

Interaction between the small-nuclear-RNA cap hypermethylase and the spinal muscular atrophy protein, survival of motor neuron

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The biogenesis of spliceosomal small nuclear ribonucleoproteins (snRNPs) requires the cytoplasmic assembly of the Sm-core complex, followed by the hypermethylation of the small nuclear RNA (snRNA) 5' cap. Both the Sm-core complex and the snRNA trimethylguanosine cap are required for the efficient nuclear import of snRNPs. Here, we show that trimethylguanosine synthase 1 (TGS1), the human homologue of the yeast snRNA cap hypermethylase, interacts directly with the survival of motor neuron (SMN) protein. Both proteins are similarly distributed, localizing in the cytoplasm and in nuclear Cajal bodies. The interaction between TGS1 and SMN is disrupted by a mutation in *SMN* that mimics the predominant isoform of the protein that is expressed in patients with the neurodegenerative disease, spinal muscular atrophy. These data indicate that, in addition to its function in cytoplasmic Sm-core assembly, the SMN protein also functions in the recruitment of the snRNA cap hypermethylase.

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INTRODUCTION

Small nuclear ribonucleoproteins (snRNPs) are essential eukaryotic molecules that are required for the splicing of nuclear pre-messenger RNA (Yu *et al.*, 1999). In metazoans, the assembly of spliceosomal snRNPs is an ordered, multistep process that involves both nuclear and cytoplasmic phases (for a review, see Will & Lührmann, 2001). After transcription, the Sm-class small nuclear RNAs (snRNAs; U1, U2, U4 and U5) are exported to the cytoplasm. This step requires the binding of the snRNA 7-methylguanosine (m⁷G) cap

structure to the PHAX/CBC complex (Izaurralde *et al.*, 1995; Ohno *et al.*, 2000). In the cytoplasm, the snRNAs then associate with the survival of motor neuron (SMN) complex, which directs the assembly of the seven related Sm proteins, SmB/B', SmD1–3, SmE, SmF and SmG (Paushkin *et al.*, 2002; Meister *et al.*, 2002; Kambach *et al.*, 1999). The m⁷G cap is then hypermethylated to form a 2,2,7-trimethylguanosine (m₃G) cap structure. Both the assembled Sm core and the m₃G cap structure of the snRNAs provide signals for the efficient nuclear import of the newly assembled snRNPs (Fischer & Lührmann, 1990; Hamm *et al.*, 1990; Plessel *et al.*, 1994). Last, the m₃G cap structure is recognized by the snurportin1 (SPN1) protein, which enhances the nuclear import of U snRNPs (Huber *et al.*, 1998).

In mammals, the SMN complex is required for the assembly of the Sm core. Mutations in the human *SMN1* gene are responsible for the neuromuscular disease, spinal muscular atrophy (SMA; for a review, see Frugier *et al.*, 2002). SMN interacts directly with three of the Sm proteins by binding to the Arg- and Gly-rich carboxy-terminal tails of the SmB, SmD1 and SmD3 proteins (Friesen & Dreyfuss, 2000). Interestingly, these domains are dimethyl-arginine-modified, and such modifications are essential for strong interactions with SMN (Friesen *et al.*, 2001; Brahms *et al.*, 2000, 2001).

The methyltransferase that is responsible for m₃G cap formation has recently been identified (Mouaikel *et al.*, 2002; Verheggen *et al.*, 2002), but the mechanism linking Sm-core assembly to cap hypermethylation is unknown. The observation that snRNA cap hypermethylation takes place in the cytoplasm after Sm-core assembly, and the crucial function of the SMN complex in bringing snRNAs and Sm proteins together (Fischer *et al.*, 1997; Meister *et al.*, 2001; Pellizzoni *et al.*, 2002), led us to examine whether the SMN protein might also be involved in snRNA m₃G cap formation. Here, we show that the SMN protein and the human snRNA cap hypermethylase associate *in vivo* and *in vitro*, indicating that, in addition to its role in cytoplasmic Sm-core protein assembly, the SMN protein also functions in the cap hypermethylation process.

