Gemin proteins are required for efficient assembly of Sm-class ribonucleoproteins

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Spinal muscular atrophy (SMA) is a neurodegenerative disease characterized by loss of spinal motor neurons. The gene encoding the survival of motor neurons (SMN) protein is mutated in >95% of SMA cases. SMN is the central component of a large oligomeric complex, including Gemins2–7, that is necessary and sufficient for the *in vivo* assembly of Sm proteins onto the small nuclear (sn)RNAs that mediate pre-mRNA splicing. After cytoplasmic assembly of the Sm core, both SMN and splicing snRNPs are imported into the nucleus, accumulating in Cajal bodies for additional snRNA maturation steps before targeting to splicing factor compartments known as ''speckles.'' In this study, we analyzed the function of individual SMN complex members by RNA interference (RNAi). RNAi-mediated knockdown of SMN, Gemin2, Gemin3, and Gemin4 each disrupted Sm core assembly, whereas knockdown of Gemin5 and Snurportin1 had no effect. Assembly activity was rescued by expression of a GFP-SMN construct that is refractive to RNAi but not by similar constructs that contain SMA patient-derived mutations. Our results also demonstrate that Cajal body homeostasis requires SMN and ongoing snRNP biogenesis. Perturbation of SMN function results in disassembly of Cajal bodies and relocalization of the marker protein, coilin, to nucleoli. Moreover, in SMN-deficient cells, newly synthesized SmB proteins fail to associate with U2 snRNA or accumulate in Cajal bodies. Collectively, our results identify a previously uncharacterized function for Gemin3 and Gemin4 in Sm core assembly and correlate the activity of this pathway with SMA.

coilin | DEAD box proteins | RNA helicases | small nuclear ribonucleoprotein biogenesis | spinal muscular atrophy

Spinal muscular atrophy (SMA) is a severe autosomal recessive disease characterized by degeneration of motor neurons in the anterior horn of the spinal cord, resulting in subsequent atrophy of skeletal muscle (1). The disease has an incidence rate of 1 in ≈8,000 live births and a carrier frequency of ≈1 in 50 (1). SMA patients can be divided into three classes based on phenotypic severity. Type I, Werdnig-Hoffmann, or infantile SMA is characterized by onset within 6 months of birth and death before 2 years of age. Type II, or intermediate, SMA patients exhibit onset at 6 months of age and survive into adolescence. Type III, Kugelberg-Welander, or juvenile SMA patients typically display a late onset (after 18 months of age) and can survive into adulthood (1).

The *Survival of Motor Neurons 1* (*SMN1*) gene was identified as the SMA disease-causing gene by Melki and colleagues (1). This region of the genome has undergone a duplication to create a second copy of the gene, *SMN2*. The key difference between *SMN1* and *SMN2* is a C to T transition within exon 7 (2). This mutation causes skipping of exon 7 in a majority of *SMN2* transcripts, resulting in a dearth of functional protein. Notably, gene conversion events can increase *SMN2* copy number and reduce SMA severity (1). Although a majority of SMA cases (92%) result from homozygous deletions of *SMN1*, a growing list of point mutations identified in *SMN1* account for 3% of SMA patients (3, 4). Overall, total levels of functional SMN protein correlate with a reduction in SMA severity, establishing the basis for phenotypic variation among affected individuals (1).

Whereas the SMN protein shows strong, diffuse cytoplasmic localization, the protein also accumulates in discrete nuclear foci known as Cajal bodies (CBs) (5, 6). In fetal tissues and a small subset of cell lines, SMN localizes to distinct nuclear structures known as Gemini bodies (gems), so named because of their typical close proximity to CBs (6). SMN is the central member of a large macromolecular complex (7, 8). Members of this so-called SMN complex are termed ''Gemins'' because they colocalize with SMN in gems and CBs. Some of the most notable members of this complex are Gemin2 (alias SMN interacting protein 1, SIP1), Gemin3 (DP103 and Ddx20), and Gemin4 (GIP1) (7, 8). Gemin2 forms a very stable direct interaction with SMN, whereas Gemin3 is a putative DEAD box RNA helicase/unwindase that directly interacts with both SMN and Gemin4.

Critical insight into SMN function came from the identification that the protein interacts with Sm proteins, core components of small nuclear ribonucleoproteins (snRNPs) (7, 8). In metazoans, pre-snRNA (snRNA, small nuclear RNA) transcripts are exported to the cytoplasm for assembly into stable Sm-core particles. *In vivo*, this assembly is mediated by the SMN complex (9, 10). After additional cytoplasmic remodeling steps, the RNPs are imported back into the nucleus, where they undergo further maturation in CBs before ultimately functioning in the spliceosome (11, 12). SMN and Gemins2–7 also localize to CBs because of a direct interaction between SMN and coilin, the Cajal body (CB) marker protein (13, 14). Thus, the biogenesis of Sm snRNPs is a multistep process that takes place in distinct subcellular compartments.

