The survival of motor neurons (SMN) protein interacts with the snoRNP proteins fibrillarin and GAR1

Livio Pellizzoni, Jennifer Baccon, Bernard Charroux* and Gideon Dreyfuss

Background: The survival of motor neurons (SMN) protein is the protein product of the spinal muscular atrophy (SMA) disease gene. SMN and its associated proteins Gemin2, Gemin3, and Gemin4 form a large complex that plays a role in snRNP assembly, pre-mRNA splicing, and transcription. The functions of SMN in these processes are mediated by a direct interaction of SMN with components of these machineries, such as Sm proteins and RNA helicase A.

Results: We show that SMN binds directly to fibrillarin and GAR1. Fibrillarin and GAR1 are specific markers of the two classes of small nucleolar ribonucleoprotein particles (snoRNPs) that are involved in posttranscriptional processing and modification of ribosomal RNA. SMN interaction requires the arginine- and glycine-rich domains of both fibrillarin and GAR1 and is defective in SMN mutants found in some SMA patients. Coimmunoprecipitations demonstrate that the SMN complex associates with fibrillarin and with GAR1 in vivo. The inhibition of RNA polymerase I transcription causes a transient redistribution of SMN to the nucleolar periphery and loss of fibrillarin and GAR1 colocalization with SMN in gems. Furthermore, the expression of a dominant-negative mutant of SMN (SMNΔN27) causes snoRNPs to accumulate outside of the nucleolus in structures that also contain components of gems and coiled (Cajal) bodies.

Conclusions: These findings identify fibrillarin and GAR1 as novel interactors of SMN and suggest a function for the SMN complex in the assembly and metabolism of snoRNPs. We propose that the SMN complex performs functions necessary for the biogenesis and function of diverse ribonucleoprotein complexes.

Address: Howard Hughes Medical Institute and Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, 19104, USA.

Correspondence: Gideon Dreyfuss
E-mail: gdreyfuss@hhmi.upenn.edu

Present address: *Labororatoire de Génétique et Physiologie du Développement, UMR 6545 CNRS-Université, IBDM CNRS-INSERM-Université de la Mediterranée, Campus de Luminy Case 907, F-13288 Marseille, Cedex 09, France.

Received: **17 April 2001**
Revised: **21 May 2001**
Accepted: **22 May 2001**

Published: **24 July 2001**

Current Biology 2001, 11:1079–1088

0960-9822/01/$ – see front matter

Background

Spinal muscular atrophy (SMA) is an autosomal recessive neurodegenerative disease characterized by progressive loss of motor neurons in the anterior horn of the spinal cord, resulting in muscular weakness and atrophy [1]. The survival of motor neurons gene (*SMN*) is the SMA-determining gene [2]. In humans, the *SMN* gene is duplicated as an inverted repeat on chromosome 5, and only deletions or mutations in the telomeric copy, *SMN1*, result in SMA [3]. The centromeric copy, *SMN2*, does not protect from SMA because, as a result of a single nucleotide difference from *SMN1*, it mostly produces an alternatively spliced SMN mRNA lacking the sequences encoded by exon 7 [2, 4]. The amount of full-length SMN produced by *SMN2* is sufficient to overcome lethality in most cells, but not in motor neurons in which the amount of SMN inversely correlates with the severity of the disease [5–9].

The SMN protein is found both in the cytoplasm and in the nucleus where it is concentrated in gems, which are nuclear bodies similar in size and number to Cajal (coiled) bodies (CBs) and often associated with them [10]. SMN is tightly associated with Gemin2 (formerly SIP1), the DEAD box RNA helicase Gemin3, and Gemin4 in a large multiprotein complex [11–13]. In the cytoplasm, the SMN complex functions in the assembly of spliceosomal snRNPs [11, 14–16]. In the nucleus, SMN functions in transcription and pre-mRNA splicing [15, 17, 18], possibly in the assembly and regeneration of components of the RNA polymerase II transcription-processing machinery. SMN forms large oligomers, and this appears to be essential for its activity, since a defect in SMN oligomerization correlates with SMA [19, 20]. SMN oligomerization is necessary for efficient interaction with the arginine- and glycine-rich carboxyl termini of SmB, SmD1, and SmD3, while SMA-causing SMN mutants found in SMA patients are deficient in both splicing regeneration and the interaction with Sm proteins and Gemin3 [12, 15, 16, 20, 21].

Preribosomal RNA (rRNA) transcription and processing and the assembly of ribosomes take place in the nucleolus, where rRNAs are transcribed, cleaved into the 28S, 18S, and 5.8S rRNA molecules, and further modified by base methylation and pseudouridylation. These processes are

mediated by small nucleolar ribonucleoprotein particles (snoRNPs), which are conserved throughout evolution from yeast to humans [22–24]. SnoRNPs can be divided into two classes, the box C/D class and the box H/ACA class, based on the presence of conserved RNA sequence motifs and on different protein compositions [25]. Box C/D snoRNPs are necessary for cleavage and site-specific methylation of rRNA [26–29]. Box H/ACA snoRNPs guide the pseudouridylation of rRNA [30–32]. The specificity of the site of modification is established by short regions of complementarity between each snoRNA and the rRNA. To understand the biogenesis of these RNPs, much work has been focused on the identification of snoRNP-specific proteins [33]. Fibrillarin, a common autoantigen of human autoimmune diseases, is a specific component and a marker of box C/D snoRNPs [34]. GAR1 is a specific component and a marker of box H/ACA snoRNPs [35, 36]. Interestingly, mammalian telomerase RNP is also a member of the H/ACA snoRNPs, although it is apparently not involved in rRNA metabolism [36].

Here, we investigated the possible relationship between the SMN complex and snoRNP metabolism suggested by the earlier identification of an interaction between SMN and fibrillarin in the yeast two-hybrid system [10] and the observation of a nucleolar localization of Gemin4 [13]. We show that fibrillarin and GAR1 are novel direct targets of SMN interaction both in vitro and in vivo. Our findings provide evidence for a link between the SMN complex and snoRNP metabolism and further extend the range of possible cellular defects associated with SMA.

Results

SMN interacts directly with the snoRNP proteins fibrillarin and GAR1

We first examined the capacity of the known components of the SMN complex to interact with the snoRNP-specific proteins fibrillarin and GAR1. SMN, Gemin2, Gemin3, and Gemin4 were translated in vitro in the presence of [^{35}S]methionine and incubated with purified recombinant fibrillarin fused to GST, GAR1 fused to GST, or GST alone as a control. Figure 1a shows that, of these proteins, only SMN binds efficiently to both fibrillarin and GAR1, but not to GST. Fibrillarin and GAR1 bind RNA in vitro [37, 38]. It was important to exclude the possibility that these interactions with SMN were nonspecifically mediated by RNAs present in the translation system because SMN can also bind to nucleic acids [39, 40]. To do so, in vitro-translated [^{35}S]methionine SMN was treated with RNase A before being added to the binding reaction. RNase A treatment had no effect on SMN interaction with both fibrillarin and GAR1 (Figure 1b). As a reference, the weak SMN interaction with hnRNPA1 is abolished by RNase treatment, indicating that this interaction is bridged by RNAs. To rule out the possibility that some components in the rabbit reticulocyte lysate mediate the interactions with SMN described above, we performed in vitro binding experiments using purified recombinant his-tagged SMN. Figure 1c shows that SMN, detected by Western blotting using an anti-T7 tag monoclonal antibody, interacts directly and efficiently with both fibrillarin and GAR1.

