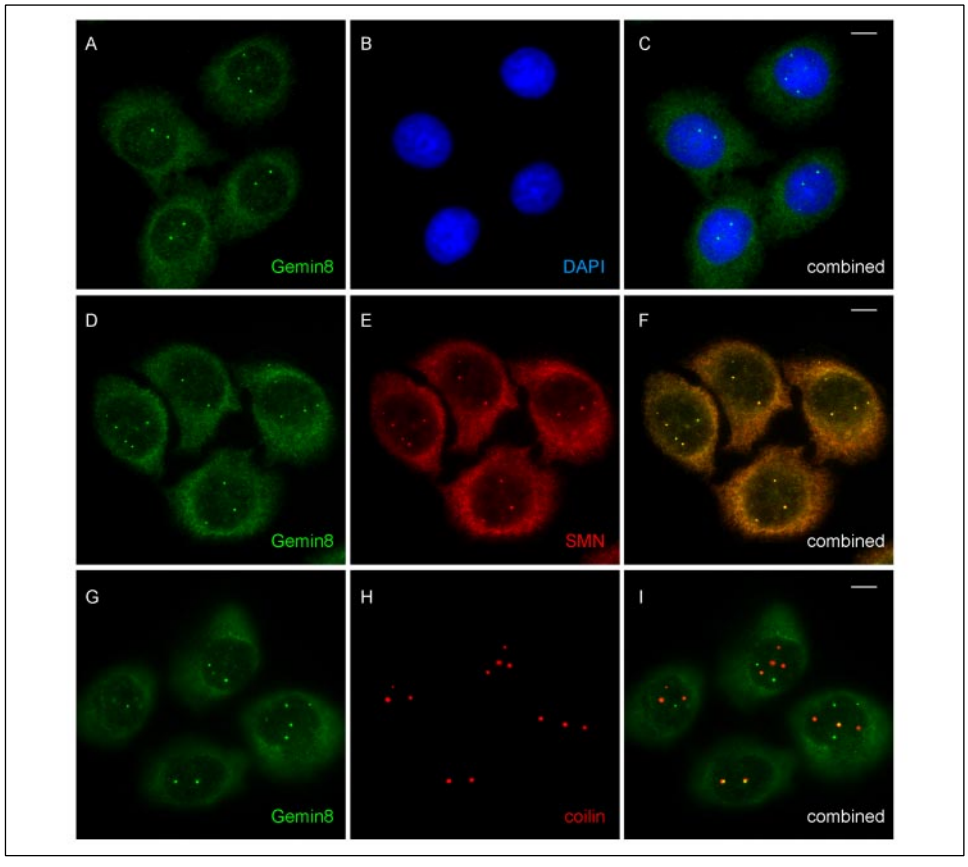

FIGURE 5. **Subcellular localization of Gemin8.** *A–C*, indirect immunofluorescence analysis of HeLa PV cells labeled with anti-Gemin8 antibodies. Nuclei are stained with 4,6-diamidino-2-phenylindole (*DAPI*). *D–F*, indirect immunofluorescence analysis of HeLa PV cells double labeled with anti-Gemin8 and anti-SMN antibodies. *G–I*, indirect immunofluorescence analysis of HeLa PV cells double labeled with anti-Gemin8 and anti-coilin antibodies. Combined images are shown in *C*, *F*, and *I*. *Scale bars* represent 5 μm.