To identify the roles of individual SMN complex proteins in the process of snRNP biogenesis, we have used RNA interference (RNAi) to ablate the expression of SMN complex proteins in HeLa cells. Our results demonstrate that SMN, Gemin2, Gemin3, and Gemin4 are required for efficient Sm core assembly. In addition, we show that loss of SMN protein leads to disassembly of nuclear CBs and a redistribution of coilin to the nucleolus. Furthermore, we found that various SMA-causing point mutations failed to rescue Sm core assembly *in vitro*, consistent with the idea that snRNP biogenesis defects underlie the pathogenesis of the disease.

Materials and Methods

RNAi. HeLa American Type Culture Collection cells were transfected with short interfering RNAs (siRNAs) targeting SMN, Gemins2–7, Snurportin, or a control sequence (Ambion). Cells were transfected by using the DharmaFECT1 lipofection reagent (Dharmacon Research, Lafayette, CO), as directed. The mRNA sequences targeted by siRNA duplexes were as follows: SMN, GGAGCAAAAUCUGUCCGAU; Gemin2, GGUUUCGAUCCCUCGGUAC; Gemin3, GGAAAUAAGUCAUACUUGG; Gemin4, GGCACUGGCAGAAUUAACA; Gemin5, GGGUCUCUGGCUUCACAUU; Gemin6, GGAUGGGUUUUAACUACAG; Gemin7, GGCCAGAGGUUCCUGAAAU; Snurpor-

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Abbreviations: CB, Cajal body; siRNA, short interfering RNA; SMA, spinal muscular atrophy; SMN, survival of motor neurons; snRNA, small nuclear RNA; snRNP, small nuclear ribonucleoprotein.

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tin, GGAATGGATTGTGGTCGTG. Control siRNAs that do not target human mRNAs include Silencer Negative Control no. 1 siRNA (Ambion) or mouse Gemin3 siRNAs: GGATTAGAATGTCATGTCT. The specificity of SMN and Gemin3 knockdown was confirmed by Western blotting and Sm core assembly assays by using a second set of siRNAs targeting each message [GAAGAAUACUGCAGCUUCC for SMN (15) or GGCUUAGAGUGUCAUGUCU for Gemin3]. The siRNA transfections were allowed to proceed for 48–60 h before analysis. GFP-SmB DNA was electroporated 48 h after the initial siRNA transfection (Bio-Rad GenePulser XCell), and cell lysates were collected 24 h later. SMN siRNAs and GFP-SMN rescue constructs were cotransfected by using Lipofectamine 2000 (Invitrogen) and allowed to proceed for 48 h.

Western Blotting and Immunofluorescence. Cytoplasmic cellular lysates were collected by using Ne-Per Nuclear/Cytoplasmic extraction kit (Pierce). Ten micrograms of cytoplasmic lysate were loaded per lane and blotted with α-Gemin4 (Santa Cruz Biotechnology), α-SMN, α-Gemin2, α-Gemin3, or α-Karyopherinα (all from BD Transduction Labs). Western blots were exposed to film and quantified by using the QUANTITY ONE (Bio-Rad) software package. Cells were fixed with 4% paraformaldehyde and extracted with 0.5% Triton X-100. Immunofluorescence was performed with α-SMN (BD Transduction Labs), α-Gemin3 (BD Transduction Labs), α-coilin (pAb R124), or α-Fibrillarin (mAb 72B9).

Sm Core Assembly Assays. U1 snRNA was transcribed *in vitro* by standard procedures in the presence of m7G cap analogue (Promega) and [^{32}P]UTP. One hundred thousand counts of U1 snRNA were incubated with 40 μg of cytoplasmic lysate for 20 min at 30°C. Sm core assembly reactions were precleared with protein-G beads (Pierce) followed by immunoprecipitation with αY12 (Labvision) in RSB-100 buffer (10). Immunoprecipitates were run on a 6% acrylamide 90 mM Tris/90 mM boric acid/2.0 mM EDTA, pH 8.3 (TBE)-Urea denaturing gel and exposed to a PhosphoImager. Sm core assembly assays and Western blots were quantified with QUANTITY ONE (Bio-Rad). U2 snRNA IP northerns were carried out by following GFP-SmB transfection in NET buffer (150 mM NaCl/5 mM EDTA/50 mM Tris, pH 7.5/0.5% Nonidet P-40) with α-GFP (Roche). Products were run on 10% TBE-Urea gels. Northern probes were generated by random-primed labeling of a U2 snRNA PCR product with [^{32}P]dCTP.

DNA Constructs. pT7U1, GFP-Spn, and GFP-SMN were cloned as described in refs. 16–18. GFP-SMN* (siRNA target mutant) was generated by using the QuikChange PCR mutagenesis kit (Stratagene), along with 5′-AGAACAGAACTTAAGTGACCTACTTTCCCCAATCTGTGAAGTAGC-3′ and 5′-GTCACTTAAGTTCTGTTCTTCTCTATTTCCATATCCAGTGTAAAC-3′ primers. SMA point mutations were developed by mutagenesis of GFP-SMN* with primers spanning 15 nt on each side of the amino acid codon change.