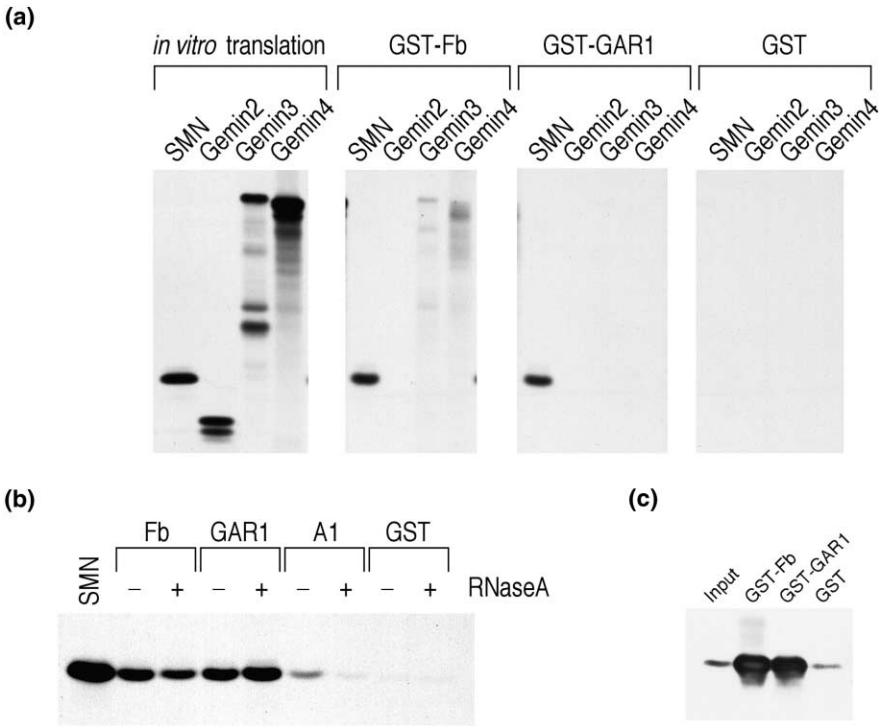
The interaction of SMN with fibrillarin and GAR1 requires the conserved Y/G box and is defective in SMN mutants found in some SMA patients

SMN contains two evolutionarily conserved regions (black boxes in Figure 2a): the one close to the amino terminus is involved in SMN binding to Gemin2, and the one close to the carboxyl terminus is involved in SMN binding to itself, Sm proteins, and Gemin3 [11, 12, 19, 20]. We analyzed the deletion mutants of SMN indicated in Figure 2a for their capacity to interact in vitro with fibrillarin and GAR1. Figure 2b shows that a deletion of the Y/G box abolishes SMN interaction with fibrillarin and GAR1. Conversely, the dominant-negative mutant SMNΔN27 binds as well as wild-type SMN. The *SMN2* gene mostly produces a truncated version of SMN lacking the amino acids encoded by exon 7 (see Background). In addition, some SMA patients carry point mutations that cluster in the Y/G box, the most common of which is the Y272C mutation [2, 41]. Figure 2c shows that the binding of SMN to both fibrillarin and GAR1 is strongly impaired by either the Y272C point mutation or the SMNΔEx7 deletion. These results demonstrate that the conserved Y/G box is necessary for SMN interaction with fibrillarin and GAR1. Moreover, SMN mutations found in SMA patients severely impair the interaction with these snoRNP-specific proteins.

The arginine- and glycine-rich domains of fibrillarin and GAR1 are necessary for SMN interaction

Fibrillarin and GAR1 contain arginine- and glycine-rich domains, also known as RGG boxes and GAR domains (Figure 3a,c). To determine the domains of fibrillarin and GAR1 that are involved in SMN interaction, we generated a series of deletion mutants of fibrillarin and GAR1. A schematic representation of the fibrillarin domain structure and the positions of the amino- and carboxyl-terminal deletions are shown in Figure 3a. Wild-type fibrillarin and the indicated deletion mutants were translated in vitro in the presence of [^{35}S]methionine and tested for their ability to bind purified recombinant SMN fused to GST. Figure 3b shows that wild-type fibrillarin and a deletion of the first 23 amino acids (ΔN23) bind efficiently to SMN. In contrast, the deletion of the RGG domain (ΔN79) and all additional amino-terminal deletions abolish binding to SMN. Therefore, the RGG domain of fibrillarin is necessary for its interaction with SMN. However, the RGG domain of fibrillarin is not sufficient for SMN interaction. The RGG domain fused to GST does not bind SMN (data not shown), and only the carboxyl-terminal α helix domain of fibrillarin appears to be dispensable for the

Figure 1



SMN interacts directly with fibrillarin and GAR1 in vitro. **(a)** In vitro-translated [^{35}S]methionine SMN, Gemin2, Gemin3, or Gemin4 were incubated with either GST, GST-fibrillarin (GST-Fb), or GST-GAR1 as indicated. Bound proteins were analyzed by SDS-PAGE and autoradiography. Ten percent of the input is shown. **(b)** In vitro-translated [^{35}S]methionine SMN, previously treated or untreated with RNaseA as indicated, was incubated with GST-fibrillarin (Fb), GST-GAR1 (GAR1), GST-hnRNPA1 (A1), or GST. Bound proteins were analyzed by SDS-PAGE and autoradiography. Ten percent of the input is shown. **(c)** Purified recombinant his-tagged SMN was incubated with GST-fibrillarin, GST-GAR1, or GST. Bound SMN was detected by Western blotting using anti-T7 tag antibodies. Five percent of the input is shown.

interaction, as longer carboxyl-terminal deletions abolish SMN binding (ΔC53, Figure 3b).

GAR1 contains a conserved domain in the middle of the protein flanked by amino- and carboxyl-terminal arginine- and glycine-rich domains (Figure 3c). We generated two deletion mutants of GAR1 lacking either the amino- or the carboxyl-terminal RGG domains. Wild-type GAR1 and deletion mutants were translated in vitro in the presence of both [^{35}S]methionine and [^{35}S]cysteine and tested for their ability to bind purified recombinant SMN fused to GST. Figure 3d shows that wild-type GAR1 binds to GST-SMN, but not to GST. Conversely, the deletion of either RGG domain abolished the interaction with SMN, indicating that they are both necessary for SMN interaction.

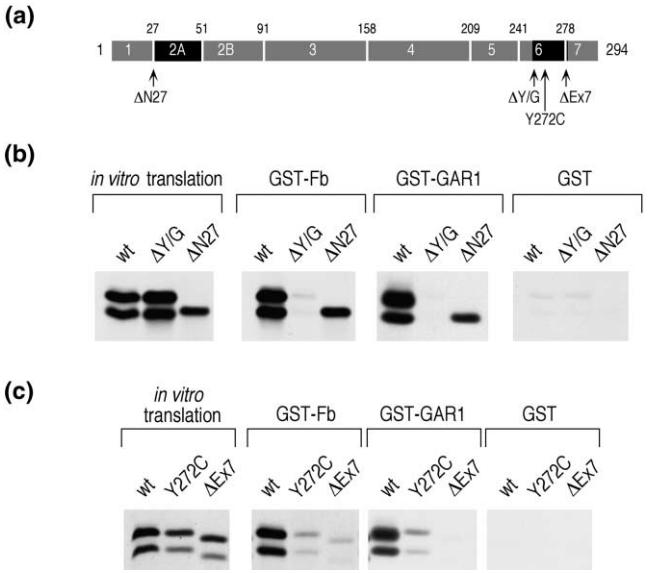
Association of the SMN complex with fibrillarin and GAR1 in vivo

We further examined the association between snoRNPs and the SMN complex in vivo by immunoprecipitation experiments. To do so, we transiently transfected into 293T cells DNA constructs for the expression of myc-tagged fibrillarin (mycFb), GAR1 (mycGAR1), or the vector alone as a control (mock). Nucleoplasmic extracts were prepared from transfected cells and subjected to immunoprecipitation using anti-myc antibodies. Immunoprecipitated proteins and an aliquot of the respective extracts were analyzed by SDS-PAGE and by Western blotting with several antibodies. As shown in Figure 4, myc-tagged fibrillarin and GAR1 were expressed and could be specifically immunoprecipitated with anti-myc antibodies. All the known components of the SMN complex, namely SMN, Gemin2, Gemin3, and Gemin4, but not the abundant nuclear RNA binding protein hnRNPA1, were coimmunoprecipitated with both fibrillarin and GAR1. None of these proteins were detected in the control immunoprecipitate from mock-transfected extracts. Consistent with the in vitro binding experiments described above, these findings indicate that a subset of SMN complexes is associated with fibrillarin and GAR1 in vivo. It appears that a higher proportion of Gemin4 compared to the other components of the SMN complex is immunoprecipitated with both fibrillarin and GAR1. Together with its unique nucleolar localization [13], this observation suggests that Gemin4 may be associated with snoRNPs also in the absence of the other components of the SMN complex. This possibility is currently being investigated further.