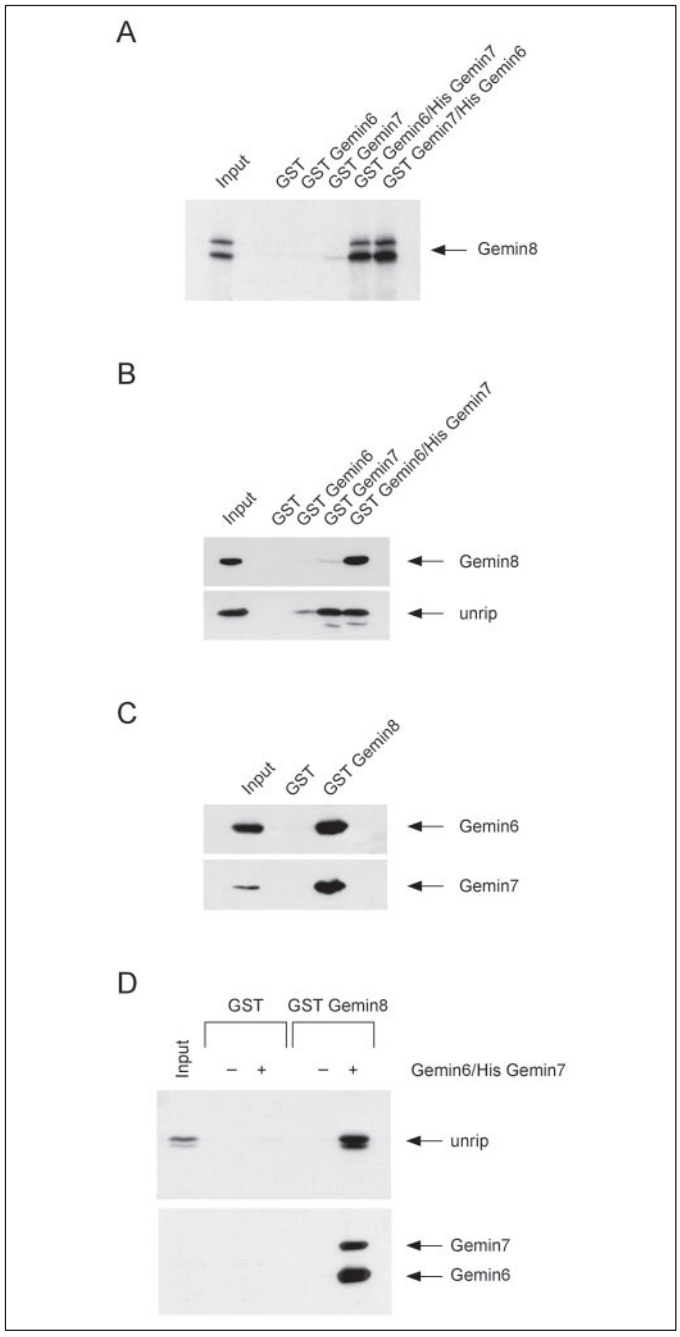

FIGURE 6. **Gemin8 interacts directly with the Gemin6-Gemin7 heterodimer.** *A*, *in vitro* translated [^{35}S]methionine-labeled Gemin8 was incubated either with GST or the indicated GST fusion proteins immobilized on beads. Input (10%) and bound proteins were analyzed by SDS-PAGE and autoradiography. *B*, purified recombinant His-tagged Gemin8 or unrip proteins were incubated with GST and the indicated GST-fusion proteins immobilized on beads. Input (5%) and bound proteins were analyzed by SDS-PAGE and Western blot with anti-T7 tag antibodies. *C*, purified recombinant Gemin6/His-Gemin7 heterodimers were incubated with GST or GST-Gemin8 immobilized on beads. Input (10%) and bound Gemin6 and His-Gemin7 proteins were analyzed by SDS-PAGE and Western blot with anti-Gemin6 and anti-T7 tag antibodies, respectively. *D*, *in vitro* translated [^{35}S]methionine-labeled unrip was added to GST and GST-Gemin8 immobilized on beads after they were preincubated with purified recombinant Gemin6/His-Gemin7 heterodimers or buffer alone as indicated. Bound Gemin6 and His-Gemin7 proteins were analyzed as in *C*. Input (10%) and bound unrip were analyzed by SDS-PAGE and autoradiography.

Following extensive washing to remove unbound Gemin6-Gemin7, *in vitro* translated [^{35}S]methionine-labeled unrip was added to the beads and analyzed for binding. Fig. 6*D* shows that unrip interacts specifically and efficiently with Gemin8 only when the Gemin6-Gemin7 heterodimer is bound to GST-Gemin8. These results indicate that Gemin8 and unrip proteins can simultaneously associate with Gemin6-Gemin7, forming a heteromeric multiprotein subunit of the SMN complex.