Results and Discussion

SMN and Gemin Protein Levels Are Interdependent. We used RNAi to systematically knock down expression of SMN complex proteins in human cells by transfection of siRNA triggers (19). Western blotting demonstrated efficient reduction of SMN, Gemin2, Gemin3, and Gemin4 protein levels as compared to the importin-α loading control (Fig. 1*A*). Control siRNAs that do not recognize a cellular target (Ctl) or those targeting Snurportin1 (SPN), a protein that is not involved in Sm core assembly, had no effect on the levels of SMN complex proteins. Interestingly, SMN knockdown also resulted in a concomitant decrease in the levels of Gemin2 protein and, to a lesser extent, Gemin3. Moreover, knockdown of Gemin4 significantly reduced Gemin3 protein levels. Immunofluorescence after RNAi of SMN and Gemin3 displayed effective knockdown in

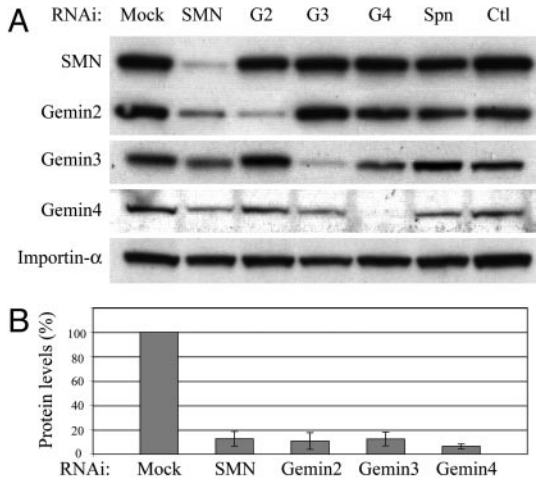

Fig. 1. SMN and Gemin protein levels are interdependent. (*A*) siRNAs were transfected and cytoplasmic cell lysates were collected 60 h later for Western analysis. Columns denote siRNA transfections with mock (no siRNA), SMN, G2 (Gemin2), G3 (Gemin3), G4 (Gemin4), Spn (Snurportin), or Ctl (control) siRNAs. Rows were blotted with the corresponding antibodies; importin-α was used as a loading control. (*B*) Quantification of protein levels from three separate siRNA transfections. Protein levels were normalized to importin-α, then graphed as a fraction of the mock transfection. The siRNA transfections effectively reduced SMN (13%), Gemin2 (11%), Gemin3 (12%), and Gemin4 (6%) protein levels.

97–98% of transfected cells (data not shown). Quantification of these results confirmed the fact that we achieved efficient knockdown of SMN, Gemin2, Gemin3, and Gemin4 (Fig. 1*B*).

It is pertinent to note that the interdependence of protein levels within the SMN complex has been illustrated by using *Smn* knockout mice. Heterozygous mutant mice exhibit a corresponding reduction of Gemin2 protein levels (20). Furthermore, SMA patients often display reduced Gemin2 and Gemin3 levels in addition to those of SMN (21). One likely explanation is that formation of

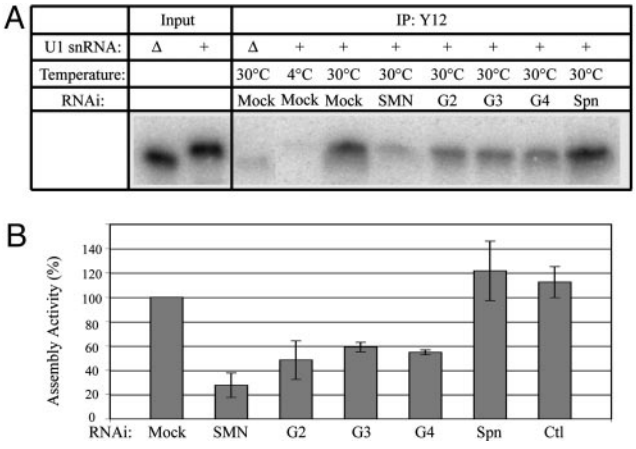

Fig. 2. SMN and Gemins2–4 are required for efficient Sm core assembly. (*A*) Cytoplasmic extracts were collected 60 h after mock, SMN, Gemin2, Gemin3, Gemin4, or Snurportin siRNA transfections. Extracts were incubated with wild-type U1 snRNA (+) or U1ΔSm snRNA (Δ, containing an Sm site deletion), at either nonpermissive (4°C) or permissive (30°C) temperatures for 20 min. Sm core assembly reactions were immunoprecipitated with monoclonal antibody Y12, run on a denaturing gel, and exposed to a PhosphoImager. (B) The results of three separate assembly assays were graphed as a fraction of the mock transfection. Sm core assembly activity was significantly inhibited ($P < 0.04$) after RNAi for SMN (28%), Gemin2 (48%), Gemin3 (59%), Gemin4 (55%), but not by Snurportin (122%) or control (112%) siRNA transfections ($P > 0.7$).