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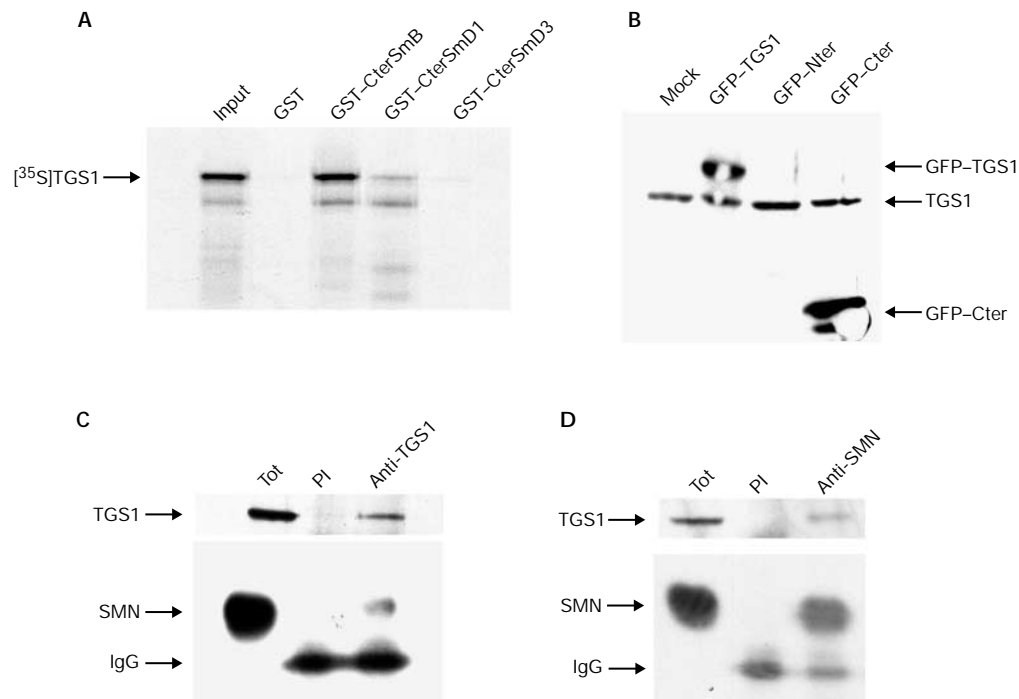


Fig. 1 | Interaction of human trimethylguanosine synthase 1 with the carboxyl tail of SmB and the survival of motor neuron protein. **(A)** The glutathione-*S*-transferase (GST) fusion proteins indicated were incubated with $[^{35}\text{S}]$ -labelled human trimethylguanosine synthase 1 (TGS1), which was prepared by *in vitro* transcription and translation using rabbit reticulocyte lysate. Bound proteins were analysed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). ‘Input’ indicates aliquots of radioactive proteins that corresponded to 10% of that used in each binding reaction. **(B)** Anti-TGS1 recognizes endogenous TGS1 as well as the green fluorescent protein (GFP)–TGS1 fusion protein and a fusion protein consisting of the carboxyl terminal of TGS1 tagged with GFP (GFP–Cter). The GFP fusion proteins indicated were transfected into HeLa cells and the extracts were separated by SDS–PAGE. Western blotting analysis was performed using anti-TGS1 antibodies. **(C)** The survival of motor neuron (SMN) protein was immunoprecipitated from a cytoplasmic extract using anti-TGS1 antibodies. Immunoprecipitation was performed using HeLa S100 extract and anti-TGS1 or preimmune serum (PI), and the proteins were immunoblotted with anti-TGS1 (upper panel) and anti-SMN (lower panel) antibodies. ‘Tot’ indicates 10% of the input. **(D)** TGS1 was immunoprecipitated from a cytoplasmic extract using anti-SMN antibodies. Immunoprecipitation was performed using HeLa cytoplasmic S100 extract and anti-SMN or PI, and the proteins were immunoblotted with anti-TGS1 (upper panel) and anti-SMN (lower panel) antibodies.

RESULTS

SMN and TGS1 proteins form a complex *in vivo* and *in vitro*

To analyse the relationship between the SMN protein and snRNA cap hypermethylation, we first cloned the human orthologue of the yeast trimethylguanosine synthase, Tgs1 (data not shown). The full-length *TGS1* open reading frame encodes an 852 amino-acid protein, which comprises a carboxy-terminal region that contains conserved methyltransferase motifs, and a large amino-terminal domain that has no obvious similarities to other known proteins (Mouaikel *et al.*, 2002). In yeast, the cap hypermethylase associates preferentially with the C-terminal extension of the SmB protein (Mouaikel *et al.*, 2002). We therefore tested the interactions of TGS1 using *in vitro* glutathione-*S*-transferase (GST) pull-down assays. $[^{35}\text{S}]$ methionine-labelled TGS1 strongly bound a GST fusion that contained the C-terminal tail of human SmB, bound less efficiently to a fusion containing the SmD1 C terminus, and did not bind significantly to a fusion containing the C terminus of SmD3 (Fig. 1A). These data indicate that TGS1, like its yeast counterpart, binds preferentially to the C-terminal extension of SmB.

