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SECIS-binding protein 2 interacts with the SMN complex and the methylosome for selenoprotein mRNP assembly and translation

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ABSTRACT

Selenoprotein synthesis requires the co-translational recoding of a UGA_{Sec} codon. This process involves an RNA structural element, called Selenocysteine Insertion Sequence (SECIS) and the SECIS binding protein 2 (SBP2). Several selenoprotein mRNAs undergo unusual cap hypermethylation by the trimethylguanosine synthase 1 (Tgs1), which is recruited by the ubiquitous Survival of MotoNeurons (SMN) protein. SMN, the protein involved in spinal muscular atrophy, is part of a chaperone complex that collaborates with the methylosome for RNP assembly. Here, we analyze the role of individual SMN and methylosome components in selenoprotein mRNP assembly and translation. We show that SBP2 interacts directly with four proteins of the SMN complex and the methylosome core proteins. Nevertheless, SBP2 is not a methylation substrate of the methylosome. We found that both SMN and methylosome complexes are required for efficient translation of the selenoprotein GPx1 *in vivo*. We establish that the steady-state level of several selenoprotein mRNAs, major regulators of oxidative stress damage in neurons, is specifically reduced in the spinal cord of SMN-deficient mice and

that cap hypermethylation of GPx1 mRNA is affected. Altogether we identified a new function of the SMN complex and the methylosome in selenoprotein mRNP assembly and expression.

INTRODUCTION

Selenoproteins are a family of proteins, characterized by the presence of the 21st amino acid selenocysteine (Sec) in their active site, whose members exhibit a wide range of essential functions (1). Twenty-five genes coding for selenoproteins have been identified in the human genome (2). Key players of oxidative stress protection, they also play roles in thyroid hormone metabolism, immune function, male fertility and muscle development (3,4). Selenoprotein synthesis requires the co-translational recoding of an in-frame UGA_{Sec} codon into selenocysteine that would otherwise be read as a stop codon (5,6). Selenoprotein mRNAs are thus submitted to distinctive translation but also biogenesis pathways (7,8). In mammals, this process involves the assembly of RNA–protein (RNP) complexes on specific stem-loops located in the 3' untranslated region (3'UTR) of selenoprotein mRNAs, called Selenocysteine Insertion Sequences (SECIS) (9–12). Essential to this process is the SECIS binding protein 2 (SBP2) that interacts with the SECIS RNA and recruits translation and assembly factors to the mRNP (9,13–14); amongst them, the specialized elongation factor eEF_{Sec} in complex with the selenocysteyl-tRNA^{Sec} (15–18).

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Other SECIS-RNA binding proteins, such as the ribosomal L30 protein, translation initiation factor 4A3 (eIF4A3) and nucleolin, have been identified and are suggested to be modulators of the recoding mechanism (19–21). Correct assembly of proteins on the 3'UTR of selenoprotein mRNAs is a pre-requisite for their translation (5–7). We have previously shown that this assembly mechanism is similar to that of several small non-coding RNPs, such as spliceosomal snRNPs (small nuclear RNPs), snoRNPs (small nucleolar RNPs) involved in ribosome biogenesis and telomerase RNPs (7). Indeed, SBP2 shares a common RNA binding domain with core proteins of the sn- and snoRNPs (22–24) and proper selenoprotein mRNP assembly relies on interactions between SBP2 and the conserved RNP assembly machinery linked to the protein chaperone Hsp90 (7). More recently we have demonstrated that like UsnRNAs and some snoRNAs, several selenoprotein mRNAs also undergo 5' cap maturation events and bear hypermethylated m₃^{2,2,7}G cap structures instead of the classical 7-methylguanosine (m⁷G) cap. This modification is required for the expression of selenoprotein GPx1 *in vivo* (8). The trimethylguanosine synthase 1 (Tgs1) (25,26) is responsible for cap hypermethylation of selenoprotein mRNAs (8) and is recruited *via* interactions with the same assembly chaperones and core proteins that are devoted to sn- and snoRNP cap maturation (27,28). The assembly chaperone survival of motor neuron (SMN) plays a major role in this process by interacting with both SBP2 and Tgs1 (8).

SMN is ubiquitously expressed, and reduced levels or loss-of function of this protein are responsible for spinal muscular atrophy (SMA), a severe motor neuron disease characterized by the degeneration of the lower motor neurons leading to muscular weakness and atrophy (29). In vertebrates, the SMN protein is mainly found associated with proteins Gemin2-8 and Unrip to form a large stable complex called the SMN complex (30–32). The best-characterized function of the SMN complex is its role in the biogenesis of snRNPs (31,33). The SMN complex mediates the association of Sm core proteins into a ring-shaped structure onto snRNAs (31,33–37). This assembly is performed in collaboration with the methylosome complex (38,39), which is composed of the protein arginine methyl transferase PRMT5, pICln and the WD-repeat protein MEP50 (39–42). PRMT5 and MEP50 constitute the core components of the methylosome (43). Sm proteins are recruited via the pICln subunit and PRMT5 symmetrically dimethylates arginines in the C-terminal tail of Sm proteins B-B', D1 and D3. This enhances their transfer onto the SMN complex and contributes to Sm core proteins rearrangement and assembly (36,39–40,44–45). Many other roles and interaction partners have been attributed to this complex (46). Among them, a novel protein partner RioK1 was found to interact with PRMT5 and to recruit nucleolin to the methylosome for its symmetrical methylation (47). RioK1 and pICln bind to PRMT5 in a mutually exclusive fashion (47). SMN-methylosome association prevents the misassembly of Sm proteins to non-target RNAs and blocks the aggregation of Sm proteins (48). SMN deficiency was shown to alter the stoichiometry of snRNAs and cause widespread tissue-specific pre-mRNA splicing defects in SMA mice models (49,50). The SMN complex was also shown to play roles in

the assembly of other RNPs such as H/ACA and C/D box snoRNP and the signal recognition particle (SRP) (51,52). It is also involved in the axonal localization of mRNPs for translation (53).

In this study, we analyze the role of the SMN and methylosome complexes in the selenoprotein mRNP assembly and translation. We show that SBP2 interacts with different components of both the SMN complex and the methylosome. Using inducible cell lines allowing conditional expression of GPx1, we established that components of the SMN and methylosome complexes are required *in vivo* for efficient translation of this selenoprotein. Importantly, we show that the repertoire of selenoprotein mRNAs is altered in the spinal cord but not in the brain of SMN-deficient mice. Altogether we bring evidence that the SMN complex and the methylosome are key players of selenoprotein mRNP assembly and expression.

MATERIALS AND METHODS

Cell culture

HEK293FT and HeLa cells were cultured at 37°C in 5% CO₂ in Dulbecco's Modified Eagle Media (DMEM) containing 10% foetal calf serum (FCS), 1% penicillin-streptomycin (50 u/ml penicillin, 50 µg/ml streptomycin, Invitrogen) and 10 nM sodium selenite. A total of 500 µg/ml geneticine was added to HEK293FT cells. Cells were transfected with pEGFP: fusion plasmids using Turbofect (Fermentas) and extracted with HNTG (20 mM HEPES-NaOH pH 7.9, 150 mM NaCl, 1% Triton, 10% glycerol, 1 mM MgCl₂, 1 mM EGTA, 1 mM PMSF, anti-protease cocktail from Roche).

siRNA inactivation and protein synthesis analysis by pulse labeling

The Flp-In™ stable cell lines HA-GPx1, HA-GPx1Cys and HA-GPx1CysΔSECIS were generated as described in (8). A custom siRNA library consisting of pools of four different dual strand modified siRNAs per target (ON-TARGET plus SMART pools Custom Library, Dharmacon) or individual siSMN1 (Dharmacon) were transfected with Lipofectamine 2000 (Invitrogen) following the manufacturer's conditions. siRNAs used for qRT-PCR are listed in Supplementary Table S1. The efficiency of siRNA inhibition was tested by qRT-PCR and western blot analysis. Forty-eight hours after transfection the expression of HA-GPx1, HA-GPx1Cys and HA-GPx1CysΔSECIS was induced using DMEM/10% FCS containing 0.5 µg/ml Doxycycline and 3 nM of Na₂SeO₃. After 3 h of induction, cells were washed with Met-free medium (DMEM Glutamax Gibco). The medium was replaced with Met-free DMEM containing 100 µCi/ml [³⁵S]-methionine for 1 h. The radioactive medium was removed and cells were lysed immediately, HA-tagged proteins were purified using a µMACS HA isolation kit (Miltenyi Biotec) and analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