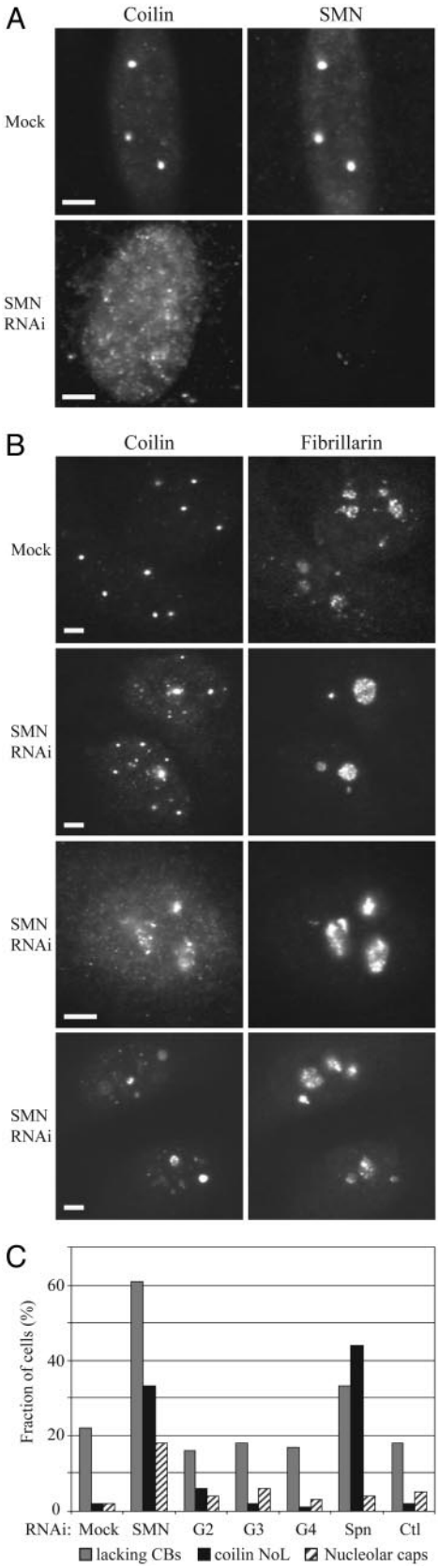

Fig. 3. Loss of SMN and Snurportin results in breakdown of nuclear CBs. (*A*) CB localization was detected by using anticoilin immunofluorescence (*Left*) in mock (*Upper*) or SMN siRNA (*Lower*) transfections. In *Upper*, note the prominent CBs, whereas in *Lower*, no CBs were detected and coilin was localized in a granular pattern. (Scale bars: 2.5 μm.) (*B*) We observed three different types of coilin nucleolar phenotypes after SMN siRNA treatment. CBs were visualized by coilin immunofluorescence (*Left*), whereas antifibrillarin stains both CBs and nucleoli (*Right*). Mock transfections exhibited typical coilin localization in CBs (*Top*). After SMN RNAi, CBs appear to break into smaller fragments (*Center Top*), and coilin is partially relocalized to the nucleolus. (*Center Bottom*) A cell is similar to the one in *A*, where the coilin signal granular in appearance, with prominent nucleolar accumulations. (*B Bottom*) SMN siRNA treatment results in coilin redistribution to nucleolar caps. (*C*) Quantification of cellular phenotypes. Cells transfected with mock, SMN, Gemin2, Gemin3, Gemin4, Snurportin, or control siRNAs were scored ($n = 160$ cells each) for the fraction of cells lacking CBs (gray bars), localizing coilin within the nucleolus (coilin NoL, black bars), or containing coilin in nucleolar caps (hatched bars).

the SMN complex is required for protein stability. Reduction in the amount of SMN might disrupt formation of the entire complex, leading to degradation of several other proteins (e.g., Gemin2 and Gemin3). Loss of Gemin4 may not be as critical for SMN complex formation, however, because Gemin4 forms an independent, stable complex with Gemin3 (22). Thus, an absence of Gemin4 might lead to specific degradation of Gemin3 due to breakdown of this independent complex.

SMN and Gemins2–4 Are Required for Efficient Sm Core Assembly. To determine the relative contribution of each member of the SMN complex to the process of Sm core assembly, we performed *in vitro* assays with siRNA-treated cytosolic extracts. Radiolabeled U1 snRNA was incubated with cytoplasmic extracts to allow for Sm core assembly. The reaction was then immunoprecipitated with monoclonal antibody Y12, which is specific for a subset of methylated Sm proteins (23). Whereas mock and SPN siRNA transfections had little effect on Sm core assembly, siRNAs targeting SMN, Gemin2, Gemin3, and Gemin4 each displayed significant defects (Fig. 2*A*). SMN knockdown showed the most pronounced effect, whereas siRNAs targeting Gemin2, Gemin3, and Gemin4 had moderate Sm core assembly defects (Fig. 2*B*). We also tested siRNAs targeting Gemins5–7 in this assay, although we were unable to confirm knockdown of these proteins because of a lack of the appropriate antibodies. Targeting of Gemin6 and Gemin7 had modest effects on Sm core assembly, whereas siRNAs against Gemin5 had no effect (Fig. 6, which is published as supporting information on the PNAS web site).

Using similar procedures (but different siRNAs and transfection reagents), Feng *et al.* (15) recently showed that RNAi-mediated knockdown of SMN, Gemin2, and Gemin6 inhibited Sm core assembly, however, siRNAs targeting Gemin3, Gemin4, and Gemin5 had little effect and Gemin7 was not tested. Thus, the major difference between the two sets of experiments is that we observed a significant defect in Sm core assembly upon loss of Gemin3 or Gemin4 (Fig. 2*B*); the results for other members of the SMN complex are in good agreement. In reconciling these differences, it is important to note that Feng *et al.* (15) were unable achieve efficient knockdown of Gemin3 (≈70%) or Gemin4 (≈55%). In contrast, we were able to reduce Gemin3 and Gemin4 levels by ≈90% (Fig. 1*B*). We therefore conclude that Gemin3 and Gemin4 are required for efficient assembly of Sm core particles.