Next, we used antibodies against the C-terminal domain of the human hypermethylase to characterize a possible interaction between SMN and TGS1 in mammalian cells. As expected, western blot analysis (Fig. 1B) showed that these antibodies specifically recognize the endogenous TGS1 protein as well as green fluorescent protein (GFP)–TGS1 and the GFP–Cter fusion, which carries the C-terminal domain of TGS1 (amino acids 478–852). By contrast, the GFP–Nter fusion protein, which carries the amino-terminal domain (amino acids 1–477), was not detected. Immunoprecipitation experiments that used these anti-TGS1 antibodies and a cytoplasmic extract showed that the SMN protein coprecipitated with TGS1 (Fig. 1C). The TGS1 protein was also co-immunoprecipitated by an anti-SMN antibody (Fig. 1D). Thus, these proteins form part of an interrelated complex *in vivo*.

To show that TGS1 and SMN interact directly, we performed *in vitro* GST pull-down assays. As shown in Fig. 2A, $[^{35}\text{S}]$ -labelled SMN bound quantitatively to GST–TGS1 and GST–Cter, whereas no significant interaction was detected with the GST–Nter fusion. By contrast, the N-terminal domain of TGS1 interacted strongly with the human SmB protein, whereas the C-terminal domain did not

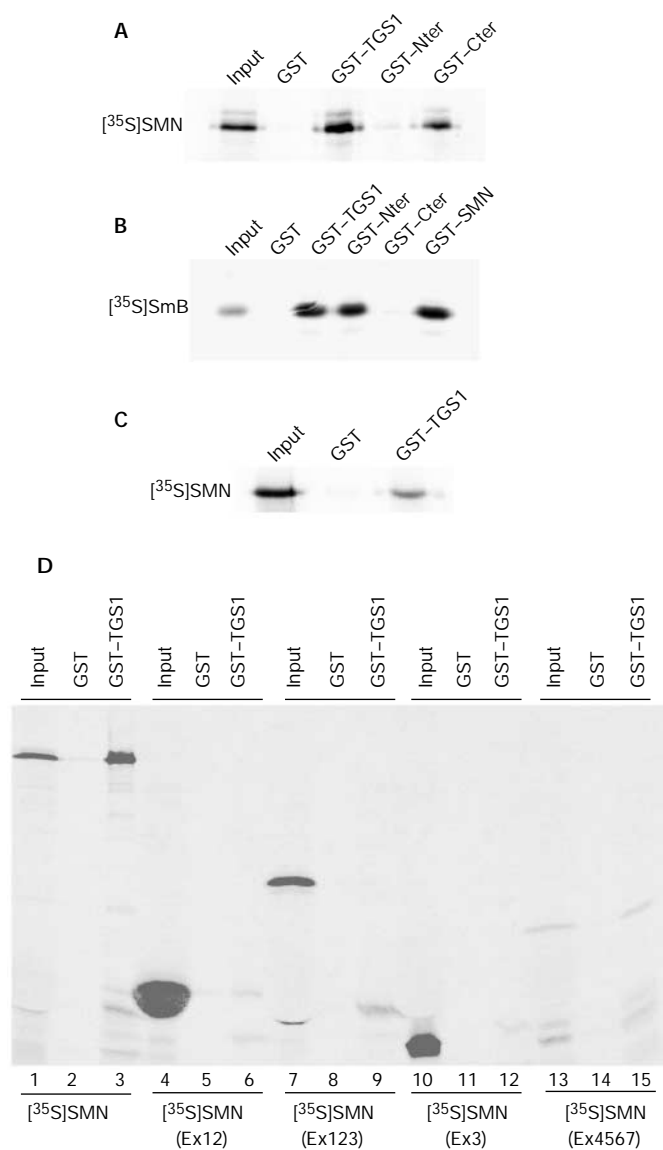


Fig. 2 | Direct binding of human trimethylguanosine synthase 1 to the survival of motor neuron protein. **(A)** Binding assays using recombinant glutathione-S-transferase (GST)–trimethylguanosine synthase 1 (TGS1) and fusions of GST to the amino and carboxyl termini of TGS1 (GST–Nter and GST–Cter, respectively) with [³⁵S]-labelled survival of motor neuron (SMN) that was *in vitro* translated using rabbit reticulocyte lysate. Bound proteins were analysed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and autoradiography. **(B)** The GST fusion proteins indicated were tested for binding with [³⁵S]-labelled SmB protein and treated as described for **(A)**. **(C)** Binding of the GST–TGS1 protein to *in vitro* translated [³⁵S]-labelled SMN using an S30 bacterial extract. Proteins bound to GST beads were analysed by SDS–PAGE and autoradiography. **(D)** The C-terminal region of SMN mediates the interaction with TGS1. The indicated [³⁵S]-labelled SMN proteins were mixed with the GST–TGS1 or GST proteins, and bound proteins were analysed by SDS–PAGE and autoradiography. Note that the amount of [³⁵S]-labelled SMN (Ex4567) input is lower compared with the other [³⁵S]-labelled SMN domains. ‘Input’ indicates aliquots of radioactive proteins that corresponded to 10% of that used in each binding reaction.