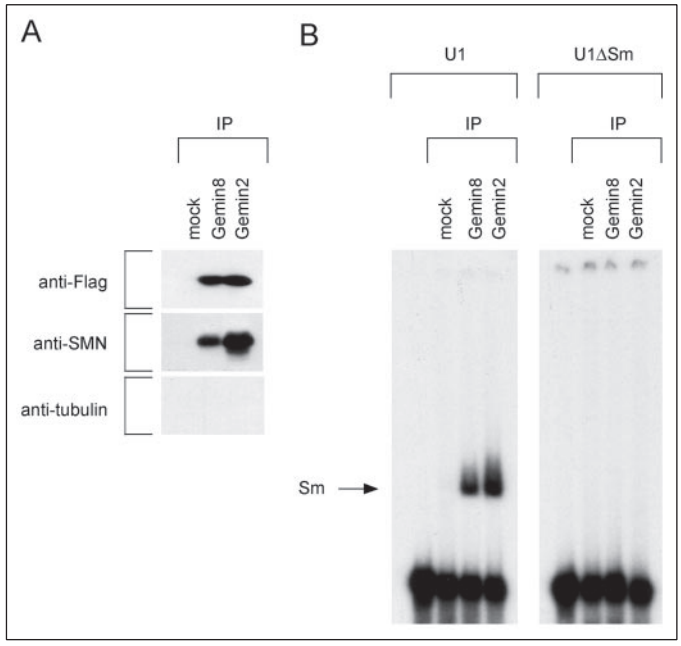

FIGURE 7. **Gemin8-containing SMN complexes mediate snRNP assembly.** *A*, total cell extracts from 293T cells transiently transfected with either pcDNA3 vectors encoding FLAG-Gemin8 (*Gemin8*) and FLAG-Gemin2 (*Gemin2*) or an empty pcDNA3 vector (*mock*) were immunoprecipitated (*IP*) with anti-FLAG antibodies. Immunoprecipitated protein complexes were analyzed by SDS-PAGE and Western blot with the indicated antibodies. *B*, the same protein complexes as in *A* were analyzed by snRNP assembly with [α-^{32}P]UTP-labeled U1 or U1ΔSm snRNAs followed by electrophoresis on native gels and autoradiography. U1 RNP complexes containing the Sm core are indicated.

Gemin8 Is a Component of SMN Complexes Active in snRNP Assembly—Cytoplasmic SMN complexes bound to the seven Sm proteins are responsible for the ATP-dependent formation of the heptameric Sm core on U snRNAs (21, 22). We found that Gemin8 can be depleted from cell extracts using anti-SMN antibodies, suggesting that most, if not all, Gemin8 is associated with SMN and may participate in snRNP assembly (data not shown). Unfortunately, however, we were unable to immunodeplete Gemin8 with anti-Gemin8 antibodies likely because they do not efficiently immunoprecipitate Gemin8 from cell extracts. To analyze directly whether Gemin8 is indeed a component of the cellular SMN complexes active in snRNP assembly, we isolated Gemin8-containing SMN complexes from 293T cells transiently transfected with a vector expressing FLAG-tagged Gemin8 using anti-FLAG antibodies. We also carried out parallel immunopurifications from 293T cells either transfected with a vector expressing FLAG-tagged Gemin2 or mock-transfected with an empty vector as a control. Western blot analysis shows that SMN is found specifically in both the FLAG-Gemin8 and the FLAG-Gemin2 immunoprecipitates but not in the control immunoprecipitates (Fig. 7*A* and see also Fig. 3*A*). We then tested the capacity of these protein complexes to assemble Sm cores on *in vitro* transcribed [^{32}P]UTP-labeled U1 snRNAs using snRNP assembly reactions and gel shift experiments. U1ΔSm, a U1 that bears a mutated Sm site and cannot assemble the Sm core, was used as a control of specificity. Gemin8-containing SMN complexes, similar to those isolated via FLAG-Gemin2, are competent for snRNP assembly as they specifically and efficiently carry out Sm core formation on U1 but not on U1ΔSm (Fig. 7*B*). These results demonstrate that Gemin8 is a component of cellular SMN complexes active in snRNP assembly.