Loss of SMN and Snurportin Results in Breakdown of Nuclear CBs. CBs are thought to be sites of posttranscriptional snRNP modification (5, 12, 24). To determine the consequence of reduction in the levels of SMN complex proteins on CB homeostasis, we performed RNAi followed by immunofluorescence microscopy with antibodies against the CB marker protein, coilin. As shown in Fig. 3*A*, RNAi for SMN often resulted in the complete loss of coilin foci, whereas mock treatment had little effect on CB number. Of all of the SMN complex proteins tested, only knockdown of SMN had a significant effect on the number of cells displaying CBs. The results are quantified in Fig. 3*C* (gray bars), showing that control cells or those

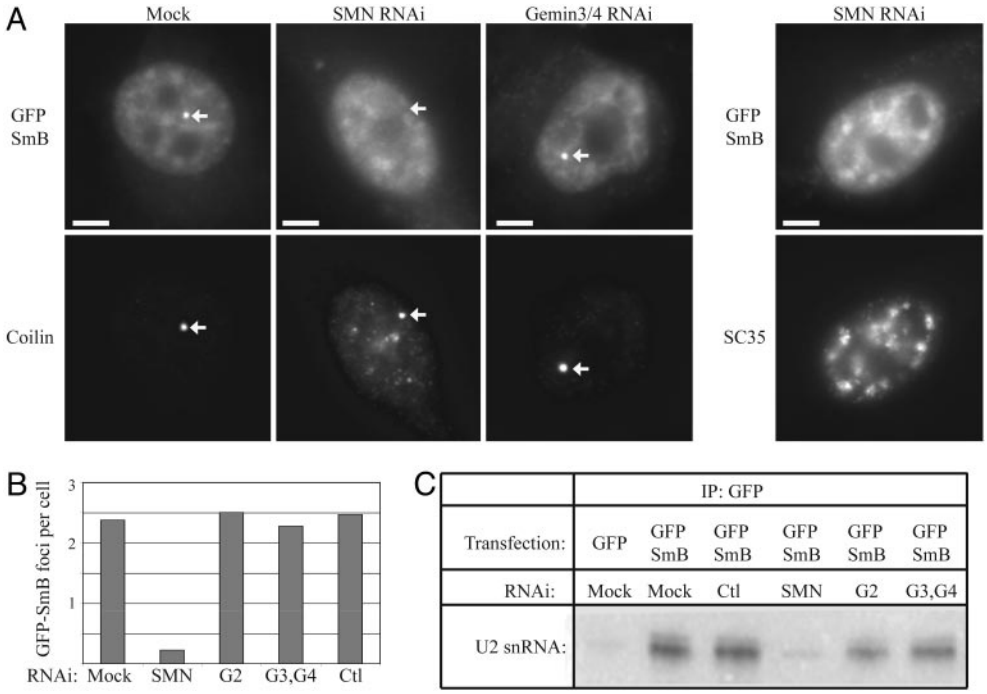

Fig. 4. SMN and Gemins regulate Sm core assembly *in vivo*. (*A*) Cells were transfected with mock, SMN, or a mixture containing Gemin3 and Gemin4 siRNAs. After 48 h of siRNA treatment, a GFP-SmB reporter construct was transfected; the cells were fixed after 66 h of total incubation time. Immunofluorescence was performed to visualize CBs (coilin, *Left*) or speckles (SC35, *Right*). The GFP-SmB reporter construct exhibited a normal distribution in CBs (arrows) and nuclear speckles after mock or Gemin3/4 siRNA treatments (*Left* and *Middle Right*). In contrast, RNAi for SMN failed to localize GFP-SmB to CBs (*Middle Left*) but did localize the reporter to large speckles (*Right*). (Scale bars: 2.5 μm.) (*B*) Quantification of GFP-SmB nuclear foci after mock, SMN, Gemin2, Gemin3/Gemin4, or control siRNA treatment ($n = 90$ cells; SMN RNAi $P < 10^{-17}$, Gemin3/Gemin4, or control RNAi, $P > 0.7$). (*C*) The experiment in *A* was repeated, and cytoplasmic cell extracts were collected after 66 h. IP-northerns were performed by using anti-GFP antibodies and blotted for U2 snRNA. SMN RNAi greatly reduced GFP-SmB association with U2 snRNA compared to the controls, whereas Gemin2 or Gemin3/Gemin4 siRNA transfections had an intermediate effect. A GFP-only reporter was used as a negative control.

treated with siRNAs targeting Gemins2–4 displayed roughly the same number of coilin foci (i.e., 80–85% of cells showed at least one CB). SMN knockdown reduced CB numbers significantly from 2.5 CBs per cell to 0.8 CB per cell ($P < 10^{-20}$) and from 80–85% of cells containing a CB to only 35–40% (Fig. 3*C*).