Immunopurification, co-immunoprecipitation and western blotting

Immunopurification of endogenous SBP2 complexes was performed as described in (7). Co-immunoprecipitation experiments were performed after transfections of Green Fluorescent Protein (GFP) fusions of the target proteins. For SMN complex analyses cells were extracted in HNTG buffer (20 mM HEPES pH 7.9, 150 mM NaCl, 1% Triton, 10% glycerol, 1 mM MgCl₂, 1 mM EGTA and protease inhibitors) for 30 min at 4°C. Cellular debris were removed by centrifugation (10 min at 9000 g and at 4°C). Extracts were put on coated beads for 2 h at 4°C (GFP-Trap, Chromotek). Beads were washed four times in HNTG. For methylosome analyses cell extracts (200 µl) were incubated with 50 µl of anti-GFP magnetic beads (Miltenyi) in IPP150 (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% NP-40, 0.5 mM PMSF, 2 mM VRC). GFP alone was used as a control. Beads were washed with IPP150. For protein analyses, pelleted materials were resuspended in Laemmli buffer and analyzed by western blotting using the following antibodies: anti-SMN (BD transduction laboratories) mouse monoclonal; anti-Gemin2 (2E17) (ThermoScientific) mouse monoclonal; anti-Gemin3 (12H12) (Millipore) mouse monoclonal; anti-Gemin4 (1710) (Millipore) mouse monoclonal; anti-Gemin5 (Sigma) rabbit polyclonal; anti-Gemin6 (Proteintech) rabbit polyclonal; anti-Gemin7 (6E2) (Millipore) mouse monoclonal; anti-Gemin8 (Proteintech) rabbit polyclonal; anti-Unrip (3G7) (gift from L. Pellizzoni) mouse monoclonal; anti-NUFIP (Proteintech) rabbit polyclonal; anti-Rvb2 (Proteintech) rabbit polyclonal; anti-SRP19 (Proteintech) rabbit polyclonal; anti-AKT (Cell Signalling) rabbit polyclonal; anti-SBP2 rabbit polyclonal (7); anti-PRMT5 goat polyclonal (Santa Cruz); anti-MEP50 mouse monoclonal (Santa Cruz); anti-pICln (Q10) goat polyclonal (Santa Cruz); anti-RioK1 rabbit polyclonal (Novus Biologicals); anti-Sm (Y12) mouse monoclonal (Abcam); anti-GFP (Roche) mouse monoclonal.

Expression and purification of the methylosome using a baculovirus expression system

Recombinant baculoviruses allowing the expression of GST-SBP2, PRMT5, MEP50 and HA-pICln were used to infect Sf9 (*Spodoptera frugiperda*) insect cells. Infected cells were cultured in TNM-FH supplemented with 10% FCS and 50 mg/ml gentamycin at 27°C for 72 h. Cells were harvested and lysed in NT2 buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM MgCl₂, 0.05% NP40, 1 mM DTT, 400 µM VRC, 100 U RNasin/ml, anti-protease cocktail). Extracts were clarified by 10 min of centrifugation at 9500 rpm. A total of 300 µl of cell extracts were incubated at 4°C for 3 h with 50 µl Glutathione Sepharose 50% (GE Healthcare). Beads were washed five times with NT2 buffer, resuspended in 25 µl Laemmli buffer, boiled and analyzed by SDS-PAGE.

In vitro methylation assay

A total of 40 pmol of purified recombinant SBP2 protein or (H3-H4)₂ were incubated with 5 pmol of the PRMT5/MEP50 complex in 8 µl of methylation buffer (50

mM Tris-HCl pH 8, 200 mM NaCl, 0.25 mM EDTA, 0.25 mM DTT, 1% BSA) and 1 µl of ¹⁴C-S-adenosylmethionine (0.4 mM 50 mCi/mmol, Perkin Elmer) for 30 min at 37°C. The reaction was stopped by addition of 3 µl of Laemmli buffer, boiled and analyzed by Coomassie staining of the SDS-PAGE. The radioactive methylation signal was revealed by phosphorImager after overnight exposure.

Yeast-two hybrid interaction tests

For Y2H assays, appropriate pGBKT7 (DB) or pGADT7 (AD) plasmids were cotransformed into AH109 (Clontech Laboratories, Inc.) and plated on triple selective media (-Leu -Trp -Ade).

RNA isolation from mice and real-time RT-PCR

A SMA mouse model (SMN2^{+/+}, SMNΔ7^{+/+}, Snn^{-/-}) reproducing the phenotype of a SMA type II pathology (Jackson's laboratory, #5025) was used for this study and called SMNΔ7 (54). Ten-day-old SMNΔ7 mice (*n* = 3) and age-matched wild-type (WT) controls (*n* = 3) were anesthetized and sacrificed as described in (51). Brain and spinal cord total RNAs were extracted. After DNase treatment, RNAs were reverse transcribed using M-MLV (Promega). Levels of mRNAs were measured by quantitative RT-PCR (qRT-PCR). Reactions were carried out on a LightCycler (Roche) using the Maxima SYBR Green PCR kit (Fermentas). Oligonucleotides used for qRT-PCR are listed in Supplementary Table S2. Data were calculated according to the ΔΔCT method. Results were expressed as mean ± standard error of an average of three measurements. Trimethyl-capped RNAs immunoprecipitations (TMG RIP) experiments were adapted from Wurth *et al.* (8) using the strictly specific rabbit polyclonal anti-m³G (TMG) serum (Synaptic Systems). A total of 5 µl of serum were coupled to 20 µl of protein A-Sepharose beads saturated with 10 µg of both purified BSA and total yeast tRNA in NT2 buffer for 18 h at 4°C. The immobilized antibody was incubated with 10 µg of pre-cleared total spinal cord RNAs prepared as described above in a total volume of 250 µl for 2 h at 4°C. Beads were washed six times in NT2 buffer, the bound RNA was extracted by phenol/chloroform and precipitated. After DNase treatment, RNAs were reversed transcribed using AMV-RT (Q-Biogen) and cDNAs were amplified by quantitative RT-PCR (qRT-PCR) as described above.

RESULTS

The SMN complex interacts with SBP2 *in vivo*

We have previously shown that SBP2 associates with the SMN protein to recruit Tgs1 for the cap maturation of selenoprotein mRNAs (8). We tested whether other components of the SMN complex also associate with SBP2. To do so, endogenous proteins associated with transfected GFP-SBP2 were immunoprecipitated from HEK293FT cells using anti-GFP antibodies. Full length SBP2 is generally produced with low efficiency in any cell type (55). GFP-SBP2 fusions are nevertheless expressed efficiently in eukaryotic cells and were used successfully to characterize SBP2 protein partners (7,8). GFP was chosen because it shows minimal nonspecific binding to mammalian cell proteins (56).

Western blotting revealed that SMN, Gemin2, 3, 4, 5, 8 and Unrip associate with GFP-SBP2, while almost no binding to GFP alone was detected (Figure 1A). Gemin6 and 7 were not detectable in the immunoprecipitation, possibly due to their naturally low abundance in the extracts. The association with the SMN complex components is globally weaker than that observed for Nufip and Rvb2, two proteins that we have previously characterized as interaction partners of SBP2 (7). No interaction was detected for AKT and SRP19, used as negative controls. Moreover, yeast two-hybrid (Y2H) assays were performed to analyze the ability of each individual SMN complex components to interact with SBP2. To this end, plasmids expressing fusion proteins with a GAL4 DNA binding domain (GAL4-BD) or a GAL4 activation domain (GAL4-AD) were co-transformed into yeast AH109. Nufip was chosen as a positive control for SBP2 interaction and Alix as a negative control (7). We found that SBP2 strongly interacts with Gemin4, Gemin7 and moderately with Gemin3 and Gemin8 (Figure 1B and Supplementary Figure S1A) on selective media. Although present in the GFP-SBP2 immunoprecipitation experiments, Gemin2, 5, 6 and Unrip did not interact directly with SBP2 in this Y2H assay, suggesting that their interaction may be mediated by other components of the SMN complex. In each case, the interactions were only detected in one of the two possible Y2H vector combinations, this well known drawback of Y2H analysis may be related to the folding and three dimensional structure of the proteins assayed (57). Nevertheless, these interactions are most likely direct since the SMN complex, SBP2 and all factors involved in selenoprotein synthesis are absent in *Saccharomyces cerevisiae*. Y2H interactions between SMN and SBP2 could not be tested since SMN alone is capable of activating the transcription of the GAL4 promoter (data not shown). However, we have previously shown by GST-SMN pull-down experiments that the association between SMN and SBP2 is direct *in vitro* and that GFP-SMN interacted with SBP2 *in vivo* (8). Altogether, these results show the existence of a network of interactions between SBP2 and the components of the SMN complex.