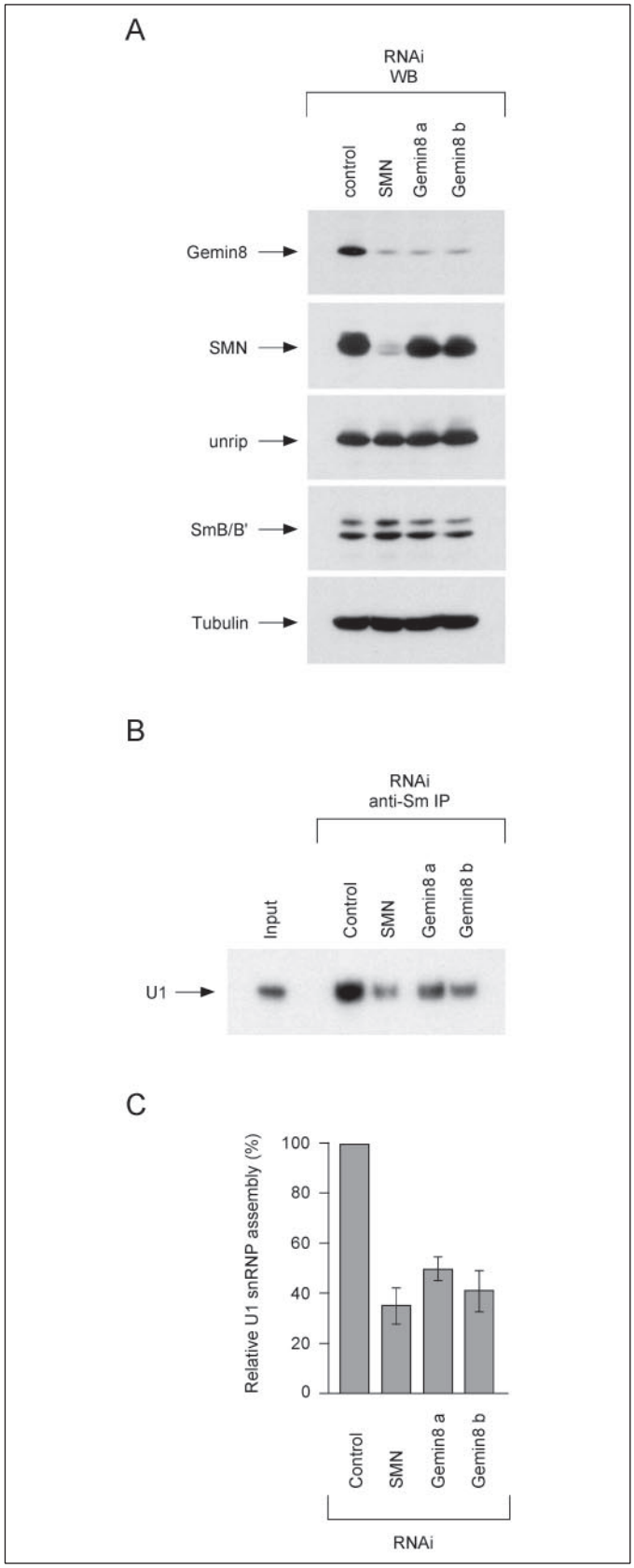

FIGURE 8. **Gemin8 knock-down impairs snRNP assembly activity.** *A*, RNAi experiments were carried out by transfection of the indicated siRNA duplexes into HeLa cells. Control cells were transfected with siRNAs against luciferase. Extracts competent for snRNP assembly were prepared 72 h post-transfection, and equal amounts of proteins (25 μg) were analyzed by Western blot (*WB*) with antibodies against the proteins indicated on the *left*. *B*, snRNP assembly reactions were carried out using the same extracts as in *A* and [α-^{32}P]UTP-labeled U1 snRNA. Following immunoprecipitation with anti-Sm (Y12) antibodies, input (10%) and immunoprecipitated U1 snRNAs were analyzed by electrophoresis on 10% polyacrylamide, 8 M urea denaturing gels and autoradiography. *C*, quantification of relative U1 snRNP assembly activity. The intensity of signals corresponding to U1 snRNAs immunoprecipitated as in *B* was quantified using a STORM 860 PhosphorImager (Amersham Biosciences) and ImageQuant version 4.2. *Gray bars* show the percentage of U1 snRNP assembly in SMN or Gemin8 knock-down extracts relative to that in the control extract, which is set as 100%. Results from five independent experiments ($n = 5$) are presented as mean values ± S.E.

Gemin8 Is Required for Efficient snRNP Assembly Activity—Next, we employed RNAi to study whether Gemin8 has a role in the activity of the SMN complex in Sm core formation. To do so, we transfected HeLa cells with two different siRNA duplexes designed to specifically target Gemin8 mRNAs and analyzed the knock-down efficiency by Western blot (Fig. 8*A* and supplemental Fig. S1). We used also siRNA duplexes against SMN, which were previously reported to knock down both SMN expression and snRNP assembly activity (35), and against luciferase as a control. Compared with control cell extracts, Gemin8 and SMN expression levels are reduced ~4-fold by transfection with their respective siRNAs (Fig. 8*A*). Similar levels of tubulin, which is used as a normalization control for equal amounts of proteins, as well as of unrip and SmB/B′ proteins are found in all extracts and indicate the specificity of the RNAi. Most strikingly, however, knock down of SMN causes a strong reduction in the levels of Gemin8. This observation further strengthens the identification of Gemin8 as an integral component of the SMN complex and suggests that a similar reduction in the levels of Gemin8 may occur in the cells of SMA patients. Indeed, a reduction of the amounts of several Gemin proteins was observed previously in cells that express reduced levels of SMN, including those of SMA patients, and likely reflects a decrease in their stability (35, 44–46). These results may suggest a potential, albeit speculative, involvement of Gemin8 dysfunction in the pathophysiology of SMA either as a candidate modifier of disease severity or as a putative disease gene product for the rare SMA cases without mutations in the *SMN1* gene.