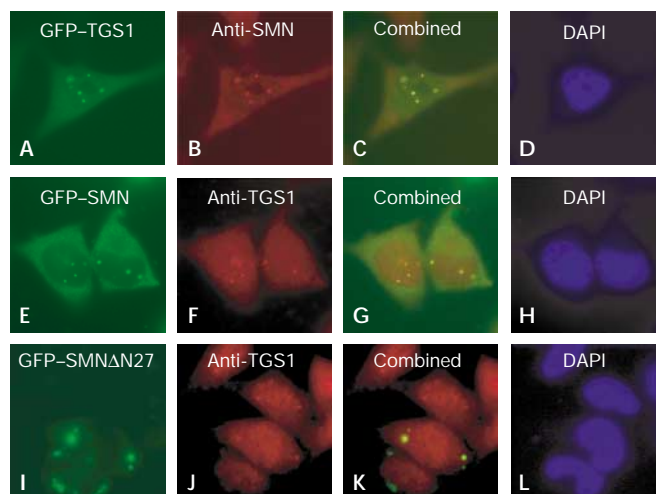


Fig. 3 | Colocalization of the human trimethylguanosine synthase 1 and survival of motor neuron proteins. HeLa cells carrying green fluorescent protein (GFP)–trimethylguanosine-synthase-1 (TGS1; upper row), GFP–survival-of-motor-neuron (SMN; middle row) or GFP–SMNΔN27 (lower row) constructs were examined by immunofluorescence microscopy using anti-SMN or anti-TGS1 antibodies. Colocalization of the GFP fusion proteins (green) and SMN or TGS1 (red) is indicated by the yellow colour in the combined panels. DNA was stained with 4,6-diamidino-2-phenylindole (DAPI; blue).

(Fig. 2B). The separability of the two binding activities suggested that the interaction between TGS1 and SMN was not mediated by Sm proteins. To definitively rule out the possibility that the interaction between TGS1 and SMN was due to other proteins in the rabbit reticulocyte lysate, we performed pull-down assays using [³⁵S]-labelled SMN that was translated from a bacterial extract. As shown in Fig. 2C, [³⁵S]-labelled SMN bound efficiently to GST–TGS1. These results show that TGS1 can interact directly and simultaneously with the SMN and SmB proteins, as the C-terminal domain of TGS1 binds to SMN and to the N-terminal domain of SmB.

To characterize the domains of the SMN protein that are involved in the interaction with TGS1, we tested the ability of various SMN deletion mutants to bind recombinant GST–TGS1. Fig. 2D shows that both wild-type SMN (lane 3) and the SMN(Ex4567) mutant (lane 15) bind the GST–TGS1 fusion. By contrast, the SMN(Ex12), SMN(Ex123) and SMN(Ex3) constructs (Fig. 2D, lanes 6, 9 and 12, respectively) failed to bind efficiently to GST–TGS1. These experiments show that the region containing exons 4–7 of the SMN protein is sufficient for interaction with TGS1.

Similar subcellular distributions of SMN and TGS1

As described above, SMN is distributed diffusely throughout the cytoplasm, and also accumulates in nuclear Cajal bodies (Liu & Dreyfuss, 1996; Matera & Frey, 1998; Carvalho *et al.*, 1999). Fluorescence microscopy of HeLa cells after transient transfection of a GFP–TGS1 construct shows that the fusion protein localizes similarly to SMN: it is present in Cajal bodies and is distributed diffusely in the cytoplasm (Fig. 3A–D). As expected, a GFP–SMN fusion also localizes diffusely throughout the cytoplasm, and also accumulates in nuclear Cajal bodies (Fig. 3E). Immunocytochemistry using TGS1