The methylosome interacts with SBP2 *in vivo*

The arginine methyl transferase PRMT5 and the WD40 protein MEP50 form a hetero-octameric complex that constitutes the core unit of the methylosome and can interact with partner proteins such as pICln or RioK1 (43,47,58). Interestingly, when cytoplasmic HeLa cell extracts were passed through an affinity column with immobilized anti-SBP2 peptide antibodies, we found by mass spectrometry analysis that PRMT5 and MEP50 copurified with SBP2 (Figure 2A). Previously characterized binding partners of SBP2 and selenoprotein mRNPs such as NSEP1 (7,59) were also present in the purification. To validate these interactions, we transfected HEK293FT cells with GFP-SBP2 and immunoprecipitated the total cell lysates with anti-GFP antibodies. As shown in Figure 2B and C respectively, endogenous PRMT5 and MEP50 associated with GFP-SBP2 *in vivo*. Neither pICln nor RioK1 co-immunoprecipitated under high salt conditions (300 mM NaCl) but a significant interaction between pICln and GFP-SBP2 was de-

tected in lower salt conditions (150 mM NaCl) (Figure 2B, lower panel); no interaction with GFP alone was detected. Conversely, GFP-PRMT5, GFP-MEP50 and GFP-pICln also interacted with transfected SBP2 *in vivo*, in low salt conditions (Figure 2D). Altogether these results reveal that SBP2 strongly associates with the core proteins of the methylosome, most likely within a complex comprising pICln, rather than RioK1. Moreover, we observed individual protein-protein interactions between SBP2 and these methylosome components by Y2H (Figure 2E and Supplementary Figure S1B).

Reconstitution of the SBP2/methylosome complex

To reconstitute the complex that we characterized by immunoprecipitation and Y2H, we co-expressed GST-SBP2, PRMT5, MEP50 and HA-pICln in Sf9 (*Spodoptera frugiperda*) insect cells infected with the corresponding recombinant baculoviruses. Sf9 cells, unlike *Escherichia coli*, allow the expression of non proteolyzed full length SBP2 (55), and therefore the simultaneous overexpression and purification of GST-SBP2 with methylosome components. None of the proteins were retained non-specifically in the absence of GST-SBP2 on Glutathione Sepharose. Recombinant core proteins PRMT5 and MEP50 co-purified with GST-SBP2 in 150 mM salt, which confirms that these three proteins form a stable complex. However, HA-pICln did not co-purify with GST-SBP2 and appears to be a more labile component (Figure 3A). This is consistent with our observation that in HEK293FT cells, pICln does not remain associated with SBP2 in high salt conditions. Binding of pICln in physiological conditions may be favoured by the presence of additional factors that are absent in Sf9 cells.

Some protein substrates of the SMN complex contain RG domains (32,40–41). In the case of Sm proteins, the arginines of the RG domain need to be converted to symmetric dimethylarginines (sDMAs) by PRMT5 in order to interact with SMN (40). We tested if SBP2 could also be a methylation substrate of PRMT5. SBP2 does not contain RG repeats, but has three unique RG sites at positions 120, 176 and 494 (see Figure 3B). An *in vitro* methylation assay of SBP2 was performed with purified PRMT5/MEP50 complex in the presence of ¹⁴C-S-adenosylmethionine. A known PRMT5 substrate, the (H3-H4)₂ histone tetramer was used as a positive control and revealed a clear methylation signal for H4. Results show that SBP2 is not methylated by PRMT5/MEP50 *in vitro* (Figure 3B). If SBP2 is not the direct target of the methylosome, one possibility may be that the methylated Sm proteins are present in the selenoprotein mRNPs. We therefore checked whether Sm proteins could be assembled onto selenoprotein mRNAs, by performing immunoprecipitation experiments of RNA-protein complexes using anti-Sm antibodies in HEK293FT cell extracts. RNAs associated with endogenous Sm proteins were detected by qRT-PCR (Figure 3C). In contrast to U1 and U2 snRNAs that were strongly enriched in the anti-Sm IP, none of the selenoprotein mRNAs that we tested, namely SelR, GPx4, SelM, SelT, TrxR1 or Sel15 were found associated with Sm proteins. These results strongly suggest that SBP2 is not a methylation substrate of the methylosome

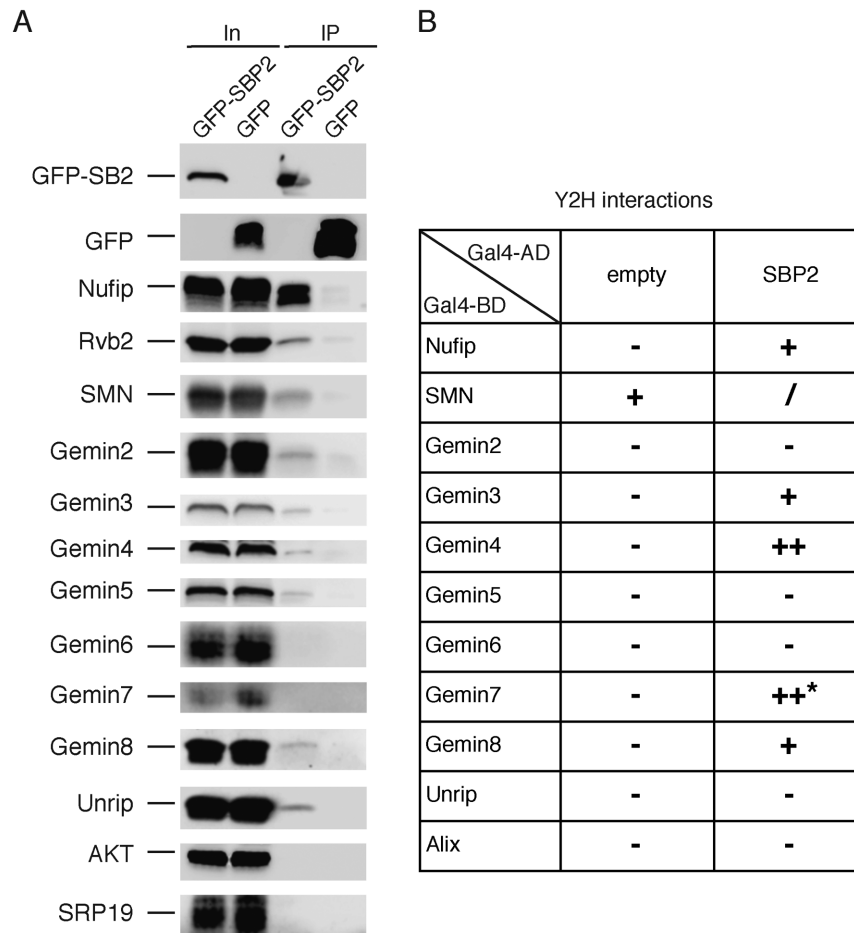


Figure 1. The SMN complex interacts with SBP2. (A) Co-immunoprecipitations using anti-GFP beads and HEK293FT cells transfected by GFP-SBP2 or GFP alone. The endogenous immunoprecipitated proteins were analyzed by SDS-PAGE and western blotting using antibodies against the indicated proteins. In: input (5% of total). (B) Summary of Y2H interaction results performed in *Saccharomyces cerevisiae* AH109 between SBP2 and the SMN complex components Gemin2-8 and Unrip. '+' and '-' indicate the presence or absence of interactions. Nufip was used as a positive interaction control with SBP2 and Alix as a negative control. Empty DNA binding (GAL4-DB) or activation domain (GAL4-AD) fusion vectors were used to verify that proteins alone do not activate the GAL4 promoter. For Gemin7 the interaction was detected in opposite orientation, this is indicated by '*'. Data are shown in Supplementary Figure S1A.

and that Sm proteins are not components of selenoprotein mRNPs.

