

SHORT REPORT

The SMN Tudor SIM-like domain is key to SmD1 and coilin interactions and to Cajal body biogenesis

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ABSTRACT

Cajal bodies (CBs) are nuclear organelles involved in the maturation of spliceosomal small nuclear ribonucleoproteins (snRNPs). They concentrate coilin, snRNPs and the survival motor neuron protein (SMN). Dysfunction of CB assembly occurs in spinal muscular atrophy (SMA). Here, we demonstrate that SMN is a SUMO1 target that has a small ubiquitin-related modifier (SUMO)-interacting motif (SIM)-like motif in the Tudor domain. The expression of SIM-like mutant constructs abolishes the interaction of SMN with the spliceosomal SmD1 (also known as SNRPD1), severely decreases SMN–coilin interaction and prevents CB assembly. Accordingly, the SMN SIM-like-mediated interactions are important for CB biogenesis and their dysfunction can be involved in SMA pathophysiology.

KEY WORDS: Cajal body, SMN, SIM, SUMO1, Sm complex

INTRODUCTION

Sumoylation is an important post-translational modification that regulates protein activity, subcellular localization, stability and protein–protein interactions (Flotho and Melchior, 2013). Covalent SUMO modification occurs between the C-terminal glycine of small ubiquitin-related modifier (SUMO) and a lysine residue corresponding to a consensus sequence (ψ KxE/D) in the target protein (Hay, 2005; Flotho and Melchior, 2013). Some substrates have also SUMO-interacting motifs (SIMs) that mediate non-covalent interaction with SUMO1 (Song et al., 2004; Hecker et al., 2006). SIMs contain a hydrophobic core (V/I-x-V/I-V/I), generally flanked by acidic and/or phosphorylated residues, that ensure the affinity for SUMO1 (Kerscher, 2007).

Cajal bodies (CBs) are nuclear organelles discovered by Ramón y Cajal (Lafarga et al., 2009). They are enriched in coilin, survival motor neuron protein (SMN) and small nuclear and Cajal body ribonucleoproteins (snRNPs and scaRNPs) involved in pre-mRNA processing (Ciocco and Lamond, 2005; Nizami et al., 2010; Machyna et al., 2013). Endogenous SMN conjugates to

Gemin2–Gemin8 and Unrip (also known as STRAP) to form the SMN complex, which is involved in the biogenesis of spliceosomal snRNPs. In the cytoplasm, the SMN complex assembles proteins of the Sm family on the snRNAs to produce snRNPs that are imported into the nucleus, where they localize to CBs to culminate their maturation (Fischer et al., 2011). Disruption of the *SMN1* gene produces spinal muscular atrophy (SMA), a major genetic cause of infantile mortality (Lefebvre et al., 1997).

Previously, we have shown that a subset of CBs concentrates active SUMO1 (Navascues et al., 2008). Here, we report that SMN is a new SUMO1 target. We further show that SMN has a SIM-like motif in the Tudor domain that regulates its binding with the SmD1 protein (also known as SNRPD1) of the Sm complex and coilin. This interaction is essential for controlling CB assembly and could potentially be involved in SMA pathophysiology caused by point mutations in the Tudor domain.

RESULTS AND DISCUSSION**SMN is a novel SUMO1 substrate**

Our previous results showing the presence of endogenous SUMO1 in a subset of CBs prompted us to investigate putative SUMO targets in CBs, particularly in coilin and SMN proteins. We confirmed in MCF7, 293T and UR61 cells that some CBs are enriched in SUMO1 (supplementary material Fig. S1A–C). In UR61 cells, we observed that active SUMO1 only concentrated in SMN-containing nuclear bodies, but not in coilin-positive and SMN-negative bodies (supplementary material Fig. S1D–L). This prompted us to investigate whether SMN is a SUMO1 target. The localization of SUMO1 in CBs was also confirmed in MCF7 cells transfected with a GFP-tagged wild-type (wt) human SMN construct (GFP–SMNwt), used here for mutagenesis experiments. Thus, co-immunostaining for coilin and either the Sm complex or SUMO1 showed that GFP–SMNwt was always recruited to canonical CBs, some of them immunoreactive for SUMO1 (Fig. 1A–H). Next, by co-immunoprecipitation assays, we demonstrated that both endogenous monomeric SMN and GFP–SMNwt interact with SUMO1 in a covalent manner (Fig. 1I,J). This interaction was validated with a sumoylation assay (Fig. 1K). Interestingly, the conjugating enzyme Ubc9 was not detected in CBs (supplementary material Fig. S1M–R), indicating that they are not the sumoylation sites. However, the localization of the isopeptidase USPL1 in CBs (Schulz et al., 2012) suggests that desumoylation events can occur in these bodies.