In addition to CB disassembly, we found that SMN knockdown often resulted in relocalization of coilin to nucleoli and/or nucleolar caps. As shown in Fig. 3*B*, several different cellular phenotypes were observed. In some cells, coilin was fragmented into smaller foci, whereas in others, the protein was localized throughout the nucleolus (Fig. 3*B Center Top* and *Center Bottom*). Cells were also scored for nucleolar coilin accumulation after RNAi of other SMN-associated proteins, including Gemins2–4 and SPN. SMN and SPN knockdown had the greatest effect, with 35–45% of the cells relocalizing coilin to the nucleolus. Gemin2, Gemin3, and Gemin4 had no effect (Fig. 3*C*, black bars). Additionally, an increased proportion of cells transfected with SMN siRNAs localized coilin to nucleolar caps (Fig. 3 *B Bottom* and Fig. 3*C*, hatched bars). Collectively, the results suggest that SMN and SPN, but not Gemins2–4, are critical for CB homeostasis. Because SMN is important for both Sm core assembly (9, 10) and UsnRNP import (25), it is difficult to separate the two functions. However, the relocalization/disassembly of CBs upon SPN knockdown suggests that ongoing UsnRNP import is the key factor.

Our findings are consistent with the previous observations of Lamond and colleagues (17), who showed that Sm protein expression enhances the formation of CBs in cells that typically lack these nuclear suborganelles. Coilin contains a cryptic nucleolar localization signal, and, in some tissues, the protein normally forms associations with nucleoli in the form of perinucleolar caps (26, 27). Leptomycin B, a drug that disrupts the first step of snRNP biogenesis, namely snRNA export to the cytoplasm, also causes coilin localization to the nucleolus (28). Alternatively, SMN reduction might disrupt other cellular pathways (e.g., cell cycle, transcription, small nucleolar ribonucleoprotein (snoRNP) biogenesis, and splicing) in addition to snRNP biogenesis, resulting in fewer CBs per cell. Thus, assembly and nuclear import of new snRNPs are critical for CB formation and coilin subnuclear localization (29). In the absence of ongoing UsnRNP import, CBs disassemble and coilin relocalizes to the nucleolus either by default or because of a continued association and function in some other pathway, such as snoRNP biogenesis (12).

SMN Is Required for Targeting Sm Proteins to CBs. Plausibly, defects in Sm core assembly might result in either a cytoplasmic accumulation or a nuclear reduction in UsnRNP levels. RNAi experiments targeting SMN, followed by immunofluorescense against Sm proteins, failed to detect significant differences in cytoplasmic versus nuclear distributions of Sm proteins (data not shown). To visualize only newly assembled UsnRNPs, we therefore analyzed the localization of GFP-tagged Sm proteins after RNAi. Cells were treated with siRNAs targeting SMN, Gemin2, or Gemin3/Gemin4 for 48 h. and then transfected and incubated overnight with constructs that express GFP-SmB (Fig. 4*A*). Mock RNAi treatments showed the typical GFP-SmB localization patterns in both CBs and nucleoplasmic speckles. SMN RNAi followed by GFP-SmB expression did not result in a significant buildup of cytoplasmic fluorescence, however, snRNP localization in the nucleus was much more diffuse and GFP-SmB failed to concentrate in the CBs (i.e., nucleoplasmic coilin foci) that remain after SMN knockdown. However, GFP-SmB did localize to large SC35-positive speckles in the absence of