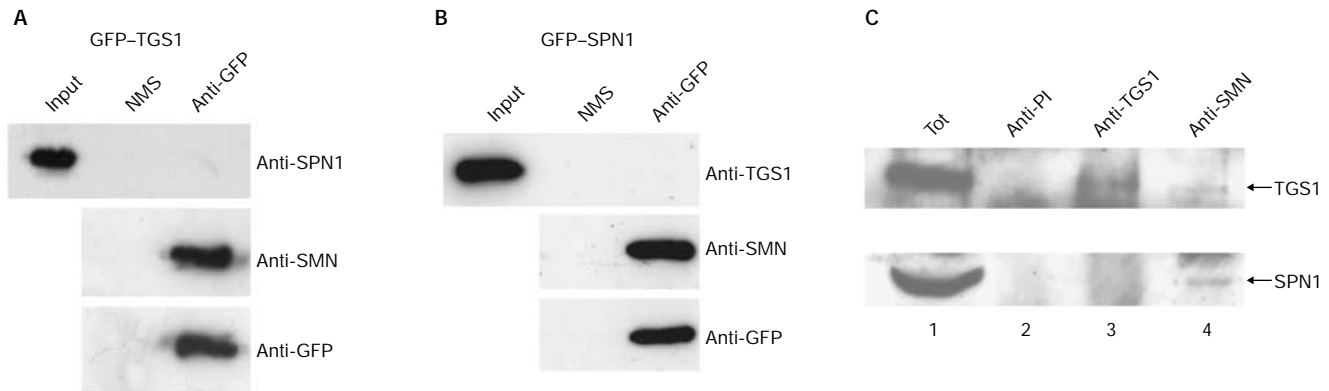


Fig. 4 | Snurportin 1 does not form a complex with human trimethylguanosine synthase 1 *in vivo*. **(A)** HeLa cells were transiently transfected with green fluorescent protein (GFP)-trimethylguanosine-synthase-1 (TGS1) and total cell lysates were immunoprecipitated with anti-GFP antibodies. The immunoprecipitates were then analysed by western blotting using the antibodies indicated. Normal mouse serum (NMS) was used as a non-immune control. **(B)** HeLa cells were transfected with GFP-snurportin 1 (SPN1) and immunoprecipitation experiments were performed either with NMS or with anti-GFP antibodies. Blots were probed with the indicated antibodies. **(C)** Cytoplasmic S100 extract was immunoprecipitated with anti-TGS1, anti-survival-of-motor-neuron (SMN) and preimmune serum (PI). Immunoprecipitates were analysed by SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-TGS1 (upper panel) and anti-SPN1 (lower panel) antibodies. 'Tot' indicates 2% of the input. 'Input' indicates aliquots of radioactive proteins that corresponded to 2% of that used in each binding reaction.

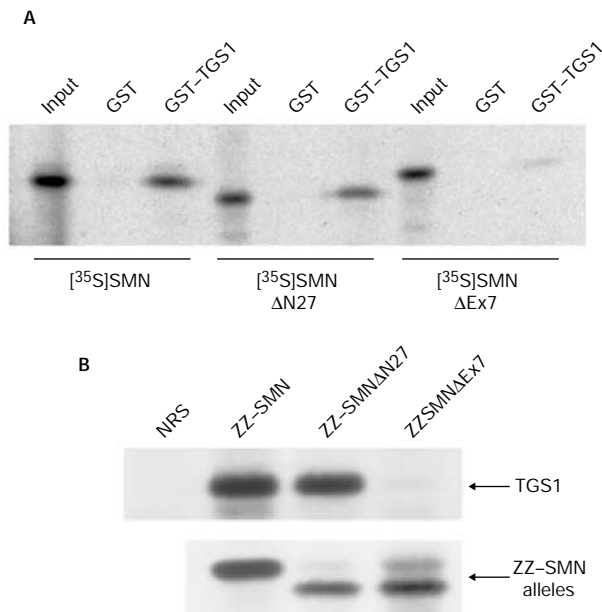


Fig. 5 | Disruption of the interaction between the SMN Δ Ex7 mutant and human trimethylguanosine synthase 1. **(A)** *In vitro* translated [35 S]-labelled SMN, [35 S]-labelled SMN Δ N27 and [35 S]-labelled SMN Δ Ex7 were incubated with recombinant glutathione-S-transferase (GST)-trimethylguanosine-synthase-1 (TGS1) or GST. Bound proteins were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. **(B)** HeLa cells were transfected with the ZZ-tagged SMN alleles indicated and immunoprecipitation experiments were performed with either normal rabbit serum (NRS) or rabbit IgG and protein-A-sepharose beads. Immunocomplexes were then separated by SDS-PAGE and immunoblotted with anti-TGS1 (upper panel) or anti-SMN (lower panel) antibodies. SMN, survival of motor neuron protein. 'Input' indicates aliquots of radioactive proteins that corresponded to 10% of that used in each binding reaction.