Transcription-dependent association of SMN with fibrillarin, GAR1, and the nucleolus

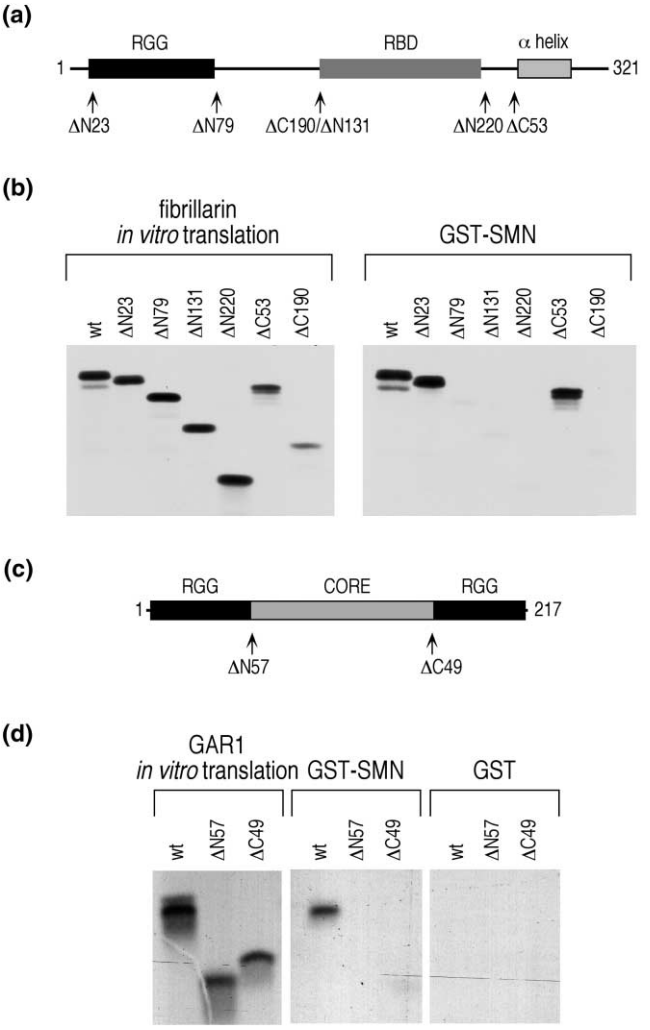
In the nucleus, SMN is particularly highly concentrated in gems [10]. Gems contain the components of the SMN complex, but not the CB marker coilin [10–13]. The fact that gems and CBs each contain distinct components indicate that they are distinct and independent nuclear structures, although there is a dynamic functional relationship between them and they are more often found colocalized

Figure 2



The mapping of SMN domains required for interaction with fibrillarin and GAR1. **(a)** A schematic representation of SMN protein structure. The position of the amino acids corresponding to each exon/exon boundary and the SMN deletion mutants are indicated. Black boxes indicate evolutionarily conserved SMN domains. **(b)** In vitro-translated [^{35}S]methionine SMN wt or the indicated deletion mutants were incubated with GST-fibrillarin, GST-GAR1, or GST. Bound proteins were analyzed by SDS-PAGE and autoradiography. Ten percent of the input is shown. **(c)** SMN mutants found in SMA patients are defective in binding to snoRNP proteins. In vitro-translated [^{35}S]methionine SMN wt, the point mutant Y272C, or the deletion ΔEx7 were incubated with GST-fibrillarin, GST-GAR1, or GST. Bound proteins were analyzed by SDS-PAGE and autoradiography. Ten percent of the input is shown.

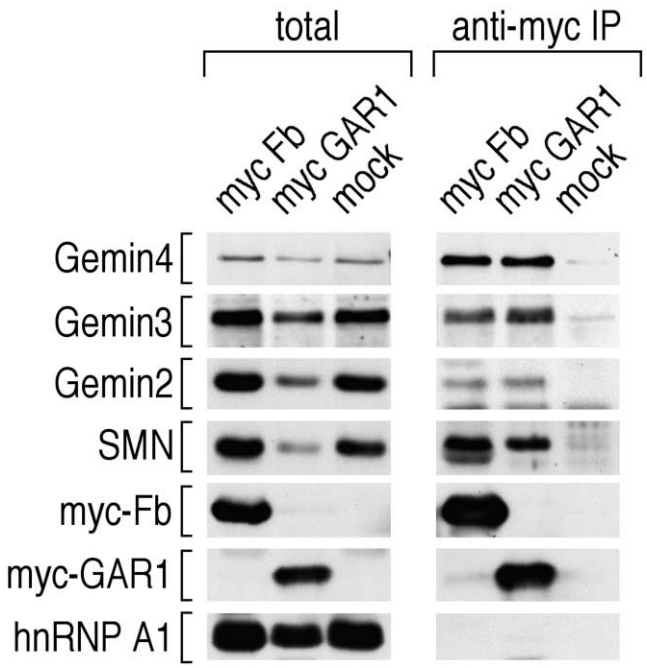
Figure 3



The RGG domains of fibrillarin and GAR1 are necessary for the interaction with SMN. **(a)** A schematic representation of the fibrillarin protein structure [60]. The RGG box, the RNA binding domain (RBD), and the α helix are indicated. The positions of the fibrillarin deletion mutants used are indicated. **(b)** In vitro-translated [^{35}S]methionine fibrillarin or the indicated deletion mutants were incubated with GST-SMN. Bound proteins were analyzed by SDS-PAGE and autoradiography. Ten percent of the input is shown. **(c)** A schematic representation of GAR1 protein structure [44]. The RGG boxes, the central core domain, and the position of the GAR1 deletion mutants used are indicated. **(d)** In vitro-translated [^{35}S]methionine and [^{35}S]cysteine GAR1 or the indicated deletion mutants were incubated with GST-SMN or GST. Bound proteins were analyzed by SDS/PAGE and autoradiography. Ten percent of the input is shown.