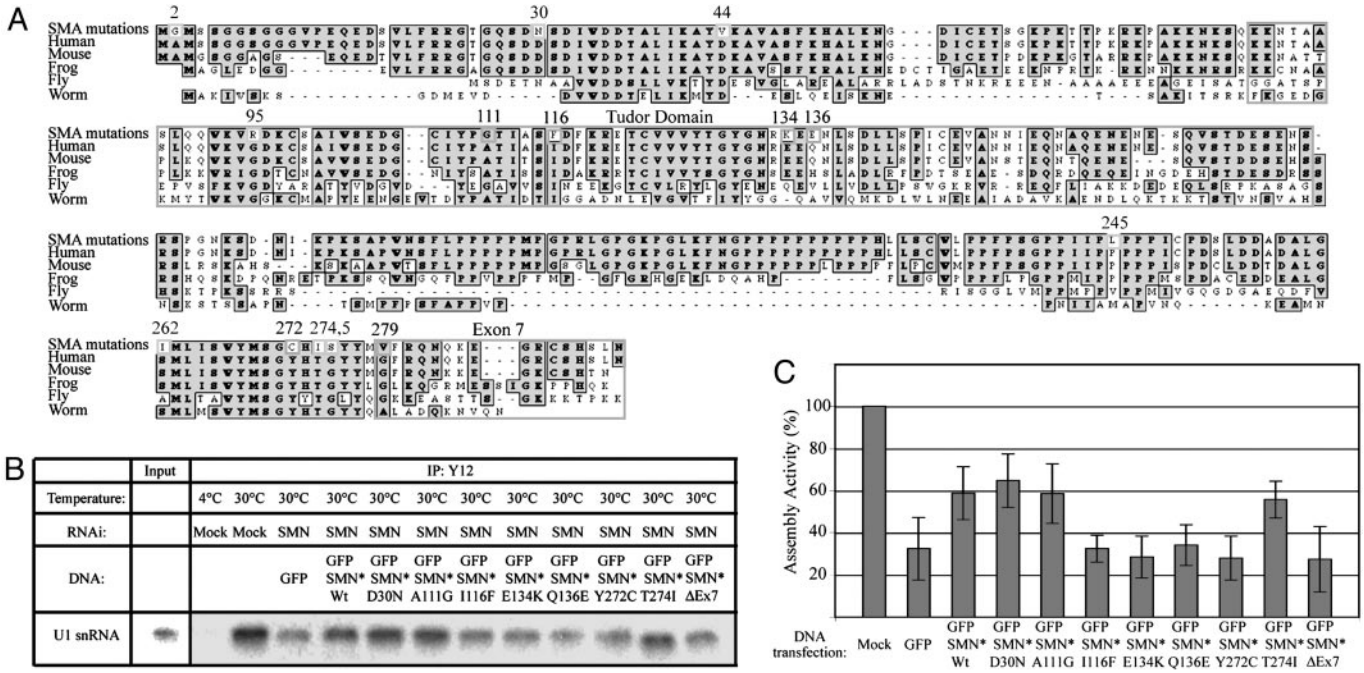

Fig. 5. SMA patient-derived SMN mutations are defective in Sm core assembly. (*A*) An alignment of SMN orthologues, showing the evolutionary conservation of SMN protein sequences from worms to humans. In the top row, the locations of SMA-causing point mutations (black boxes) are shown (SMA Type I: A111G, I116F, E134K, Q136E, Y272C, and G279V; SMA Type II or III: A2G, D30N, D44V, G95R, P245L, S262I, T274I, and G275S). The Tudor domain, responsible for Sm protein interaction, is boxed off in the middle of the protein, and the sequences encoded by human exon 7, skipped in the SMN2 major transcript, are boxed at the end of the protein. (*B*) Cells were either mock-transfected or cotransfected with SMN siRNAs and a GFP or GFP-SMN* DNA construct (containing synonymous substitutions in the siRNA target site; see text for details). Cells were collected after 48 h, and Sm core assembly assays were performed by using the various GFP-SMN* mutant cytoplasmic extracts. (*C*) Quantification of the Sm assembly activity of each GFP-SMN* construct in the absence of endogenous SMN protein. Notably, the activities of SMA type I SMN mutations I116F, E134K, Q136E, Y272C, and ΔEx7 were each significantly lower ($P < 0.03$), than the GFP-SMN*(Wt) rescue construct. Conversely, the activities of the SMA type III mutations D30N and T274I were not significantly different from the GFP-SMN*(Wt) control ($P > 0.6$).

SMN (Fig. 4*A*). In contrast, RNAi of Gemin3 and Gemin4 together did not have a significant effect on GFP-SmB localization (Fig. 4*A*).

Quantification of the number of GFP-SmB foci (corresponding to CBs and large speckles) in these experiments revealed that cells treated with control or anti-Gemin siRNAs displayed an average of 2.4 foci per cell, whereas only 0.2 foci per cell were observed after RNAi for SMN (Fig. 4*B*, $P < 10^{-16}$). To verify that the defects observed upon RNAi were correlated with a loss of Sm core assembly, we repeated the GFP-SmB reporter experiment and performed immunoprecipitation with anti-GFP antibodies, followed by northern analysis of U2 snRNA (Fig. 4*C*). As shown, SMN knockdown had the greatest effect on recovery of U2 snRNA with GFP-SmB, whereas knockdown of Gemin2 or Gemin3/Gemin4 brought down intermediate amounts of U2. Note that the results of this *in vivo* pulldown (Fig. 4*C*) are in good agreement with those of the *in vitro* assembly assay (Fig. 2).

Interestingly, even in the absence of proper snRNP assembly, GFP-SmB did not significantly accumulate in the cytoplasm. Moreover, we routinely recovered reduced amounts of GFP-SmB from cytoplasmic extracts of cells treated with siRNAs targeting SMN. To normalize the input levels of GFP-SmB in Fig. 4*C*, we transfected 1.5-fold greater amounts of GFP-SmB plasmid. The fact that we do not see a clear increase in cytoplasmic accumulation of Sm proteins suggests that the unassembled Sm subunits are either imported into the nucleus without complexing with snRNAs or they are degraded. These two options are not mutually exclusive; overexpression of GFP-SmB might swamp the machinery that normally degrades unassembled Sm proteins, allowing their import. Consistent with this scenario, recent experiments have demonstrated a nuclear localization activity associated with the basic C-terminal tails of Sm proteins (30). Our results suggest that this import pathway is independent of snRNP assembly and interaction with SMN.

Thus, a consequence of SMN reduction is that we not only detect fewer CBs, but the remaining CBs do not accumulate detectable amounts of GFP-SmB. Therefore, the decline in snRNP import precedes CB disassembly. Because GFP-SmB still can localize to large speckles after SMN RNAi, it is possible that the unassembled GFP-SmB proteins can interact with other splicing factors, leading to accumulation in this nuclear locale. Although Sm proteins localize to the nucleus in the absence of ongoing snRNP assembly, our findings illustrate that proper targeting of newly synthesized Sm proteins to CBs depends on SMN.