antibodies also shows that the hypermethylase distributes similarly to SMN, localizing in the cytoplasm and in nuclear Cajal bodies (Fig. 3F; Verheggen *et al.*, 2002). The colocalization of the SMN and TGS1 proteins was confirmed using a dominant-negative SMN mutant (SMN Δ N27), which causes a rearrangement of snRNPs and gives rise to cytoplasmic aggregates that contain endogenous SMN complexes, Sm proteins and m⁷G-capped snRNAs (Pellizzoni *et al.*, 1998). We investigated whether the expression of SMN Δ N27 had an effect on the subcellular localization of TGS1 in HeLa cells. As shown in Fig. 3I-L, TGS1 accumulated with the GFP-SMN Δ N27 protein in cytoplasmic aggregates, showing that this mutant construct prevents the formation of subnuclear bodies containing TGS1 and SMN. It is interesting to note that expression of the GFP-SMN Δ N27 fusion did not generate the nuclear inclusions seen by Pellizzoni *et al.* (1998) using Myc-SMN Δ N27. The differences might be because of variations in protein expression levels due to the vectors used in both studies.

TGS1 does not form a complex with snurportin 1

It has been reported recently that the SMN complex is associated with m³G-capped snRNPs bound to the SPN1 protein (Narayanan *et al.* 2002; Massenet *et al.*, 2002). We therefore tested whether TGS1 is also part of this complex. To test this, we transfected HeLa cells with GFP-TGS1, immunoprecipitated the total cell lysates with monoclonal antibodies against GFP, and probed a western blot with anti-GFP and anti-SPN1 antibodies. As shown in Fig. 4A, no SPN1 was co-immunoprecipitated, indicating that TGS1 and SPN1 do not form a complex *in vivo*. Similarly, in cells transfected with a GFP-SPN1 construct, immunoprecipitation with anti-GFP antibodies did not recover TGS1 (Fig. 4B). By contrast, in both experiments, the SMN protein was immunoprecipitated with anti-GFP antibodies (Fig. 4A,B), confirming that SMN associates with both GFP-TGS1 and GFP-SPN1 *in vivo*. The idea that TGS1 and SPN1 do not form a complex *in vivo* is further supported by the inability of anti-TGS1 antibodies to pull down endogenous SPN1. As shown in Fig. 4C (lane

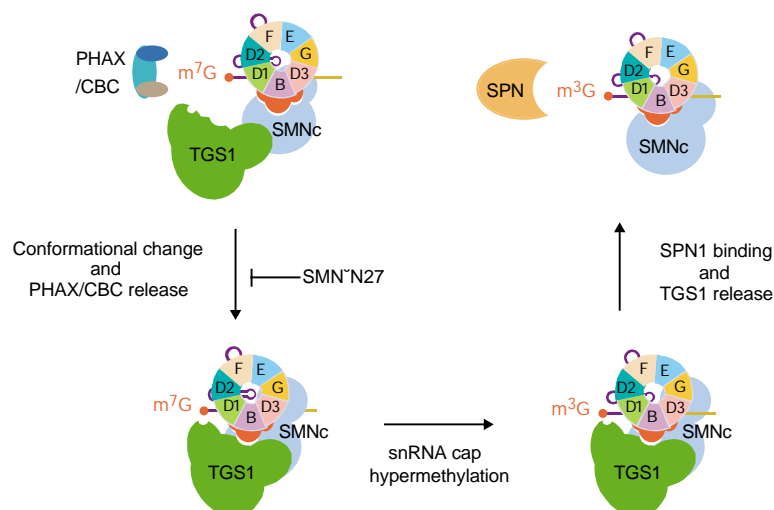


Fig. 6 | Model of TGS1 and SMN interactions in cytoplasmic snRNP biogenesis. Filled red circles represent the carboxy-terminal extensions of SmB, SmD1 and SmD3 proteins. The defect of the SMN Δ N27 mutant is also indicated. See text for more details. m₃G, 2,2,7-trimethylguanosine cap; m⁷G, 7-methylguanosine cap; PHAX/CBC; SMNc, survival of motor neuron complex; snRNP, small nuclear ribonucleoprotein; SPN1, snurportin 1; TGS1, trimethylguanosine synthase 1.

3), anti-TGS1 antibodies failed to immunoprecipitate SPN, whereas in control experiments anti-SMN (Narayanan *et al.*, 2002; Massenet *et al.*, 2002) recovered both TGS1 and SPN1 (Fig. 4C, lane 4).