[10, 15, 42, 43].We studied by indirect immunofluorescence microscopy the localization of fibrillarin and GAR1 relative to SMN in untreated HeLa cells or in cells treated with actinomycin D at a concentration (0.04 μg/ml) that inhibits transcription by RNA polymerase I, but not RNA polymerase II. Figures 5a–c and 6a–c show that SMN, fibrillarin, and GAR1 colocalize in gems/CBs, but not in the nucleolus, where SMN is not detectable under normal conditions. After actinomycin D treatment, fibrillarin and GAR1, and very likely snoRNPs, display an overall identical pattern of reorganization (Figures 5 and 6). After 1.5 hr of actinomycin D treatment, fibrillarin and GAR1 accumulate at the nucleolar periphery. In addition, their nucleoplasmic pool increases, and their colocalization with SMN in gems is barely detectable (Figures 5d–f and 6d–f, arrows in 5e and 6e). Gems are more resistant than CBs to inhibition of RNA polymerase I transcription, as CBs are disrupted under these conditions and the CB marker p80 coilin localizes at the nucleolar periphery (data not shown). Interestingly, in approximately 10%–20% of the cells, some of the SMN, in addition to its presence in gems, colocalizes with fibrillarin and GAR1 at the periphery of nucleoli (arrowheads in Figures 5f and 6f). SMN association with nucleoli is transient because after longer actinomycin D treatment (3 hr), SMN is no longer concentrated at the periphery of nucleoli, displaying a more diffuse and weaker nucleolar localization in addition to strong labeling of gems (Figures 5g–i and 6g–i). At this time point, no residual fibrillarin or GAR1 staining is

Figure 4



The association of the SMN complex with fibrillarin and GAR1 in vivo. 293T cells were transiently transfected with DNA constructs expressing myc-tagged fibrillarin (myc-Fb), myc-tagged GAR1 (myc-GAR1), or with vector alone (mock). Immunoprecipitation (IP) experiments were carried out from nucleoplasmic extracts using anti-myc antibodies. Immunoprecipitates were analyzed by SDS-PAGE and Western blotting with the antibodies to the indicated proteins. Five percent of the input is shown.

detected within gems. After 4.5 hr of actinomycin D treatment, the weak nucleolar staining of SMN is no longer detectable (Figures 5j–l and 6j–l). These results indicate that the association of SMN with snoRNPs in vivo is dynamic and related to RNA polymerase I transcription.

Expression of SMNΔN27 causes accumulation of snoRNPs outside the nucleolus

To further address the involvement of the SMN complex in snoRNP metabolism, we examined the subcellular localization of fibrillarin and GAR1 in cells expressing SMNΔN27. Transfection in HeLa cells of this dominant-negative mutant of SMN blocks snRNP assembly in the cytoplasm and causes a selective accumulation of several components of the RNA polymerase II transcription/processing machinery in large nuclear structures that also contain the known components of gems and CBs [15, 18]. Concomitantly, SMNΔN27 inhibits mRNA and rRNA transcription. Similar to untransfected cells, fibrillarin is localized in the nucleolus and CBs of cells transfected with SMNwt (Figure 7a–c). Gems that are completely separate from CBs are indicated by the arrows in Figure 7c. Strikingly, in cells transfected with SMNΔN27, fibrillarin is relocalized and accumulates with the mutant protein in large structures (Figure 7d–f). Often, these structures are found at the periphery of nucleoli (Figure 7g), and sometimes higher local concentrations of fibrillarin are observed at the interface, or the connecting region, between the two structures (Figure 7f). It was then important to determine whether fibrillarin alone or the entire snoRNP accumulated in these structures. To do so, we carried out in situ hybridization to localize U3 snoRNA, the most abundant member of box C/D snoRNPs. Figure 7g–i shows that U3 snoRNA, like fibrillarin, also accumulates outside of the nucleoli in the SMNΔN27-containing structures.

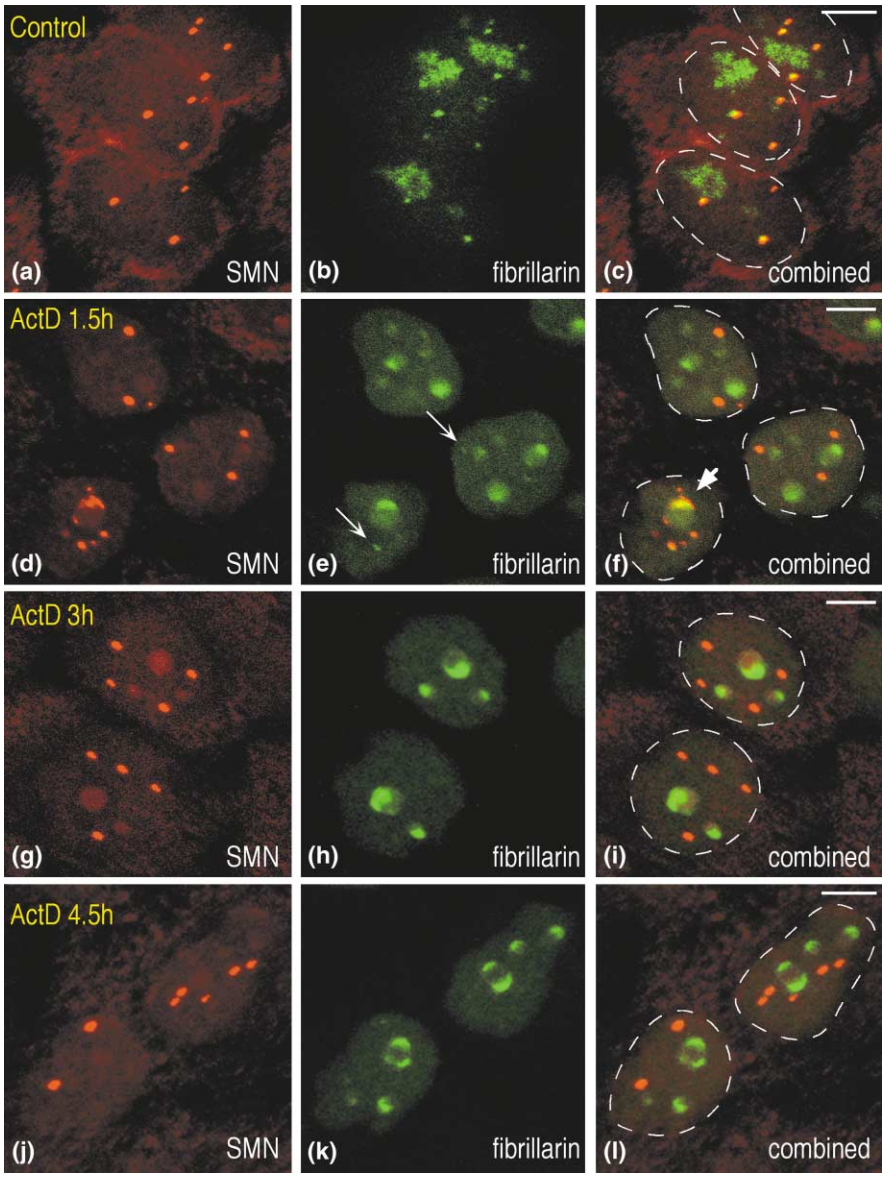
We also tested the effect of SMNΔN27 expression on the localization of GAR1. Consistent with its reported localization in untransfected cells [44], in cells transfected with SMNwt, GAR1 is localized to the nucleolus and CBs (Figure 7j–l). Figure 7m–o shows that GAR1 is also chased out of the nucleolus and accumulates in the SMNΔN27-containing structures. As observed for fibrillarin, higher local concentrations of GAR1 are observed at the interface between the two structures (Figure 7n).

Discussion

We demonstrated here that SMN binds directly to fibrillarin and GAR1, constituents of box C/D and box H/ACA snoRNPs, respectively. An interaction between SMN and fibrillarin was initially observed in yeast two-hybrid screening [10]. We showed that SMN binds directly to fibrillarin and GAR1 in vitro and that the evolutionarily conserved Y/G box, near the carboxyl terminus of SMN, is required for these interactions. Moreover, both SMN Y272C, the most common point mutant found in non-*SMN1*-deleted SMA patients, and SMNΔEx7, the main product of the *SMN2* gene (that lacks the amino acids encoded by exon 7), are strongly defective in these interactions. These results suggest that a defect in the association of SMN with snoRNP proteins likely contributes to the pathogenesis of SMA.