SMN and methylosome components are required *in vivo* for the translation of GPx1

To determine the functional importance of the components of both SMN and methylosome complexes for selenoprotein synthesis *in vivo*, we analyzed the effect of their depletion by siRNAs. To this end we made use of the stable Flp-In T-Rex cell lines HA-GPx1, HA-GPx1Cys and HA-GPx1Cys Δ SECIS that we have developed previously and that allow the inducible expression of the corresponding HA-tagged proteins (8). In these cell lines, the HA-GPx1 expression cassettes are integrated at the same locus, which ensures homogeneous levels of gene expression. In the HA-GPx1 cell line, the synthesis of GPx1 relies on translational recoding of the UGA_{Sec} codon; in HA-GPx1Cys, the Sec to Cys mutation renders the translation independent of recoding events (Figure 4A). Finally, in the HA-GPx1Cys Δ SECIS cell line, the SECIS element has been

deleted from the 3'UTR. This construct is therefore unable to recruit selenocysteine specific synthesis factors. Inhibition of individual SMN and methylosome components was performed for 48 h using pools of four different non-overlapping dual-strand modified siRNAs to reduce potential off-targets effects (60). The inhibition level of the expression of each protein was verified by western blot (Figure 4B and Supplementary Figure S2). At this stage, *de novo* expression of HA-tagged proteins was selectively induced for 3 h and followed by 1 h of [³⁵S]-methionine pulse labeling. Trans-acting factors inactivation by siRNA did not impact the relative mRNA steady-state accumulation of the reporter constructs (see Supplementary Figure S3). The resulting ³⁵S-HA-GPx1 proteins were immunoprecipitated using anti-HA antibodies and quantified. Results were normalized against those obtained with the HA-GPx1Cys Δ SECIS cell line. For each RNAi experiment, we selected conditions with similar protein inhibition levels for the three cell lines. Knockdown of SBP2 and eEFSec, two key factors of the selenoprotein translational recoding

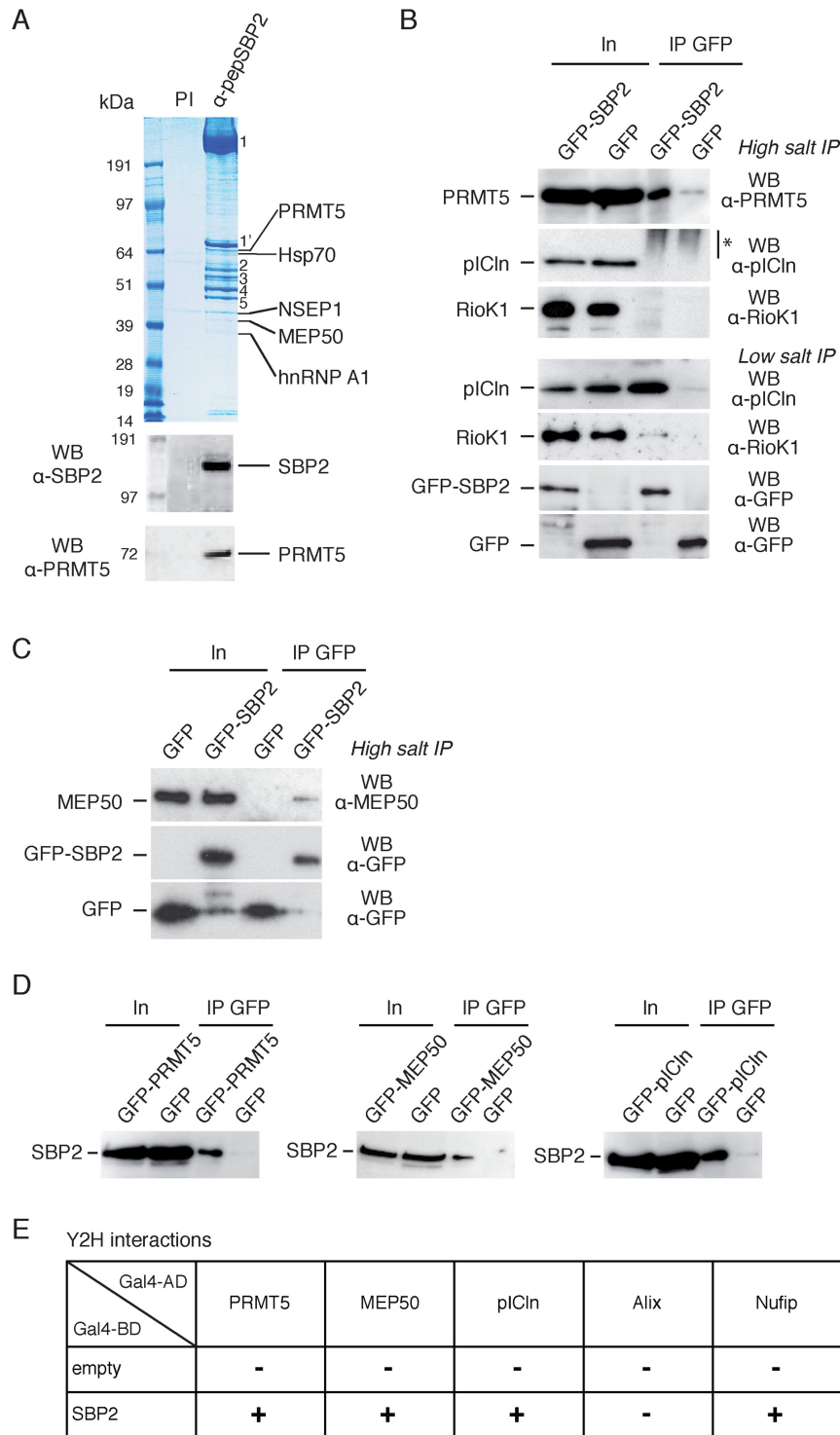


Figure 2. The methylosome complex interacts with SBP2 *in vivo*. (A) Immunoprecipitation of endogenous SBP2 from HeLa cytoplasmic extracts using antipeptide antibodies (α -pepSBP2) directed against SBP2 residues 380–852. PI: beads with preimmune serum. The immunoprecipitated proteins were identified by mass spectrometry (MS) and specific proteins are indicated on the right. Numbers represent common sepharose-matrix binding contaminants found in MS analysis (56), most of them are cytoskeletal proteins (1,1': Dynein chains, 2: Keratin, 3: Tubulin β ; 4: Tubulin α ; 5: VASP actin associated protein). Molecular weight (kDa) is indicated. (B–D) Co-immunoprecipitations using anti-GFP beads and HEK293FT cells transfected by (B and C) GFP-SBP2 and (D) GFP-PRMT5, GFP-MEP50, GFP-pICln or GFP alone. Immunoprecipitations were performed in high salt (300 mM NaCl) and low salt (150 mM NaCl) conditions. The immunoprecipitated endogenous proteins were analyzed by SDS-PAGE and western blotting using the indicated antibodies. In: input (10% of total). * smear of unknown origin always present at high salt but absent at low salt. (E) Direct interactions between SBP2 and components of the methylosome complex. Y2H interaction tests performed in *Saccharomyces cerevisiae* AH109 between SBP2 and the methylosome components PRMT5, MEP50 and pICln; Nufip was used as a positive interaction control with SBP2. Experiments were performed as described Figure 1B. Data are shown in Supplementary Figure S1A.