Bioinformatic analysis of human SMN with SUMOplot (<http://www.abgent.com/tools>) and SUMOsp 2.0 (<http://sumosp.biocuckoo.org>) servers revealed the presence of a SUMO acceptor lysine within a consensus site (F-K119-R-E) with a

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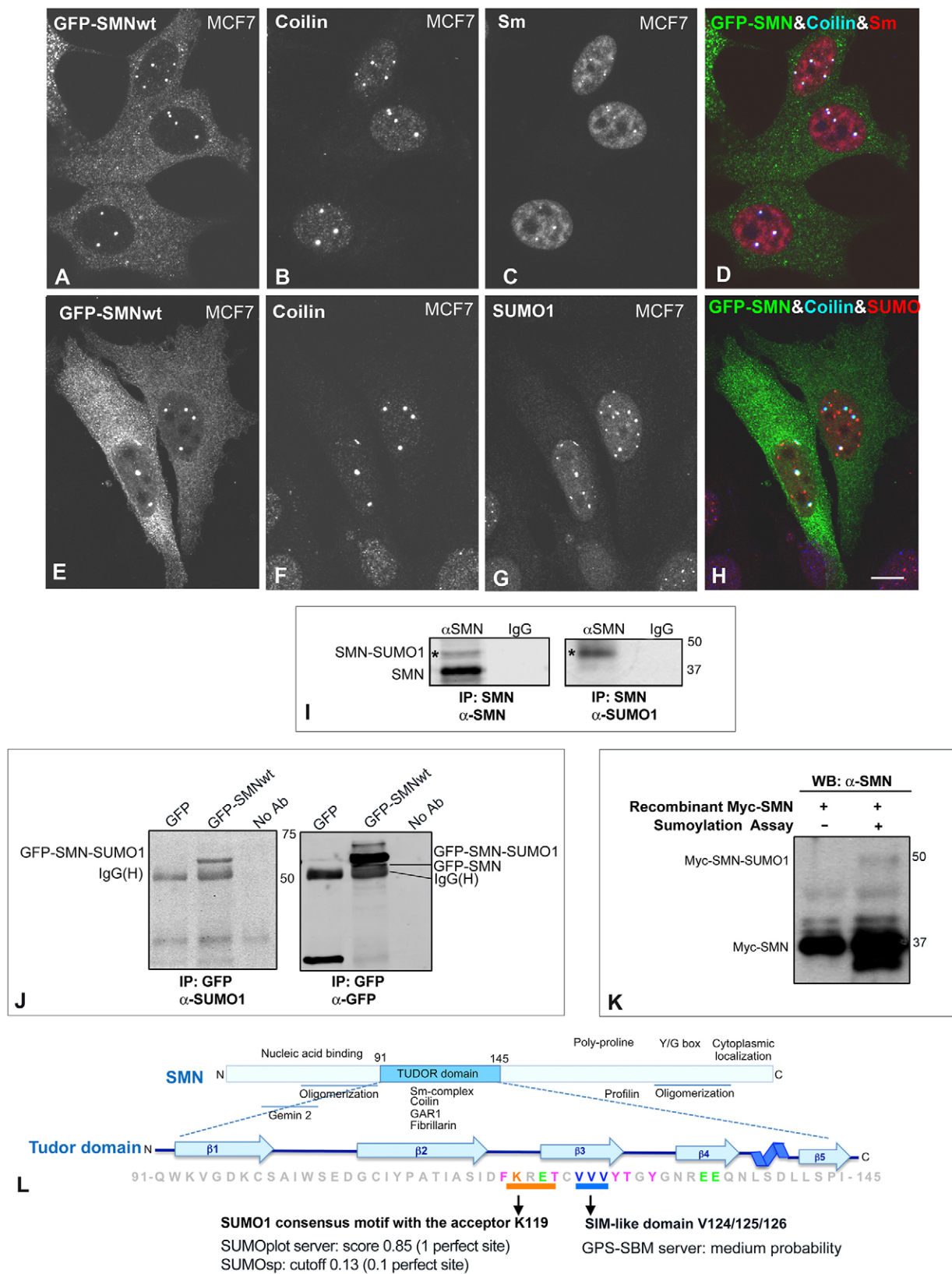