We also found that the arginine- and glycine-rich (RG) domains of both fibrillarin and GAR1 are necessary, but not sufficient, for the interaction of these proteins with SMN. These results strengthen the emerging view that SMN binds RG-rich domains. Several SMN-interacting partners such as SmB, SmD1, SmD3, LSm4, and RNA helicase A contain RG-rich regions that are necessary for SMN interaction [18, 21]. In the case of SmD1, SmD3, and LSm4, RG-rich domains are both necessary and sufficient for their interaction with SMN [21]. It is likely that SMN binds directly to the RG-rich regions of fibrillarin and GAR1, but either additional determinants in the context of the full-length proteins or the specific presentation of these domains is also critical and contributes to the avidity or specificity of the interaction. For example,

Figure 5

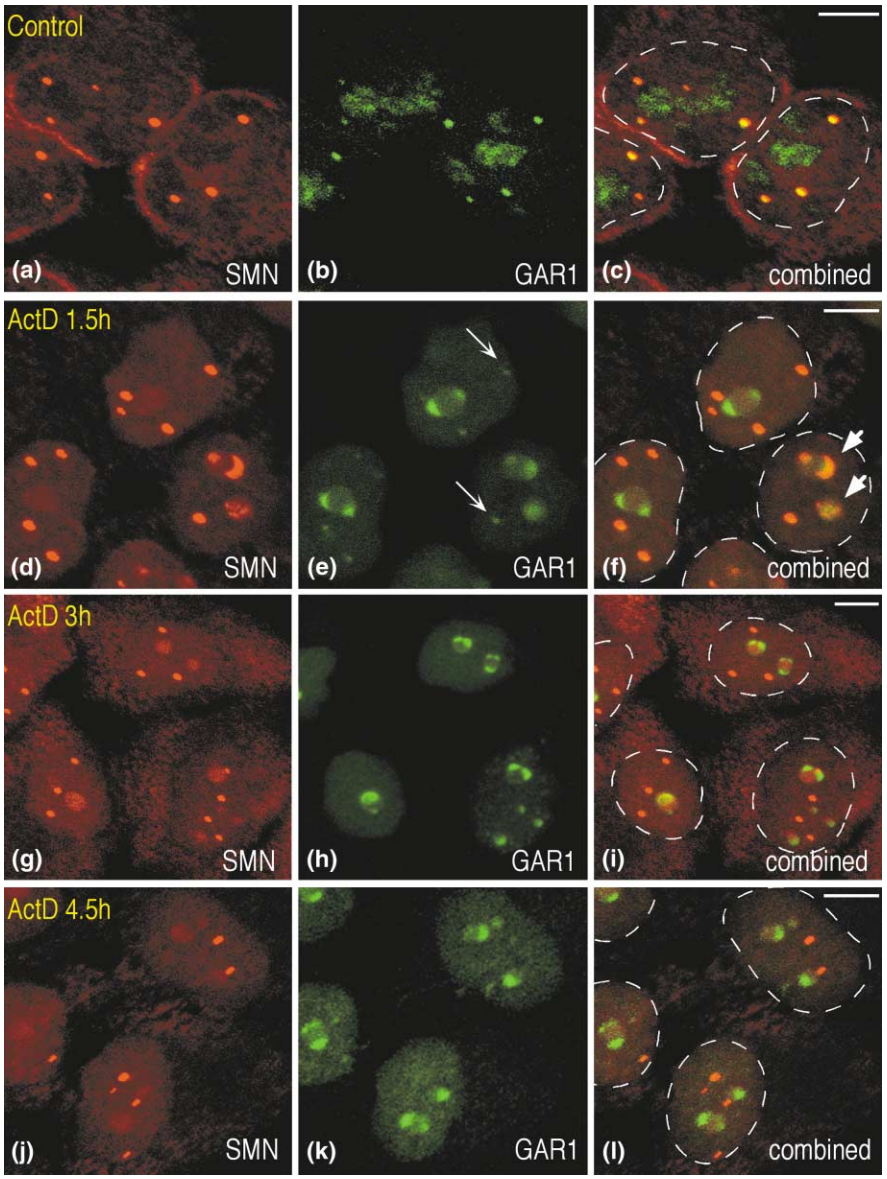


The dynamic association of SMN with fibrillarin in vivo. **(a–l)** Double-label confocal indirect immunofluorescence detection of SMN (a,d,g,j) and fibrillarin (b,e,h,k) in HeLa cells either untreated as a control (a–c) or treated with 0.04 μg/ml actinomycin D for 1.5 hr (d–f), 3 hr (g–i), and 4.5 hr (j–l). The respective combined images are shown in (c), (f), (i), and (l). Colocalization results in a yellow signal. Dashed lines demarcate the nucleus. The arrows in (e) indicate residual colocalization of fibrillarin and SMN in gems. The large arrowhead in (f) points to SMN colocalized with fibrillarin in the nucleolus. The scale bars represent 5 μm.

hnRNPA1 contains an RGG box, but it does not bind SMN (see Figure 1b).

The interaction of SMN with snoRNP proteins was further investigated in vivo. Coimmunoprecipitation experiments demonstrated that the SMN complex interacts with fibrillarin and GAR1 in vivo (see Figure 4). However, fibrillarin and GAR1 are not intrinsic components of the SMN complex and, similar to Sm and Lsm proteins and RNA helicase A [10, 18, 20, 21], we consider fibrillarin and GAR1 to be potential substrates of the SMN complex. Several pieces of evidence indicate a dynamic association between the SMN complex and snoRNPs in vivo. Fibrillarin and GAR1 (and CBs) colocalize with SMN (and gems), but this colocalization is lost upon treatment with low doses of actinomycin D, suggesting that it is dependent on RNA polymerase I transcription. Under the same treatments, a subset of SMN transiently associates with nucleoli. It is possible that the SMN complex associates with the nucleoli under normal conditions, but its association is too transient to be detectable and only becomes apparent at early times after the inhibition of RNA polymerase I transcription. The reorganization of snoRNPs upon expression of SMNΔN27 also points to a functional interaction between snoRNPs and the SMN complex in vivo. We observed that snoRNPs are depleted from the nucleolus and accumulate in the SMNΔN27-containing structures in cells expressing this dominant-negative mutant of SMN. In these cells, transcription is inhibited in both the nucleoplasm and the nucleolus [18], and the