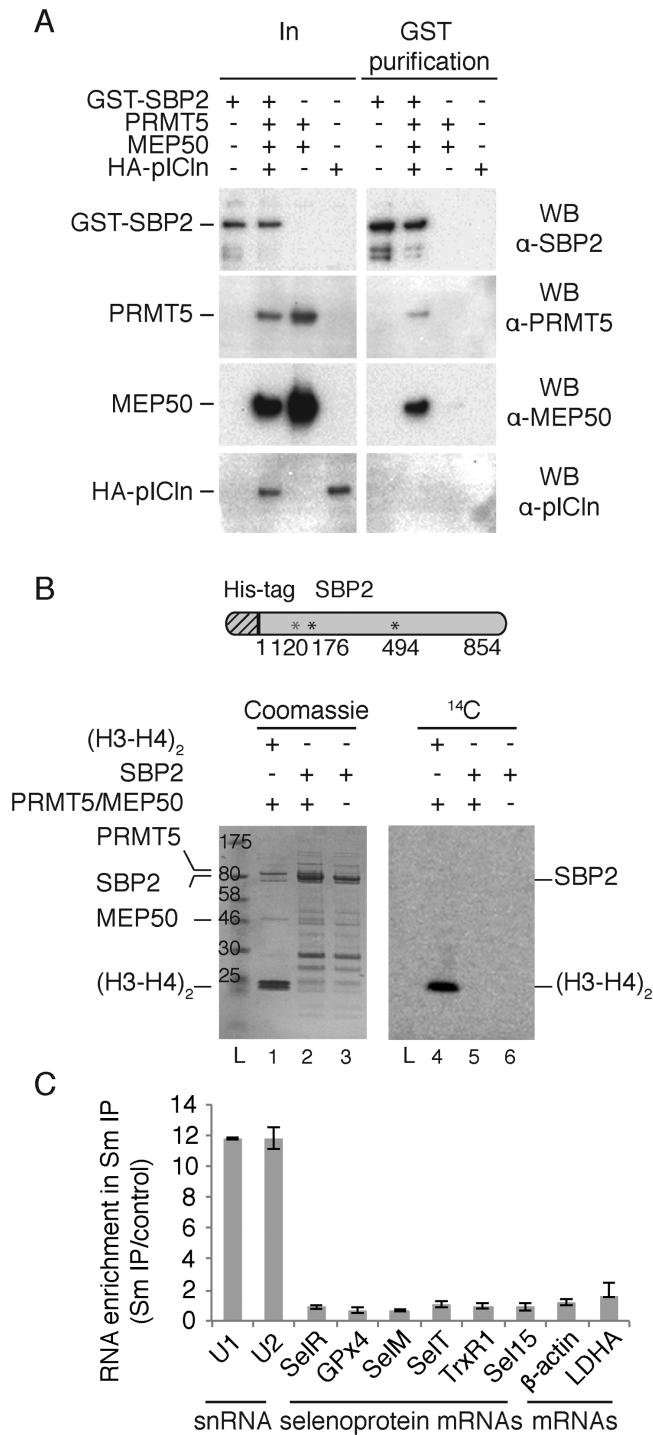


Figure 3. Reconstitution and activity of the SBP2/methylome complex and functional analysis (A) Coexpression and purification of SBP2 associated with the methylome complex. Recombinant baculoviruses allowing the expression of GST-SBP2, PRMT5, MEP50 and HA-pICln were used to infect Sf9 insect cells in the combinations indicated. Expression of PRMT5/MEP50 and HA-pICln alone served as negative controls. The complexes associated to GST-SBP2 were purified on Glutathione Sepharose, and the associated proteins were analyzed by SDS-PAGE and western blotting using the indicated antibodies. In: input (4% of the total cell extract). (B) SBP2 is not a substrate of PRMT5/MEP50. For methylation assays PRMT5/MEP50 was incubated with SBP2 or histones (H3-H4)₂ in the presence of ¹⁴C SAM for 30 min at 37°C. The (H3-H4)₂ tetramer is a methylation substrate of the methylome and therefore serves as a positive control. Proteins are analyzed on SDS-PAGE and revealed by coomassie staining (lanes 1–3). The radioactive methylation signal is revealed by phosphorImager after overnight exposure (lanes 4–6). The cartoon represents His-Tag SBP2 and the position of the RG sequences (*) that are potential sites of methylation. L: ladder. (C) Sm proteins do not bind selenoprotein mRNAs. Total RNA extracted from HEK293FT cells was immunoprecipitated with anti-Sm antibodies. qRT-PCR was used to determine the RNA enrichment in the IP experiment compared to the input extract by the $\Delta\Delta$ Ct method. SelR, GPx4, SelM, SelT, TrxR1 and Sel15 are selenoprotein mRNAs. U1 and U2 snRNAs were used as positive controls; β -actin and LDHA mRNAs are housekeeping mRNAs used as negative controls. Error bars represent standard deviation of an average of three independent experiments.

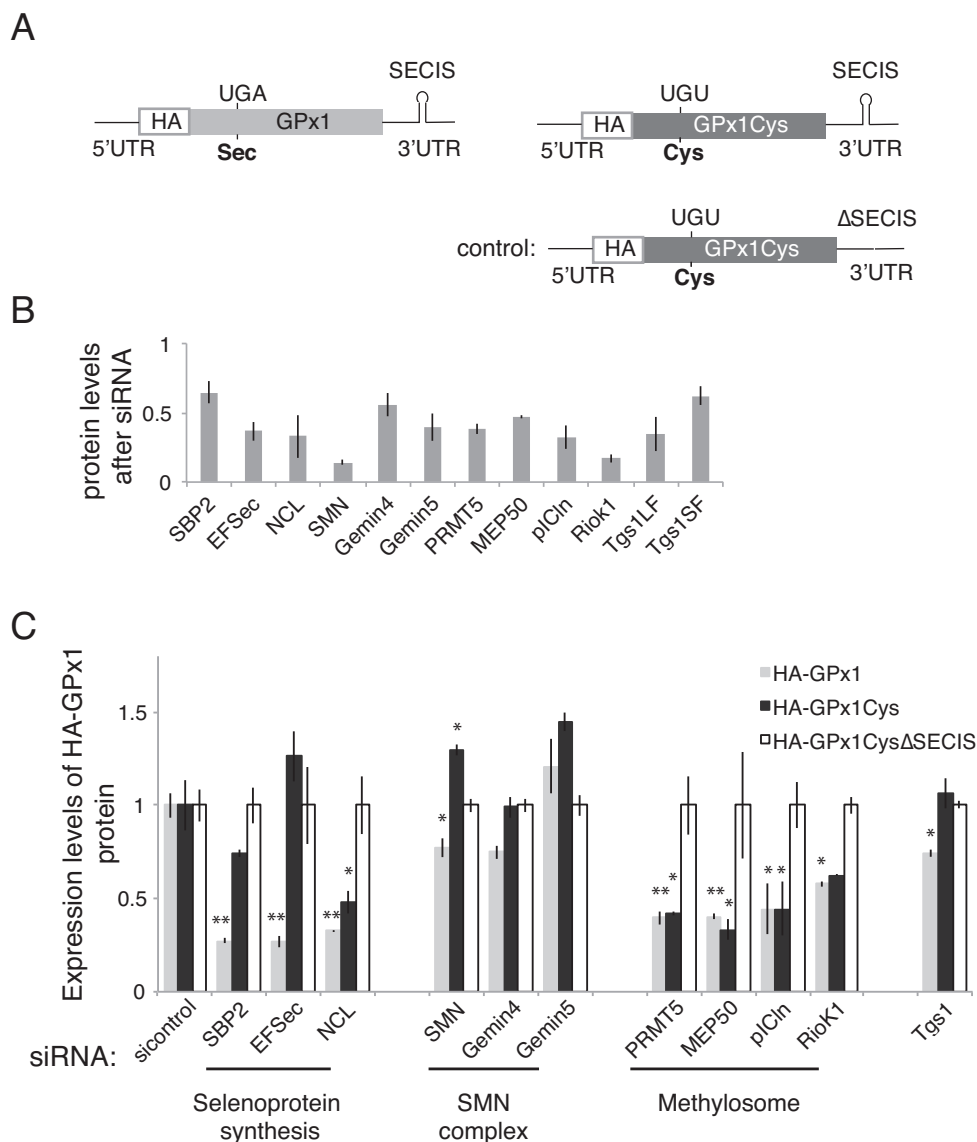


Figure 4. SMN and methylosome components are required for *de novo* synthesis of the selenoprotein GPx1. (A) Representation of the expression constructs HA-GPx1, HA-GPx1Cys and HA-GPx1CysΔSECIS integrated in HEK293FT stable cell lines, flanked by their natural GPx1 5' and 3'UTRs. The SECIS RNA is indicated. Protein expression was induced 48h after siRNA knock-down of selenoprotein synthesis factors, SMN and methylosome components. Pools of 4 independent dual-strand modified siRNAs were used in order to maximally reduce the risk of off-target gene silencing (60). After 3h of induction, *de novo* selenoprotein synthesis was monitored by ^{35}S methionine pulse labeling for 1h. (B) Quantification of protein levels by western blot after siRNA treatment. (C) Quantification of *de novo* ^{35}S labeled HA-GPx1 protein expression levels. Results were normalized against HA-GPx1CysΔSECIS that serves as a control. Gray bars: HA-GPx1; dark bars: HA-GPx1Cys; white bars: HA-GPx1CysΔSECIS. Error bars represent standard deviation of an average of 4 independent experiments. Asterisks indicate statistically significant differences between a given siRNA experiment and the corresponding sicontrol condition. * $P < 0.05$ and ** $P < 0.005$ based on Student's *t* test. Absence of labeling indicates that the result is not considered statistically significant (e.g. $P = 0.077$ for siSBP2 in HA-GPx1Cys).