SMA Type I, but Not Type III, SMN Point Mutations Are Defective in Sm Assembly. The severity of SMA is inversely proportional to SMN protein levels (1). However, in the absence of an *SMN1* gene deletion, a number of disease-causing missense mutations also have been described (3, 4, 31). As shown in Fig. 5*A*, these mutations are scattered throughout the length of the coding region. Because SMA is a recessive disease and the SMN complex is oligomeric, it has been particularly difficult to assay the effects of these patient-derived mutants by overexpressing them in a wild-type background. To assay the activities of individual SMN mutations, in the absence of the wild-type protein, we generated a wild-type SMN construct (called GFP-SMN*) containing synonymous point substitutions in the region targeted by the siRNAs, making it refractive to RNAi (data not shown). A number of SMA-causing point mutations were next introduced into this background and then assayed for their abilities to rescue Sm core formation *in vitro*.

As shown in Fig. 5*B*, cotransfection of anti-SMN siRNAs

with GFP-SMN*(Wt) substantially rescued Sm core assembly; cotransfection with the empty GFP vector had a nominal effect. Note that the SMN knockdown was slightly less effective and that cell death was slightly increased when cells were cotransfected with plasmids and siRNAs (data not shown). Thus, the partial rescue of Sm-core assembly activity detected in the GFP-only transfections (Fig. 5*B*, lane 4) is likely due to the incomplete inactivation of the SMN complex in those cells. Conversely, the incomplete rescue of Sm-core activity by the GFP-SMN*(Wt) construct (Fig. 5*B*, lane 5) may be due to a difference in the expression of the exogenous construct and/or the presence of the GFP tag. In any event, the assembly activity of the wild-type rescue construct was reproducibly higher and significantly different ($P < 0.02$) from that of GFP (Fig. 5*C*).

Next, we analyzed the abilities of eight separate SMN mutations (six SMA type I alleles and two SMA type III alleles) to rescue Sm core assembly. As shown in Fig. 5 *B* and *C*, five of the six SMA type I alleles tested (I116F, E134K, Q136E, Y272C, and ΔEx7) showed only basal levels of Sm-core assembly activity, whereas both of the SMA type III alleles (D30N and T274I) functioned as well as the wild-type construct (Fig. 5 *B* and *C*). Three of the SMA type I mutations (E134K, Y272C, and ΔEx7) have been studied previously *in vitro*. The SMN(E134K) is thought to reduce binding of SMN to Sm proteins and importin β (3, 25, 32). Moreover, the Y272C and ΔEx7 mutations have been shown to disrupt SMN oligomerization, with concomitant or downstream defects in Sm protein binding (33, 34). Consistent with these previous findings, we now demonstrate that E134K, Y272C, and ΔEx7, as well as the uncharacterized I116F and Q136E SMN mutations, are defective in Sm core assembly. Interestingly, I116F, E134K, and Q136E cluster together within the Tudor domain of SMN, which is required for high-affinity Sm protein interaction (35, 36). Recently, two other laboratories have shown that the SMN2 gene product (i.e., primarily SMNΔEx7) is partially defective in Sm core assembly by performing *in vitro* assays in extracts derived from SMA patient fibroblasts (37, 38). Furthermore, we found that SMA type I point mutations (E134K and Y272C) were unable to rescue concomitant loss of Gemin2 upon SMN RNAi (Fig. 6). In contrast, GFP-SMN*(Wt), GFP-SMN*(D30N), and GFP-SMN*(T274I) restored expression of Gemin2 to control levels (Fig. 6). Therefore, it is possible that the Type I SMA point mutations prevent proper formation of the SMN complex and, thus, are defective in Sm core assembly. Notably, we found that both of the SMA type III alleles (D30N and T274I) and one SMA type I allele (A111G) were functional in our *in vitro* assembly assay. It is possible that these regions of SMN may be required for some other aspect of SMN function, such as cap hypermethylation (39) or nuclear import (25), thus establishing a potential basis for SMA type I-type III phenotypic variation. Alternatively, the D30N, T274I, and A111G mutations might be required for some novel SMN function, such as in trafficking of messenger RNAs to neuronal growth cones (40). In summary, we have shown that SMN-deficient cells display defects in Sm core assembly activity that can be rescued by the addition of ectopically expressed GFP-SMN.

Gemin3 and Gemin4: Active Participants in Assembly of the Sm Core?

Our results highlight the importance of Gemin3 and Gemin4 in the Sm core assembly process; previous efforts to investigate this question were inconclusive (15). However, additional experiments clearly will be required to elucidate the precise functions of each of the members of the SMN complex. For example, it will be interesting to learn whether Gemin3, the putative RNA helicase/unwindase, is actively involved in adding Sm proteins to the snRNA or whether it merely aids by providing a framework for assembly. Alternatively, the participation of microRNAs (miRNAs) in the assembly of snRNPs has not been strictly ruled out. In addition to interacting with SMN, Gemin3 and Gemin4 have been shown to form independent complexes with miRNAs (22). It is unclear whether these complexes are functionally related. Future experiments, including development of *in vivo* model systems, will be essential in sorting out the different pathways that contribute to SMA pathology.

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