Fig. 1. SMN is a SUMO1 substrate. (A–H) Immunostaining for coilin and snRNPs (A–D), or coilin and SUMO1 (E–H) in MCF7 cells expressing GFP–SMNwt showing SUMO1-immunoreactive CBs. Scale bar: 15 μ m. (I, J) Interactions of both endogenous SMN and GFP–SMNwt with SUMO1 in co-immunoprecipitation assays (*SMN–SUMO1 complex). (K) Sumoylation assay of the recombinant Myc–SMN protein. (L) Protein sequence of the human SMN Tudor domain.

high probability for sumoylation. Furthermore, the GPS-SBM (<http://sbm.biocuckoo.org>) server identified a SIM consisting of three consecutive valine residues (V124–V125–V126) flanked by acidic residues (E121, E134–E135) and putative residues for phosphorylation (T122, T128, Y127 and Y130), which were conserved in several mammalian species (Fig. 1L). Given that such a sequence does not conform exactly to the proposed canonical SIMs (Song et al., 2004), we call it SIM-like. Interestingly, both the K119 residue and SIM-like motif are strategically located in consecutive sequences within the β 3-strand of the SMN Tudor domain (Fig. 1L), which is a crucial sequence for multiple interactions with several CB proteins (Selenko et al., 2001; Renvoisé et al., 2006; Tripsianes et al., 2011).

SMN has a SIM-like sequence that regulates CB assembly and coilin localization

To determine whether covalent or non-covalent SMN–SUMO1 interactions are involved in CB assembly, the GFP–SMNwt construct was mutated by replacing lysine 119 for arginine (GFP–SMN–K119R) or by replacing two or three valine residues of the SIM-like sequence for the minor hydrophobic alanine residues (GFP–SMN–V124/125A or GFP–SMN–V124/125/126A) (Fig. 2A).

Next, we expressed GFP–SMNwt in MCF7 cells previously depleted from endogenous SMN with a 3′-UTR small interfering RNA (siRNA). The efficiency of the siRNA was confirmed by immunoblotting (Fig. 2B; supplementary material Fig. S2A). SMN depletion induced dramatic changes in coilin expression, including loss of CB and relocalization of coilin within the nucleolus and in numerous nucleoplasmic microfoci (supplementary material Fig. S2B,C), as previously reported in HeLa cells (Lemm et al., 2006). This coilin reorganization is consistent with previous studies showing nucleolar accumulations of coilin upon the experimental disruption of CBs (Tapia et al., 2010; Gilder and Hebert, 2011) and with our recent observation that SMN depletion in human motor neurons from SMA patients causes CB loss and nucleolar relocalization of coilin (Tapia et al., 2012).

The expression of the mutant K119R in the SUMO acceptor site in SMN-depleted cells rescued the normal nuclear phenotype observed in MCF7 cells transfected with the GFP–SMNwt construct (Fig. 2C,D), indicating that the lysine K119 residue is not essential for CB assembly. In contrast, transfections with the SIM-like mutants were unable to rescue the normal phenotype, which displayed a dramatic loss of CBs and intranucleolar relocalization of coilin (Fig. 2E,F). Changes in CB distribution in all transfection experiments were confirmed by quantitative analysis (Fig. 2G,H; supplementary material Table S1). Collectively, these results identified that the SIM-like sequence, but not the K119, plays an important role in CB assembly. In this context, we consider it important to investigate whether the CB disruption in SMN SIM-like mutants could be explained by modifications in the structure of the Tudor domain or by an inefficient interaction of SMN with SUMO or other target proteins involved in CB biogenesis.