mechanism resulted in a drop of 73% of HA-GPx1 synthesis but did not significantly impact the synthesis of HA-GPx1Cys (Figure 4C). No effect was observed in samples treated with control siRNAs (Figure 4C). This validates our experimental conditions and is in agreement with our previously published results obtained by western blot (8). We then inhibited the expression of the core protein SMN, as well as a peripheral protein Gemin4 and the RNA binding protein Gemin5 of the SMN complex. Reduction of SMN protein level to 14% resulted in a modest but significant 23%

decrease of HA-GPx1 selenoprotein synthesis while the expression of HA-GPx1Cys was increased by 26% (Figure 4C). Deregulation of selenoprotein mRNA expression thus occurs also when the UGA_{Sec} codon is mutated to UGU_{Cys} , meaning that this effect is probably independent of recoding events. This result was confirmed using a different set of siRNAs against SMN and the quantification of HA-GPx1 and HA-GPx1Cys by western blot (Supplementary Figure S4). SMN interacts with SBP2 to recruit the cap hypermethylase Tgs1 for selenoprotein mRNA cap modification (8).

Inactivation of Tgs1 leads to a 26% decrease of HA-GPx1 levels but has no effect on HA-GPx1Cys translation (Figure 4C) and (8). In contrast, inhibition of either Gemin4 or Gemin5 mRNAs has no significant effect on HA-GPx1 and HA-GPx1Cys synthesis (Figure 4C). It is possible that the level of reduction of Gemin4 and 5 obtained by RNAi is not sufficient to reveal their involvement in selenoprotein mRNA assembly or selenoprotein synthesis. Consistently, RNAi experiments of Gemin4 and 5 have no significant effect on snRNP assembly, even though Gemins modulate the activity of the SMN complex (61,62).

Conversely, siRNA inactivation of the methylosome components PRMT5, MEP50, pICln and RioK1 reduced strongly and equally the expression levels of both HA-GPx1 and HA-GPx1Cys about 50% but not of HA-GPx1Cys Δ SECIS (Figure 4C). This indicates that the activity of the methylosome depends on the presence of the SECIS element in the 3'UTR of selenoprotein mRNAs. When PRMT5 and MEP50 are part of a complex with RioK1 (47) they are involved in the methylation of the RNA binding protein nucleolin (NCL). Nucleolin binds the SECIS RNA and was shown to be involved in the expression of selenoproteins (21,63). The inactivation of nucleolin had a similar effect as the inhibition of methylosome components and decreased both HA-GPx1 and HA-GPx1Cys synthesis (Figure 4C). This confirms the role of nucleolin in the synthesis of GPx1 but indicates that it does not necessarily act at the recoding step but at a different level of the translational regulation of this selenoprotein as it also affects the synthesis of HA-GPx1Cys. This also suggests that RioK1 associated to the methylosome complex could be involved in the recruitment of nucleolin to the SECIS elements. In conclusion, our data suggest that at least the SMN protein and the methylosome components seem to be required for the translation of selenoprotein mRNAs but are unlikely to influence directly the UGA_{Sec} recoding step.

The steady-state level of m₃G-capped selenoprotein mRNAs is altered in the spinal cord of SMA mice

Defects in SMN protein expression are at the origin of SMA, a neuromuscular disease characterized by the degeneration of the lower motor neurons, leading to muscular weakness and atrophy (29,31). Increased oxidative stress and lipid peroxidation was observed in the motor neurons of SMA patients that could explain cell death (64). Based on our data, and the fact that selenoproteins play significant roles in the antioxidative defence and the reduction of lipid peroxidation products, we hypothesized that selenoprotein mRNA levels could be affected in SMA. We therefore measured the levels of selenoprotein mRNAs in an animal model of the disease. SMA is due to recessive mutations or deletions that affect the *SMN1* gene (29). Two genes, *SMN1* and *SMN2* code for the SMN protein in humans (65,66). *SMN1* produces full-length transcripts but *SMN2* produces mostly an alternatively spliced mRNA lacking exon 7 (SMN Δ Ex7); the copy number of *SMN2* is a determinant of the disease severity (65). We analyzed total RNA from 10-day-old SMN deficient mice (SMN2^{+/+}, SMN Δ 7^{+/+}, Snn^{-/-}), which is an established severe SMA mouse model reproducing the phenotype of

the SMA type II pathology (54). These SMA mice have a mean life expectancy of 13 days. SMA is a multi-system disorder, but we focussed on the analysis of the spinal cord that contains the motor neurons that are strongly affected in SMA in a non-gender specific manner. We compared the selenoprotein mRNAs abundance from the spinal cord and the brain of three SMA mice to that of three transgenic control mice (SMN2^{+/+}, SMN Δ 7^{+/+}, Snn^{+/+}) by qRT-PCR. For the detection of selenoprotein mRNAs we used mouse specific primers complementary to 9 out of the 25-selenoprotein mRNAs characterized in mammals (Figure 5). HPRT (hypoxanthine guanine phosphoribosyltransferase), LDHA (lactate dehydrogenase A) and TBP (TATA-binding protein) mRNAs were used as non-selenoprotein mRNA controls, and U12 snRNA was used as a positive control of SMN activity. Interestingly, the levels of the mouse selenoprotein mRNAs of selenoprotein R (SelR), glutathione peroxidase 1 (GPx1), GPx4, SelM and SelW were decreased in the spinal cord between 40 and 75%, but not in the brain (Figure 5A and B). In contrast, the levels of SelT, thioredoxin reductase 1 (TrxR1), Sel15 and SelN selenoprotein mRNAs were affected neither in the spinal cord nor in the brain, compared to non-selenoprotein mRNAs (Figure 5A and B). Several exons of SBP2 can be aberrantly spliced, in particular exon3 skipping has been characterized in oxidative stress conditions (67). We therefore examined the levels of exon3 containing SBP2 mRNAs, that also reflects full-length SBP2 mRNAs and found that while this level is unchanged in the brain of SMA mice, it is reduced to 65% in the spinal cord (Figure 5A and B). Splicing defects of SBP2 mRNA could therefore also contribute to decrease selenoprotein mRNA levels in the spinal cord since SBP2 is required for the stability of these mRNAs (68). Remarkably, selenoprotein mRNAs that were most affected by SMN deficiency in the mouse spinal cord correspond precisely to those that we previously found bearing a hypermethylated cap in human cells and that are poorly recognized by eIF4E (see Figure 5C). This is consistent with the role of SMN in the recruitment of Tgs1 for the cap hypermethylation of selenoprotein mRNAs (8). To confirm this, RNA was extracted from the spinal cord of control and SMA mice and immunoprecipitated using highly specific anti-m₃G cap serum (R1131) (Figure 5D). Due to the low availability of the biological material we focused on the analysis of the abundant GPx1 mRNA and used the m₃G capped U12 snRNA as a control because it is dependent on SMN for cap hypermethylation by Tgs1. Extracted RNAs were analyzed by qRT-PCR. Results showed that 6% of GPx1 mRNA was immunoprecipitated in the control mice (Figure 5D), consistent with our previous results showing that 5–15% of selenoprotein mRNAs can be recovered in this experiment (8). TMG-IP efficiency in the SMA mice dropped to 2%, implying that the cap hypermethylation level of GPx1 mRNA is indeed affected in the spinal cord of SMA mice as a consequence of SMN inactivation. This is comparable to the results obtained for the U12 RNA control that shows a drop of TMG IP from 100 to 37% in spinal cord of SMA mice. Altogether our results support the conclusion that alteration of the steady-state levels of several selenoprotein mRNAs is linked to SMN deficiency in spinal cord of a SMA mouse model. Particularly affected are selenoprotein