Disrupted SMN Δ Ex7 and TGS1 interaction

In view of the pathology of SMA and the interaction between SMN and TGS1, we tested whether the hypermethylase is able to form a complex with SMN mutant proteins *in vitro* and *in vivo*. As shown in Fig. 5A, GST pull-down assays showed that binding of the [³⁵S]-labelled SMN Δ Ex7 construct to a GST-TGS1 protein was greatly impaired. Moreover, the [³⁵S]-labelled SMN Δ N27 mutant had a binding activity similar to that of wild-type SMN. To determine whether the association between the TGS1 and SMN Δ Ex7 proteins was disrupted *in vivo*, we transiently transfected plasmids carrying SMN, SMN Δ N27 and SMN Δ Ex7 constructs tagged with ZZ (a fragment of protein A). Whole-cell extracts were prepared from these cells and, as shown in Fig. 5B (lower panel), the ZZ-SMN, ZZ-SMN Δ N27 and ZZ-SMN Δ Ex7 fusion proteins were immunoprecipitated with rabbit IgG. The TGS1 protein was co-immunoprecipitated from cells expressing the ZZ-SMN and ZZ-SMN Δ N27 constructs (Fig. 5B, upper panel), but only a very small amount was detected in the pellet of extracts prepared from cells expressing ZZ-SMN Δ Ex7 (Fig. 5B). These results show that the interaction of the hypermethylase with SMN is disrupted by the SMN mutation that is most commonly present in SMA patients.

DISCUSSION

We show here, for the first time, that the SMN protein binds directly to the human snRNA cap hypermethylase both *in vitro* and *in vivo*. Our data suggest a role for the SMN protein complex in the recruitment of TGS1, suggesting a function for the SMN complex in the formation of the snRNA m₃G cap structure. As SMN is essential for the assembly of the Sm core complex in higher eukaryotes, our results also suggest that the SMN complex can be seen as a machine that participates in the generation of the bipartite nuclear localization signal of snRNPs.

The human snRNA cap hypermethylase, like its yeast counterpart, binds preferentially to the C-terminal extension of SmB (Fig. 1A). These findings are consistent with a previous report that suggested that the human snRNA-(guanosine-N2)-methyltransferase recognizes U1 snRNP by binding to the SmB/B' proteins (Plessel *et al.*, 1994). As the C-terminal extension of SmB also interacts with SMN (Friesen & Dreyfuss, 2000; Friesen *et al.*, 2001; Brahm *et al.*, 2001), our results show that both TGS1 and SMN recognize, at least in part, a similar interaction determinant in the heptameric Sm-core-complex. Whether SMN and TGS1 interact consecutively or simultaneously with the C-terminal extension of SmB cannot be concluded from our results and will require further competition experiments.

The interaction between the SMN and TGS1 proteins that we describe in this report are consistent with two recent studies that showed that the SMN complex is associated with snRNPs during the various cytoplasmic steps of snRNP maturation (Narayanan *et al.*, 2002; Massenet *et al.*, 2002). Indeed, SMN was shown to associate with both m⁷G- and m₃G-capped snRNAs, suggesting that the SMN complex is present before and after the snRNA cap hypermethylation process. Consistent with these results, we show that SMN interacts directly with the hypermethylase. A role for SMN in m₃G cap formation is further supported by the fact that the dominant-negative mutant, SMN Δ N27, blocks the snRNP maturation pathway in the cytoplasm in a step that precedes cap hypermethylation (Pellizzoni *et al.*, 1998). A model that shows the relationships between SMN and TGS1 in snRNP assembly is shown in Fig. 6. We suggest that after promoting the formation of the Sm ring, the SMN complex allows the engagement of TGS1 with the m⁷G-capped snRNP particle. As shown in the model, a conformational change might then allow dissociation of the interaction between the C terminus of SmB and the SMN complex, followed by the association of the SmB C terminus with the hypermethylase. Such a step could also allow the concomitant release of the PHAX/CBC complex (see below) and proper folding of the TGS1 protein in order to bring the m⁷G cap structure to the catalytic centre and allow m₃G cap formation. The

association of the SPN1 protein with the m₃G cap structure could then induce the loss of the hypermethylase from this complex and generate import-competent snRNPs. This idea is consistent with our finding that no SPN1–TGS1 complex was detected in HeLa cells in co-immunoprecipitation experiments (Fig. 4).