To test the consequence of reduced levels of Gemin8 on the SMN complex activity in Sm core formation, we carried out snRNP assembly reactions with radioactive U1 snRNA and extracts from HeLa cells after RNAi treatment. The efficiency of Sm core formation on U1 was analyzed by immunoprecipitation with anti-Sm monoclonal antibodies and electrophoresis on denaturing gels. Compared with control extracts from HeLa cells transfected with siRNAs against luciferase, knock-down of Gemin8 effectively reduces SMN complex activity and significantly impairs Sm core formation (Fig. 8*B*). As expected, reduced levels of SMN also affect snRNP assembly (Fig. 8*B*). The average decrease of snRNP assembly activity in extracts from HeLa cells transfected with siRNAs against SMN and Gemin8 is 65 and 50–60%, respectively, of that of control cells (Fig. 8*C*). Knock-down of Gemin8 affects snRNP assembly to a similar extent than reduction of either SMN, Gemin2, or Gemin6, which are the only other integral components of the SMN complex with a direct role in Sm core formation to date (35). Altogether, these results indicate that Gemin8 plays an important role within the SMN complex and is required for efficient snRNP assembly activity.

Conclusions—Here we have demonstrated that Gemin8 is a novel integral component of the SMN complex that interacts with the Gemin6-Gemin7 heterodimer and co-localizes with SMN in nuclear Gems. Gemin8 also associates with Sm proteins, and Gemin8-containing SMN complexes are competent for snRNP assembly. Furthermore, Gemin8 is required for efficient snRNP assembly and, therefore, is important for the activity of the SMN complex. These results further expand the protein composition of the SMN complex and identify Gemin8 as a novel cellular protein involved in the pathway of snRNP biogenesis.

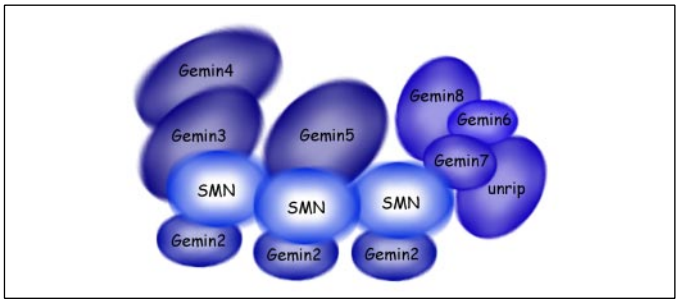

FIGURE 9. **The SMN complex.** An up-to-date schematic illustration of the known protein components of the SMN complex and their reciprocal interactions.

To date, the cytoplasmic macromolecular assembly machine that carries out the ATP-dependent formation of Sm cores on spliceosomal snRNAs contains SMN, Gemin2–8, and unrip proteins bound to the seven Sm proteins. Fig. 9 shows an updated schematic representation of the SMN complex and the protein-protein interactions that hold together its core components. For simplicity of drawing, SMN is depicted as a trimer within a multisubunit complex; however, the precise stoichiometry of the components of the SMN complex is unknown, and sucrose gradient centrifugation experiments indicate that they can form several distinct macromolecular complexes ranging in size from 20 S to 80 S (Fig. 3*D*) (14, 18). The SMN protein likely plays a central role in the structural organization of the SMN complex, as it is essential to bring together the different subunits. SMN binds directly to Gemin2, Gemin3, Gemin5, and Gemin7 (8, 9, 11, 13), whereas the interactions of Gemin3 with Gemin4 and of Gemin7 with Gemin6 bridge these proteins to the SMN complex (10, 13). We have shown here that Gemin8 interacts directly with the Gemin6-Gemin7 heterodimer and, together with the unrip protein, which also binds to Gemin6 and Gemin7 (18, 19), form a heteromeric subunit of the SMN complex.

The presence of multiple subunits and the seemingly modular organization of the SMN complex because of the ability of SMN to oligomerize, which is impaired in SMN mutants of SMA patients (47, 48), support the current view of the SMN complex as a dynamic macromolecular machine capable of multiple tasks. A future challenge will be to define the specific role(s) of the individual components of the SMN complex in the different steps of snRNP biogenesis as well as in additional cellular functions.

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Gemin8 Is a Novel Component of the Survival Motor Neuron Complex and Functions in Small Nuclear Ribonucleoprotein Assembly

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