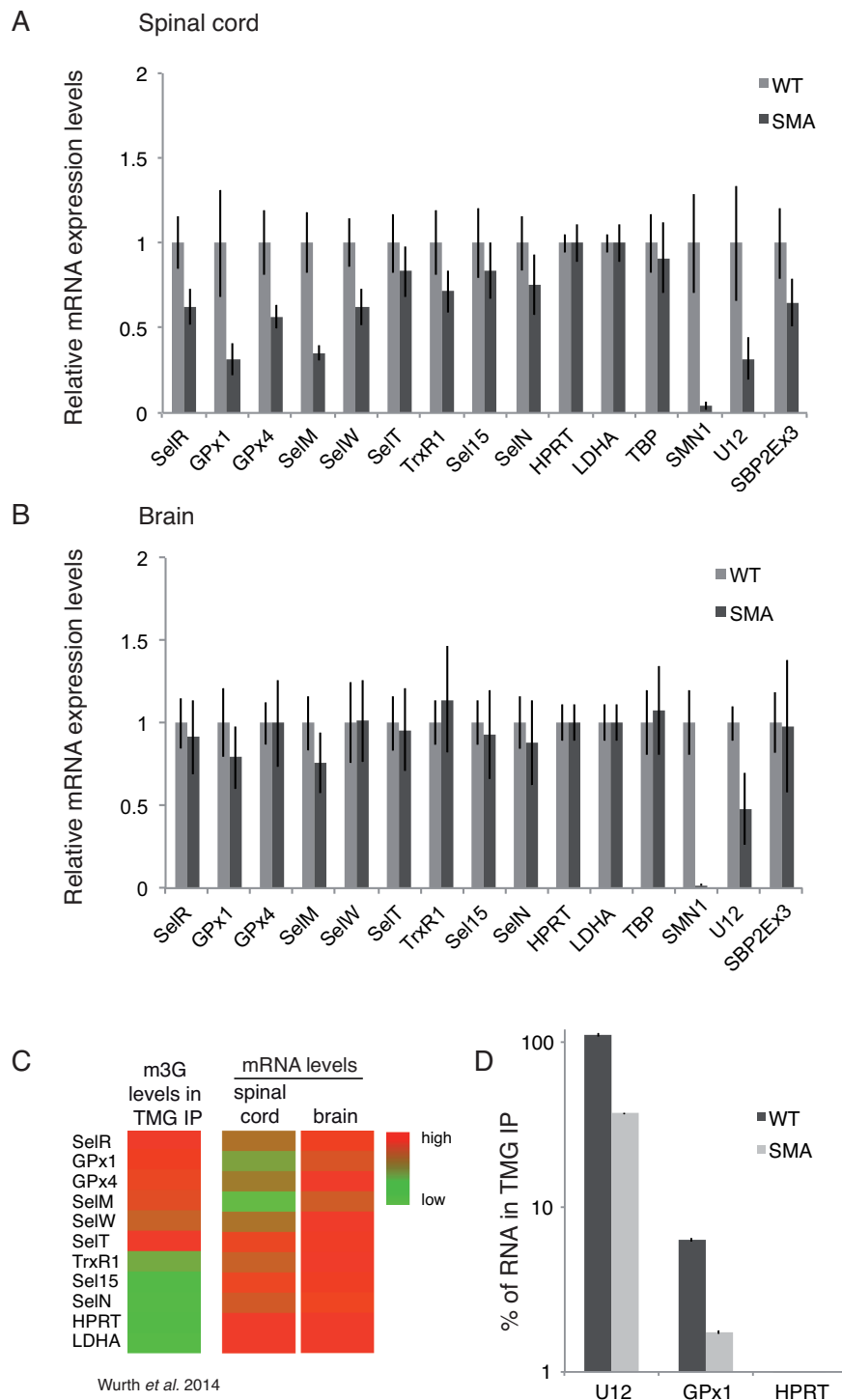


Figure 5. The steady state levels of selenoprotein mRNAs is reduced in the spinal cord of SMA mice. The relative expression levels of selenoprotein mRNAs in spinal cord (A) and brain cells (B) of three wild-type (WT) and three SMA mice (including two females, one male) was measured by qRT-PCR and determined by the $\Delta\Delta C_t$ method. For each mouse three technical replicates were performed. SelR, GPx1, GPx4, SelM, SelW, SelT, TrxR1, Sel15 and SelN are selenoprotein mRNAs. For clarity, the same nomenclature as human mRNAs was used. U12 snRNA was used as a positive control, HPRT, LDHA and TBP are housekeeping mRNAs used as negative controls. SBP2Ex3 corresponds to Exon3 containing SBP2 mRNA. Invalidation of WT mouse SMN1 mRNA in the (*smn*^{-/-}) mice is shown as a control. Error bars represent the standard deviation of the three biological replicates of WT or SMA mice. (C) Heat map representation of mRNA levels in the spinal cord and the brain of SMA mice compared to m₃G levels of human selenoprotein mRNAs measured in TMG IP experiments by Wurth et al. (8). The heat map scale is represented to the right; maximum values in each set of experiments are represented in red and minimal values in green. Heat maps were generated with the MeV software. (D) Total RNA extracted from the spinal cord of three WT or three SMA mice cells was immunoprecipitated with anti-TMG serum (α -m₃G). Bound RNA was analyzed by qRT-PCR. The graph represents the % of RNAs in IP in log scale (y-axis) normalized by the input RNA. Error bars represent standard deviations between the three independent mice analyzed.

mRNAs that bear hypermethylated caps. It is tempting to suggest that this contributes to the oxidative stress and increased lipid peroxidation in the motor neurons of SMA patients since both the glutathione peroxidase GPx1 and the phospholipid hydroperoxide GPx4 mRNAs are affected.

DISCUSSION

The SMN complex and the methylosome interact with SBP2, a key player of selenoprotein mRNA assembly, biogenesis and translation

Selenoprotein mRNA assembly and translation are heavily dependent on the interaction of SBP2 with the 3'UTR of selenoprotein mRNAs and on its ability to recruit chaperones, modification enzymes, as well as translation recoding factors. In a previous work we proposed that the cap hypermethylase Tgs1 is recruited to selenoprotein mRNAs via direct interactions between the SMN and SBP2 proteins. Here we show that in addition to its direct interaction with SMN, SBP2 interacts directly with 4 Gemins of the SMN complex in Y2H assays (Gemin3, 4, 7 and 8), indicating the existence of an important interaction network between SBP2 and components of the SMN complex (Figure 6B). This is confirmed by our observation that SBP2 associates *in cellulo* with most of the SMN complex core components (Figure 6A). Only Gemin6 and 7 were not detected in our experimental conditions, possibly due to their low abundance in cells. In view of our data, it is reasonable to assume that interactions between SBP2 and SMN take place in the context of the entire functional complex, even though one cannot completely exclude that SBP2 also forms different types of subcomplexes with components of the SMN complex. SBP2 also interacts directly with proteins of the methylosome, the partner of the SMN complex in snRNP assembly. PRMT5 and MEP50 constitute a heterooctameric functional biological module, which either binds pICln to methylate Sm proteins, or RioK1 to methylate nucleolin (43). We found that SBP2 interacts strongly with the core components PRMT5 and MEP50 *in vivo* and *in vitro*. It also interacts with pICln but the interaction seems to be weaker since it is only observed in low salt conditions. However, we did not detect an interaction between SBP2 and RioK1 in our experimental conditions. This is consistent with the fact that RioK1 and pICln are part of distinct methylosome complexes and are mutually exclusive (47). Our data support the hypothesis that (i) SBP2 is not a methylation substrate of the methylosome and that (ii) Sm proteins are not components of selenoprotein mRNPs. Thus, another modification target of the methylosome may be recruited by SBP2 to selenoprotein mRNAs but it remains to be identified.

A new function of the SMN complex and the methylosome in selenoprotein mRNA assembly and translation

The fact that SBP2 interacts with both the SMN complex and the methylosome highlights a new function of these two complexes in selenoprotein mRNA assembly and/or maturation and is expected to have an impact on selenoprotein translation. Accordingly, we found that depletion of

the SMN protein but also inactivation of any of the methylosome components resulted in alterations of *de novo* HA-GPx1 selenoprotein expression, provided that the SBP2 binding site (SECIS element) was still present in the 3'UTR of the selenoprotein mRNAs. This highlights the importance of a SBP2/SECIS dependent recruitment of both the SMN complex and the methylosome to selenoprotein mRNAs. Interestingly, depletion of SMN or of any components of the methylosome deregulated both level of mRNAs containing UGA_{Sec} or UGU_{Cys} codons, indicating that SMN and the methylosome do not act at the translational recoding step but rather contribute to the global efficiency of translation of SECIS-containing mRNAs. This is in sharp contrast with the inactivation of selenoprotein specific translation recoding factors which affects only the translation of UGA_{Sec} containing selenoprotein mRNAs (17,21,63,69). Even though we did not detect interactions between SBP2 and RioK1, its inhibition impacted the expression of HA-GPx1 selenoprotein. The RioK1-containing methylosome is involved in the methylation of nucleolin, a multifunctional protein that plays roles in processing of pre-rRNA, mRNA stability and mRNP assembly (47). Nucleolin was also shown to bind to the SECIS RNA and to regulate the translation of a subset of selenoproteins mRNAs (21,63). Our experiments confirm the implication of nucleolin in this process. Interestingly, SMN was also found to interact with nucleolin containing RNP complexes and this association is lost in the fibroblast of SMA patients (70).