Figure 6

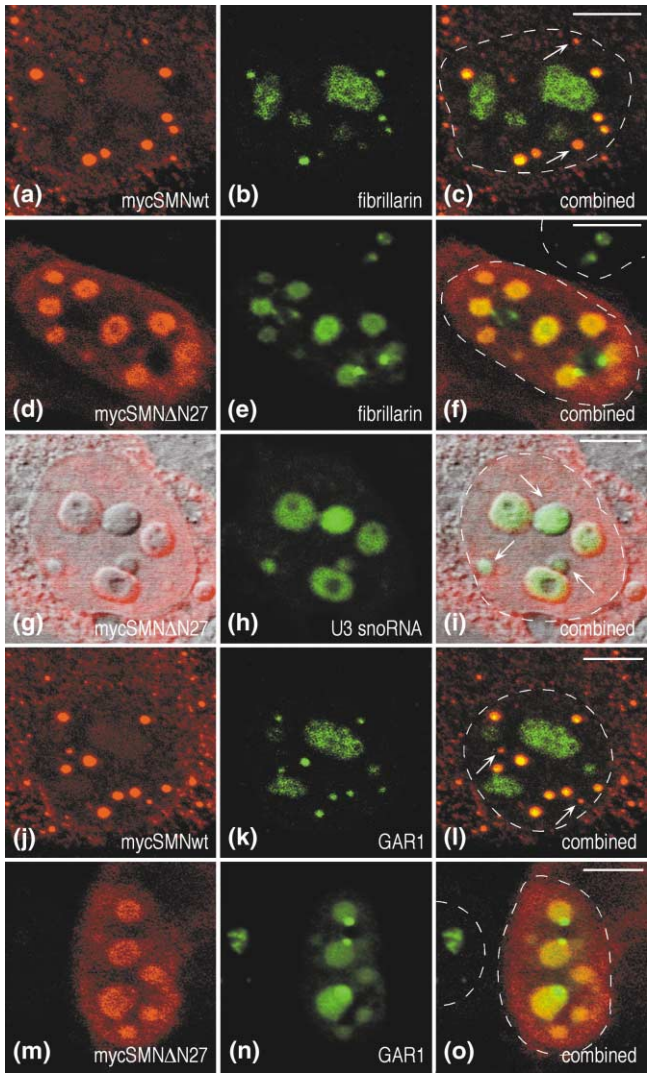


The dynamic association of SMN with GAR1 in vivo. **(a–l)** Double-label confocal indirect immunofluorescence detection of SMN (a,d,g,j) and GAR1 (b,e,h,k) in HeLa cells either untreated as a control (a–c) or treated with 0.04 μg/ml actinomycin D for 1.5 hr (d–f), 3 hr (g–i), and 4.5 hr (j–l). The respective combined images are shown in (c), (f), (i), and (l). Colocalization results in a yellow signal. Dashed lines demarcate the nucleus. The arrows in (e) indicate residual colocalization of GAR1 and SMN in gems. The large arrowheads in (f) point to SMN colocalized with GAR1 in the nucleolus. The scale bars represent 5 μm.

reorganization of snoRNPs may contribute to the inhibition of nucleolar transcription. Similarly, the expression of dominant-negative mutants of p80 coilin and Nopp140 have been shown to cause the reorganization of snoRNPs as well as inhibit transcription in the nucleolus [45, 46]. These findings pointed to a functional link between the nucleolus and CBs [47, 48], and our results suggest a functional link between the SMN complex and the function or metabolism of snoRNPs.

SnoRNAs are produced and assembled in the nucleus [47–49]. Some snoRNAs such as U3, the most abundant and well-characterized snoRNA, are transcribed by RNA polymerase II in the nucleoplasm and have a trymethylguanosine (TMG) cap structure at the 5′-end [50]. Most vertebrate snoRNAs of both classes do not contain a TMG cap, as they are excised by exonucleolytic processing from the introns of host genes coding for components of the translational machinery [51]. Specific proteins are thought to bind snoRNAs early in the snoRNP assembly pathway and contribute to their stability by protecting their extremities from exonucleolytic digestion. It has been suggested that snoRNP protein assembly takes place simultaneously, or shortly after, transcription/processing of the pre-mRNAs from which they are derived, and then they localize to the nucleolus [52]. In addition to the nucleolus, snoRNPs localize in CBs, but the functional relevance of their presence in CBs is not known. It seems plausible that snoRNPs, as part of their biogenesis pathway, associate with the SMN complex and, similar to the pathway

Figure 7



The expression of SMNΔN27 causes fibrillarin, U3 snoRNA, and GAR1 to accumulate outside the nucleolus. **(a–f)** Double-label confocal immunofluorescence experiments using anti-myc (a,d) and anti-fibrillarin (b,e) antibodies on HeLa cells transiently transfected with mycSMNwt (a–c) and mycSMNΔN27 (d–f). **(g–i)** Double-label confocal immunofluorescence experiments using anti-myc (g) and an oligonucleotide probe complementary to U3 (h) to detect U3 snoRNA by in situ hybridization of an HeLa cell transiently transfected with mycSMNΔN27. **(j–o)** Double-label confocal immunofluorescence experiments using anti-myc (j,m) and anti-GAR1 (k,n) antibodies on HeLa cells transiently transfected with mycSMNwt (j–l) and mycSMNΔN27 (m–o). Gems are indicated by arrows in (c) and (l). The differential interference contrast (DIC) image and anti-myc staining are shown combined in (g). Nucleoli are indicated by arrows in (i). The respective combined images are shown in (c), (f), (i), (l), and (o). Colocalization results in a yellow signal. Dashed lines demarcate the nucleus. The scale bars represent 5 μm.

recently proposed for the maturation of spliceosomal snRNPs [53], may transit through CBs. These nuclear structures likely represent large assemblies of functionally related macromolecules that form within the nucleus, because reciprocal interactions slow down their diffusion rates in the nucleoplasm [48]. Therefore, although high local concentrations of the components that function in this pathway (i.e., SMN complex and snoRNPs) can be observed as discrete structures at the light microscope level, their association and activities need not necessarily take place in these structures.

By analogy with the role of SMN in spliceosomal snRNP assembly [14–16], the interaction of SMN with fibrillarin and GAR1 likely reflects a function of the SMN complex in the biogenesis and assembly of snoRNPs. Moreover, this association may not only be limited to the initial assembly of new snoRNPs: because mature snoRNPs also appear to move continuously between nucleolus, nucleoplasm, and CBs [54, 55], this may reflect the need for a functional regeneration or recycling of snoRNPs akin to the one we have proposed for snRNPs after splicing [15]. We envision that this aspect of snoRNP metabolism also requires the functions of the SMN complex and is thus blocked by SMNΔN27.

Conclusions

We showed that fibrillarin and GAR1, constituents of box C/D and box H/ACA snoRNPs, respectively, bind directly to SMN, but not to SMN mutants found in some SMA patients. These findings further extend the range of targets of SMN interaction and indicate additional defects that may be associated with SMA. They also suggest a role for the SMN complex in snoRNP biogenesis and function. The observation that fibrillarin and GAR1 contain RGG domains that are required for SMN interaction strengthens the view that SMN binds arginine- and glycine-rich domains and suggests that these domains are a common feature necessary for substrate recognition by the SMN complex. The reorganization of snoRNPs in cells expressing the dominant-negative mutant SMNΔN27 points to a functional interaction of the SMN complex with snoRNPs in vivo. We propose that the functions of the SMN complex are not limited to the metabolism of spliceosomal snRNPs, but extend to snoRNPs and possibly additional RNPs, such as telomerase. We suggest that the SMN complex is a general macromolecular assembly machine for the biogenesis and functional maintenance of diverse RNPs.

Materials and methods

DNA constructs

The cDNAs encoding SMN, Gemin2, Gemin3, and Gemin4 were previously described [10–13]. Plasmids for expressing myc-tagged SMN wild-type and mutant proteins were previously described [15, 20]. The fibrillarin cDNA open reading frame (ORF) was previously isolated in a two-hybrid screening [10]. The GAR1 cDNA ORF was amplified by RT-PCR using total RNA extracted from HeLa cells. Myc-tagged wild-type and deletion mutants of fibrillarin and GAR1 were generated by PCR using appropriate oligos and cloned into pcDNA3 (InVitrogen) down-

stream of a sequence encoding to the myc (9E10) epitope. All constructs were analyzed by automated DNA-cycle sequencing.

Antibodies

Antibodies used in these experiments were as follows: mouse IgG1 monoclonal anti-SMN (2B1) [10]; mouse IgG1 monoclonal anti-Gemin2 (2E17) [11]; mouse IgG1 monoclonal anti-Gemin3 (11G9) [12]; mouse IgG1 monoclonal anti-Gemin4 (22C10) [13]; mouse IgG1 monoclonal anti-myc (9E10); affinity-purified rabbit polyserum anti-myc 9E10 epitope (A-14, Santa Cruz Biotech); affinity-purified rabbit polyserum anti-GAR1 [44]; and mouse IgG2a monoclonal anti-fibrillarin (72B9) [56].