Our subcellular localization studies (Fig. 3) show that TGS1 is found in the cytoplasmic accumulations that are generated by the expression of the SMN Δ N27 mutant in HeLa cells (Pellizzoni *et al.*, 1998). The fact that PHAX is also found in similar aggregates (Massenet *et al.*, 2002) indicates that both PHAX and TGS1 could also be part of the same functional complex. It is not yet known how and when the PHAX/CBC complex is released from m⁷G-capped snRNPs. According to our model (Fig. 6), following the initial binding of TGS1 to SMN, a conformational change is required both for the release of the PHAX/CBC complex and for the correct positioning of the hypermethylase with respect to the m⁷G cap structure. The hypermethylation defect of the SMN Δ N27 allele could therefore be a consequence of an inability of this mutant to undergo such a conformational change.

It is not yet known whether the observed weak *in vitro* and *in vivo* interactions of the hypermethylase with the SMN Δ Ex7 isoform result in a defect in snRNA cap hypermethylation. The correlation between the binding of TGS1 with SMN and snRNA m₃G cap formation remains to be demonstrated directly and will require the development of an *in vitro* hypermethylation assay. However, our finding that SMN and TGS1 interact directly strengthens the view of the SMN complex as a machine that is involved in the formation of import-competent snRNPs (Massenet *et al.*, 2002; Narayanan *et al.*, 2002). Accordingly, it has recently been shown that reduced SMN/Gemin 2 protein levels lead to reduced nuclear accumulation of U snRNPs and to enhanced motoneuron degeneration (Jablonka *et al.*, 2002). These observations suggest that impaired generation of the U-snRNP bipartite nuclear localization signal represents an important biochemical defect that is responsible for the SMA disease.

METHODS

Cloning of complete trimethylguanosine synthase 1 complementary DNA and plasmid constructs. The full-length TGS1 cDNA was obtained by PCR and RT–PCR (PCR after reverse transcription) using oligonucleotides based on the sequence of the human orthologue (GenBank accession number AY028423) of the yeast trimethylguanosine synthase, Tgs1 (Mouaikel *et al.*, 2002). A human cDNA library and Image clone 4513665 (GenBank accession number BG287526) were used to amplify the corresponding DNA fragments.

GFP-tagged and ZZ-tagged wild-type and deletion SMN constructs were generated by PCR using appropriate oligonucleotides and were cloned into the pEGFP vector (Clontech) and a version of the pEGFP vector that lacks the GFP coding region (for the ZZ-tagged constructs).

Cell culture and immunocytochemistry. HeLa cells were grown at 37 °C in 5% CO₂, in DMEM containing 10% FCS. Cells were transfected using the calcium phosphate coprecipitation procedure as described previously (Verheggen *et al.*, 2002). HeLa cell extracts were prepared as described in Narayanan *et al.* (2002).

Immunofluorescence was performed on cells grown on coverslips, washed in PBS and fixed in 4% (w/v) paraformaldehyde in PBS for 15 min at 25 °C. Permeabilization was performed using

0.5% Triton-X100 in PBS for 5 min at 25 °C or in acetone (3 min at –20 °C). Anti-SMN (Transduction Laboratories) and anti-TGS1 rabbit polyclonal antibodies (Verheggen *et al.*, 2002) were diluted 1 in 500. Incubations were performed for 1 h at 25 °C in PBS containing 2% BSA, and slides were then washed three times for 10 min in PBS. Secondary antibodies, which were coupled to Cy3, were diluted to 1 in 2000 and were used in the same conditions. Coverslips were mounted on glass slides using mounting medium (Vectorshield) and were observed as described previously (Verheggen *et al.*, 2002).

Glutathione-S-transferase pull-down assays and immunoprecipitations. Plasmids carrying GST fusions were transformed into *Escherichia coli* BL21 cells, which were grown in LB medium containing ampicillin and 20 mM glucose; expression was induced by the addition of 0.5 mM isopropyl-*D*-thiogalactoside. Recombinant proteins were purified using GST beads (Amersham Pharmacia). *In vitro* transcriptions using T7 RNA polymerase and translations using rabbit reticulocyte lysate (TnT) or bacterial extract (S30) were performed in the presence of [³⁵S]methionine, in accordance with the manufacturer's instructions (Promega). *In vitro* protein–protein interaction studies were carried out as already described (Mouaikel *et al.*, 2002). Immunoprecipitation experiments were performed as described previously (Mouaikel *et al.*, 2002; Narayanan *et al.*, 2002). Immunocomplexes were detected with horseradish-peroxidase-conjugated sheep-anti-mouse or peroxidase-conjugated immunopure protein A/G (Pierce), followed by chemiluminescence.

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