The methylosome and SMN complex are thus likely to play a role in selenoprotein mRNA assembly and processing and contribute to the recruitment of modification enzymes such as Tgs1 as well as RNA binding proteins such as nucleolin or other yet to be identified RNP binding proteins.

Alterations in the selenoprotein mRNAs repertoire may contribute to SMA

The SMA pathology is caused by the homozygous deletions of, or mutations in the *SMN1* gene. This leads to ubiquitously reduced levels of the SMN protein and selective motor neuron degeneration. Several studies showed that motor neuron cell death in SMA patients could originate from increased oxidative stress and lipid peroxidation (64,71–73). Our results show that the steady state level of several selenoprotein mRNAs is selectively affected in the spinal cord of a SMA mouse model but not in the brain. These include GPx enzymes that are recognized as the major regulators of oxidative stress damage in neurons, SelR that regulates aging processes and SelW that plays roles in the prevention of neurodegeneration (1,64,74–75). Alterations of selenoprotein mRNA levels may therefore contribute to the severity of SMA by increasing oxidative stress in the motor neurons of SMA patients. Several cellular defects may contribute to the alterations of selenoprotein mRNA levels when SMN protein is low. Firstly we observed that solely the levels of selenoprotein mRNAs bearing hypermethylated caps in human cells (8) are affected in the spinal cord of SMA mice. Reduced levels of SMN are likely to impact the recruitment of Tgs1 for cap modification. This is corroborated by our results showing that the hypermethylation

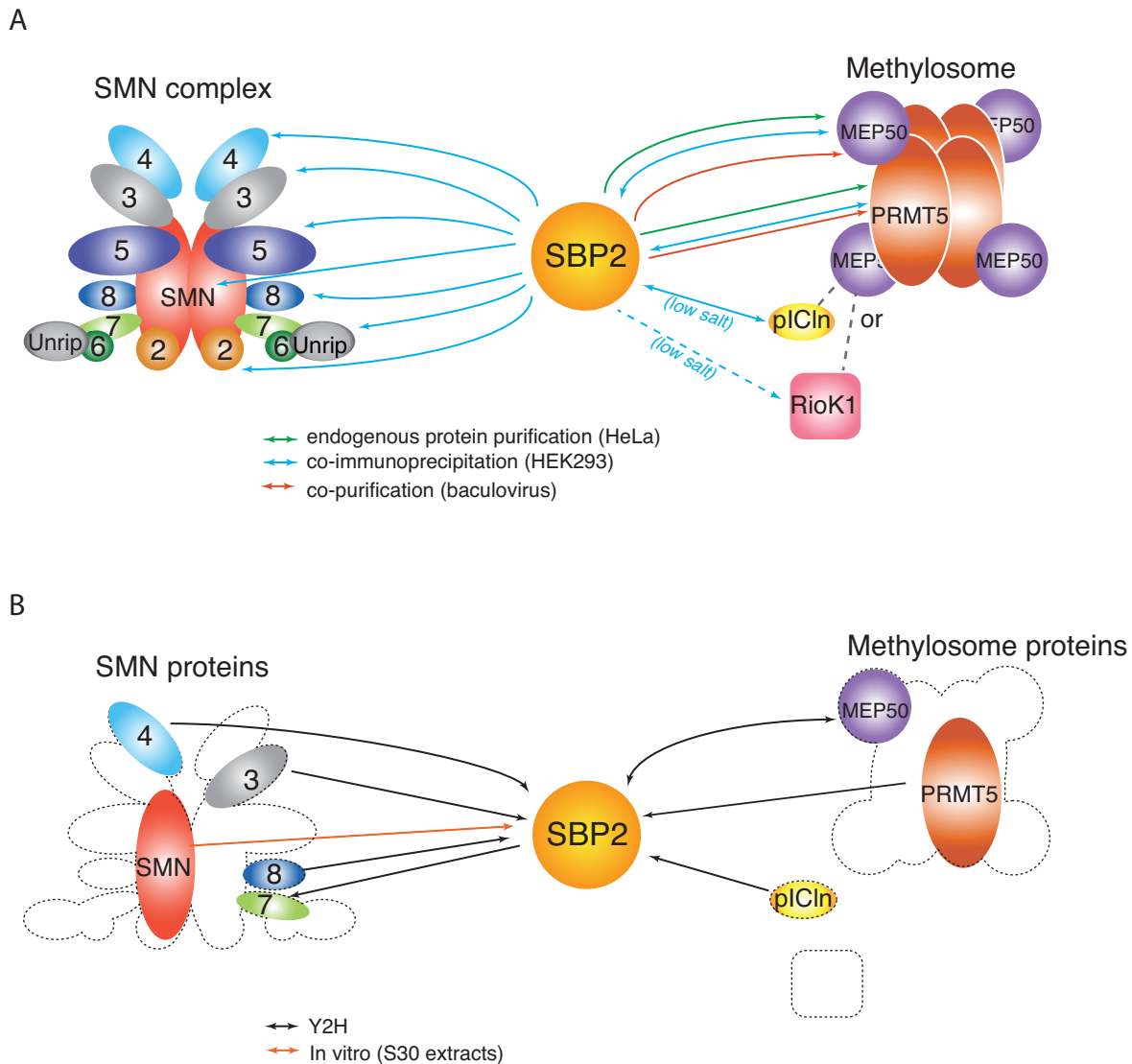


Figure 6. Overview of the interaction network between SBP2, the SMN complex and the methylosome. **(A)** Complex dependant protein associations. Green arrows represent endogenous associations between SBP2 and the methylosome in HeLa cells. *In vivo* co-immunoprecipitations between transfected SBP2 and endogenous HEK293FT proteins are represented by blue arrows. Purification of the SBP2-associated methylosome components expressed from baculovirus infected SF9 cells are represented in red. **(B)** Direct protein interactions. Y2H interactions are represented by black arrows (this study) and *in vitro* interactions of recombinant GST-SMN with SBP2 in orange (8). Single and double head arrows represent uni- and bi-directional interactions respectively. The SMN and methylosome components are represented in a non-stoichiometric manner (31,46). Numbers 2–8 represents the 7 Gemin proteins. MEP50 and PRMT5 are the core components of the methylosome; pICln and RioK1 are part of distinct methylosome complexes (32,46–47).

level of GPx1 mRNA is indeed affected in the spinal cord of SMA mice. Secondly, a possible role of the SMN complex in selenoprotein mRNA assembly or stability is also to be considered. Accordingly, functions for the SMN complex as a mRNA chaperone have been reported and deficiencies in the SMN protein were shown to impair mRNA localization and translation in motor neurons (53,76). Thirdly, during the course of this study it was shown that oxidative stress triggers aberrant splicing of several exons of SMN2 as well as SBP2 (77), for which exon3 skipping has been characterized (67). We found that exon3 containing SBP2 mRNAs are reduced in the spinal cord of SMA mice. This could additionally contribute to the reduction of selenoprotein mRNA levels. Clinical reports indicate that addi-

tional peripheral organs are affected also by low levels of SMN (78). Interestingly, mild SMA mice models show severe impairment of male reproductive organ development and WT testis have extremely high levels of SMN protein compared to other tissues (79). Selenoproteins play essential roles in male fertility, and both GPx4 (1,3) and SBP2 are abundantly expressed in spermatids (4,9). Therefore, the correlation between SMN and SBP2 may even be stronger in testis than in the spinal cord.

In conclusion, the SMN complex and the methylosome appear to be new actors in the complex mechanism of selenoprotein mRNA biogenesis and translation. Our data reveal new functions for these major cellular chaperones in selenoprotein synthesis; deciphering the mechanism of

their differential regulation may contribute to a better understanding of the SMA pathology.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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