Cell culture and immunofluorescence microscopy

HeLa cells, plated on glass coverslips, were transfected by the standard calcium phosphate method. To inhibit RNA polymerase I activity, HeLa cells were incubated in the presence of 0.04 μg/ml actinomycin D for the indicated times. Cells were fixed and processed by immunofluorescence staining and confocal microscopy as previously described [15]. In situ hybridization was performed using a biotinylated antisense oligonucleotide complementary to U3 (nucleotides 64–74) as previously described [57].

Immunoprecipitation experiments

293T cells were transfected by the standard calcium phosphate method. Immunoprecipitations were carried out in the presence of 0.25% Triton X-100 from nucleoplasmic extracts prepared 36 hr post transfection as previously described [20]. The antibody used for these experiments was anti-myc (9E10) mouse monoclonal antibody coupled to sepharose beads (Santa-Cruz). Immunoblotting was performed as previously described [11].

Recombinant protein production and in vitro protein binding assay

Purification of his-tagged SMN and the GST fusion of SMN and hnRNPA1 were described previously [10, 11, 15, 20]. Fibrillarin was cloned into pGEX-5X to obtain the amino-terminal GST fusion. Full-length GAR1 was cloned into pET-GST II vector to obtain a fusion protein containing the GST at the amino terminus and an in-frame 6His-tag at the carboxyl terminus. GST-GAR1 was purified by affinity chromatography on nickel beads and then bound to glutathione beads for the binding assays. The recombinant proteins were expressed in *E. coli* BL28 and purified by affinity chromatography on glutathione-Sepharose beads (Pharmacia) or Hybond beads (Novagen) according to the manufacturer's instructions. In vitro binding experiments were performed as previously described [20] using either purified recombinant proteins or proteins translated in vitro in the presence of [^{35}S]methionine or both [^{35}S]methionine and [^{35}S]cysteine (Amersham) as indicated.

Acknowledgements

We thank Dr. Greg Matera for the U3 antisense oligonucleotide probe and Drs. E. Tan and W. Filipowicz for anti-fibrillarin and anti-GAR1 antibodies, respectively. We are grateful to members of our laboratory for stimulating discussions and, in particular, to Drs. Westley Friesen and Zissimos Mourelatos for critical comments on this manuscript. This work was supported by a grant from the National Institutes of Health. G.D. is an Investigator of the Howard Hughes Medical Institute.

References

1. Melki J: **Spinal muscular atrophy.** *Curr Opin Neurol* 1997, **10:**381-385.
2. Lefebvre S, Burglen L, Reboullet S, Clermont O, Burlet P, Viollet L, *et al.*: **Identification and characterization of a spinal muscular atrophy-determining gene.** *Cell* 1995, **89:**155-165.
3. Burghes AHM: **When is a deletion not a deletion? When it is converted.** *Am J Hum Genet* 1997, **61:**9-15.
4. Lorson C, Hahnan E, Androphy EJ, Wirth B: **A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy.** *Proc Natl Acad Sci USA* 1999, **96:**6307-6311.
5. Coovert D, Le TT, McAndrew PE, Strasswimmer J, Crawford TO, Mendell JR, *et al.*: **The survival motor neuron protein in spinal muscular atrophy.** *Hum Mol Genet* 1997, **6:**1205-1284.
6. Lefebvre S, Burlet P, Liu Q, Bertrandy S, Clermont O, Munnich A, *et al.*: **Correlation between severity and SMN protein level in spinal muscular atrophy.** *Nat Genet* 1997, **16:**265-269.
7. Frugier T, Tiziano FD, Cifuentes-Diaz C, Miniou P, Roblot N, Dierich A, *et al.*: **Nuclear targeting defect of SMN lacking the C-terminus in a mouse model for spinal muscular atrophy.** *Hum Mol Genet* 2000, **9:**849-858.
8. Hsieh-Li HM, Chang JG, Jong YJ, Wu MH, Wang NM, Tsai CH, *et al.*: **A mouse model for spinal muscular atrophy.** *Nat Genet* 2000, **24:**66-70.
9. Monani UR, Sendtner M, Coovert DD, Parsons DW, Andreassi C, Le TT, *et al.*: **The human centromeric survival motor neuron gene (SMN2) rescues embryonic lethality in Smn$^{-/-}$ mice and results in a mouse with spinal muscular atrophy.** *Hum Mol Genet* 2000, **9:**333-339.
10. Liu Q, Dreyfuss G: **A novel nuclear structure containing the survival of motor neurons protein.** *EMBO J* 1996, **15:**3555-3565.
11. Liu Q, Fischer U, Wang F, Dreyfuss G: **The spinal muscular atrophy disease gene product, SMN, and its associated protein SIP1 are in a complex with spliceosomal snRNP proteins.** *Cell* 1997, **90:**1013-1028.
12. Charroux B, Pellizzoni L, Perkinson RA, Shevchenko A, Mann M, Dreyfuss G: **Gemin3, a novel DEAD box protein that interacts with SMN, the spinal muscular atrophy gene product, and is a component of gems.** *J Cell Biol* 1999, **147:**1181-1193.
13. Charroux B, Pellizzoni L, Perkinson RA, Yong J, Shevchenko A, Mann M, *et al.*: **Gemin4: a novel component of the SMN complex that is found in both gems and nucleoli.** *J Cell Biol* 2000, **148:**1177-1186.
14. Fischer U, Liu Q, Dreyfuss G: **The SMN-SIP1 complex has an essential role in spliceosomal snRNP biogenesis.** *Cell* 1997, **90:**1023-1029.
15. Pellizzoni L, Kataoka N, Charroux B, Dreyfuss G: **A novel function for SMN, the spinal muscular atrophy disease gene product, in pre-mRNA splicing.** *Cell* 1998, **95:**615-624.
16. Buhler D, Raker V, Luhrmann R, Fischer U: **Essential role for the tudor domain of SMN in spliceosomal U snRNP assembly: implications for spinal muscular atrophy.** *Hum Mol Genet* 1999, **8:**2351-2357.
17. Meister G, Buhler D, Laggerbauer B, Zobawa M, Lottspeich F, Fischer U: **Characterization of a nuclear 20S complex containing the survival of motor neurons (SMN) protein and a specific subset of spliceosomal Sm proteins.** *Hum Mol Genet* 2000, **9:**1977-1986.
18. Pellizzoni L, Charroux B, Rappsilber J, Mann M, Dreyfuss G: **A functional interaction between the survival motor neuron complex and RNA polymerase II.** *J Cell Biol* 2001, **152:**75-86.
19. Lorson CL, Strasswimmer J, Yao JM, Baleja JD, Hahnen E, Wirth B, *et al.*: **SMN oligomerization defect correlates with spinal muscular atrophy severity.** *Nat Genet* 1998, **19:**63-66.
20. Pellizzoni L, Charroux B, Dreyfuss G: **SMN mutants of spinal muscular atrophy patients are defective in binding to snRNP proteins.** *Proc Natl Acad Sci USA* 1999, **96:**11167-11172.
21. Friesen WJ, Dreyfuss G: **Specific sequences of the Sm and Sm-like (Lsm) proteins mediate their interaction with the spinal muscular atrophy disease gene product (SMN).** *J Biol Chem* 2000, **275:**26370-26375.
22. Bachellerie JP, Cavaille J: **Guiding ribose methylation of rRNA.** *Trends Biochem Sci* 1997, **22:**257-261.
23. Smith CM, Steitz JA: **Sno storm in the nucleolus: new roles for myriad small RNPs.** *Cell* 1997, **89:**669-672.
24. Lafontaine DL, Tollervey D: **Birth of the snoRNPs: the evolution of the modification-guide snoRNAs.** *Trends Biochem Sci* 1998, **23:**383-388.
25. Balakin AG, Smith L, Fournier MJ: **The RNA world of the nucleolus: two major families of small RNAs defined by different box elements with related functions.** *Cell* 1996, **86:**823-834.
26. Tollervey D, Lehtonen H, Carmo-Fonseca M, Hurt EC: **The small nucleolar RNP protein NOP1 (fibrillarin) is required for pre-rRNA processing in yeast.** *EMBO J* 1991, **10:**573-583.
27. Tollervey D, Lehtonen H, Jansen R, Kern H, Hurt EC: **Temperature-sensitive mutations demonstrate roles for yeast fibrillarin in pre-rRNA processing, pre-rRNA methylation, and ribosome assembly.** *Cell* 1993, **72:**443-457.
28. Kiss-Laszlo Z, Henry Y, Bachellerie JP, Caizergues-Ferrer M, Kiss T: **Site-specific ribose methylation of preribosomal RNA: a**

novel function for small nucleolar RNAs. *Cell* 1996, **85:**1077-1088.
29. Nicoloso M, Qu LH, Michot B, Bachellerie JP: **Intron-encoded, antisense small nucleolar RNAs: the characterization of nine novel species points to their direct role as guides for the 2′-O-ribose methylation of rRNAs.** *J Mol Biol* 1996, **260:**178-195.
30. Bousquet-Antonelli C, Henry Y, G'Elugne J, Caizergues-Ferrer M, Kiss T: **A small nucleolar RNP protein is required for pseudouridylation of eukaryotic ribosomal RNAs.** *EMBO J* 1997, **16:**4770-4776.
31. Ni J, Tien AL, Fournier MJ: **Small nucleolar RNAs direct site-specific synthesis of pseudouridine in ribosomal RNA.** *Cell* 1997, **89:**565-573.
32. Ganot P, Caizergues-Ferrer M, Kiss T: **The family of box ACA small nucleolar RNAs is defined by an evolutionarily conserved secondary structure and ubiquitous sequence elements essential for RNA accumulation.** *Genes Dev* 1997, **11:**941-956.
33. Weinstein LB, Steitz JA: **Guided tours: from precursor snoRNA to functional snoRNP.** *Curr Opin Cell Biol* 1999, **11:**378-384.
34. Tyc K, Steitz JA: **U3, U8 and U13 comprise a new class of mammalian snRNPs localized in the cell nucleolus.** *EMBO J* 1989, **8:**3113-3119.
35. Girard JP, Lehtonen H, Caizergues-Ferrer M, Amalric F, Tollervey D, Lapeyre B: **GAR1 is an essential small nucleolar RNP protein required for pre-rRNA processing in yeast.** *EMBO J* 1992, **11:**673-682.
36. Dragon F, Pogacic V, Filipowicz W: **In vitro assembly of human H/ACA small nucleolar RNPs reveals unique features of U17 and telomerase RNAs.** *Mol Cell Biol* 2000, **20:**3037-3048.
37. Bagni C, Lapeyre B: **Gar1p binds to the small nucleolar RNAs snR10 and snR30 in vitro through a nontypical RNA binding element.** *J Biol Chem* 1998, **273:**10868-10873.
38. Fatica A, Galardi S, Altieri F, Bozzoni I: **Fibrillarin binds directly and specifically to U16 box C/D snoRNA.** *RNA* 2000, **6:**88-95.
39. Liu Q, Siomi H, Siomi MC, Fischer U, Zhang Y, Wan L, *et al.*: **Molecular characterization of the protein products of the fragile X syndrome gene and the survival of motor neurons gene.** *Cold Spring Harb Symp Quant Biol* 1996, **61:**689-697.
40. Lorson CL, Androphy EJ: **The domain encoded by exon 2 of the survival motor neuron protein mediates nucleic acid binding.** *Hum Mol Genet* 1998, **7:**1269-1275.
41. Wirth B: **An update of the mutation spectrum of the survival motor neuron gene (SMN1) in autosomal recessive spinal muscular atrophy (SMA).** *Hum Mutat* 2000, **15:**228-237.
42. Matera AG, Frey MR: **Coiled bodies and gems: Janus or gemini?** *Am J Hum Genet* 1998, **63:**317-328.
43. Carvalho T, Almeida F, Calapez A, Lafarga M, Berciano MT, Carmo-Fonseca M: **The spinal muscular atrophy disease gene product, SMN: a link between snRNP biogenesis and the Cajal (coiled) body.** *J Cell Biol* 1999, **147:**715-727.
44. Pogacic V, Dragon F, Filipowicz W: **Human H/ACA small nucleolar RNPs and telomerase share evolutionarily conserved proteins NHP2 and NOP10.** *Mol Cell Biol* 2000, **20:**9028-9040.
45. Bohmann K, Ferreira JA, Lamond AI: **Mutational analysis of p80 coilin indicates a functional interaction between coiled bodies and the nucleolus.** *J Cell Biol* 1995, **131:**817-831.
46. Yang Y, Isaac C, Wang C, Dragon F, Pogacic V, Meier UT: **Conserved composition of mammalian box H/ACA and box C/D small nucleolar ribonucleoprotein particles and their interaction with the common factor Nopp140.** *Mol Biol Cell* 2000, **11:**567-577.
47. Lamond AI, Earnshaw WC: **Structure and function in the nucleus.** *Science* 1998, **280:**547-553.
48. Lewis JD, Tollervey D: **Like attracts like: getting RNA processing together in the nucleus.** *Science* 2000, **288:**1385-1389.
49. Filipowicz W, Pelczar P, Pogacic V, Dragon F: **Structure and biogenesis of small nucleolar RNAs acting as guides for ribosomal RNA modification.** *Acta Biochim Pol* 1999, **46:**377-389.
50. Terns MP, Dahlberg JE: **Retention and 5′ cap trimethylation of U3 snRNA in the nucleus.** *Science* 1994, **264:**959-961.
51. Maxwell ES, Fournier MJ: **The small nucleolar RNAs.** *Annu Rev Biochem* 1995, **64:**897-934.
52. Samarsky DA, Fournier MJ, Singer RH, Bertrand E: **The snoRNA box C/D motif directs nucleolar targeting and also couples snoRNA synthesis and localization.** *EMBO J* 1998, **17:**3747-3757.
53. Sleeman JE, Lamond AI: **Newly assembled snRNPs associate with coiled bodies before speckles, suggesting a nuclear snRNP maturation pathway.** *Curr Biol* 1999, **9:**1065-1074.
54. Snaar S, Wiesmeijer K, Jochemsen AG, Tanke HJ, Dirks RW: **Mutational analysis of fibrillarin and its mobility in living human cells.** *J Cell Biol* 2000, **151:**653-662.
55. Phair RD, Misteli T: **High mobility of proteins in the mammalian cell nucleus.** *Nature* 2000, **404:**604-609.
56. Reimer G, Pollard KM, Penning CA, Ochs RL, Lischwe MA, Busch H, *et al.*: **Monoclonal autoantibody from a (New Zealand black × New Zealand white) F1 mouse and some human scleroderma sera target an Mr 34,000 nucleolar protein of the U3 RNP particle.** *Arthritis Rheum* 1987, **30:**793-800.
57. Matera AG, Tycowski KT, Steitz JA, Ward DC: **Organization of small nucleolar ribonucleoproteins (snoRNPs) by fluorescence in situ hybridization and immunocytochemistry.** *Mol Biol Cell* 1994, **5:**1289-1299.