Loss of hydrophobicity in SMN SIM-like motif reduces its protein–protein affinity and aggregation capacity

To determine the biophysical properties of the SMN SIM-like motif, we performed a bioinformatics analysis by designing theoretical mutations with a loss of hydrophobicity (V124A/V125A and V124A/V125A/V126A) and with an increase in hydrophobicity (V124I/V125I). First, to correlate changes in the

hydrophobicity with the aggregation propensity we used the AGGRESCAN server. Fig. 3A illustrates the localization of two hydrophobic stretches in the wild-type SMN Tudor domain, amino acid residues A111–I116 and R120–G129. The algorithm predicts that the V124A/V125A and V124A/V125A/V126A mutations dramatically reduce hydrophobicity of the R120–G129 stretch and cause lower scores for protein aggregation, as compared with the wild-type or the SMN V124I/V125I mutant that has higher hydrophobicity (Fig. 3B). Second, the IUPred server showed that the decrease in hydrophobicity in the SIM-like mutants conveys a relative propensity to disorder within the SMN Tudor domain in comparison with both the wild-type and the mutant V124I/V125I proteins (Fig. 3C) (Conchillo-Solé et al., 2007). The propensity to disorder of the hydrophobic mutant proteins might modify the affinity of SMN for its binding targets (Uversky et al., 2008). Finally, given that the Tudor motif had been crystallized (Selenko et al., 2001), valine residues 124–126 were localized in its structure (PDB 1G5V). In the predicted structure of V124A/V125A/V126A, mutant replacement of the three valine residues for alanine residues did not affect the structure of the Tudor domain, but surface hydrophobicity was modified (Fig. 3D). Thus, this mutation probably affects protein–protein recognition. In fact, whereas nuclear bodies containing ectopic SMN were present in MCF7 cells expressing the wild-type or the more hydrophobic SIM-like construct (Fig. 3E,H,I), these tended to disappear in cells expressing mutant proteins that had loss of hydrophobicity (Fig. 3F,G,I). In these latter cells, coilin and Gemin2 colocalized in the few endogenous CBs (supplementary material Fig. S2G–J). Interestingly, a fluorescence intensity analysis of nuclear GFP–SMN proteins revealed that the reduction in hydrophobicity did not interfere with the nuclear import of mutant SMN SIM-like proteins (Fig. 3J), suggesting a defective interaction of SMN with CB-protein partners rather than a defective nuclear import (Narayanan et al., 2004). In this way, a defect in the targeting of SMN to CBs is a cardinal feature of SMA motor neurons (Hebert et al., 2001; Tapia et al., 2012).

The SIM-like domain regulates SMN interactions with spliceosomal Smd1 and coilin

To determine whether the SIM-like motif is involved in the protein–protein interactions needed for CB biogenesis we studied three processes: sumoylation of SMN, interaction of SMN with components of the snRNP biogenesis pathway, and SMN–coilin interactions in the molecular assembly of CBs (Fig. 4A).

Given that the hydrophobic interaction between the SIM of the target protein and SUMO1 often precedes and enhances covalent conjugation with SUMO (Kerscher, 2007), we have investigated whether the SIM-like domain is involved in covalent sumoylation of the SMN (Fig. 4A). To address this issue, 293T cells were transfected with GFP, GFP–SMNwt or GFP–SMN–V124/125A and total lysates were subjected to western blot analysis. Immunodetection of GFP–SMN–SUMO with anti-SUMO antibody showed that the lower hydrophobicity in the SIM-like mutant did not abrogate the SMN–SUMO1 interaction (Fig. 4B). Covalent sumoylation of GFP–SMNwt and GFP–SMN–V124/125A was confirmed by co-immunoprecipitation (Fig. 4C). Although our results indicate that the SIM-like domain is not required for covalent sumoylation of SMN, we cannot discard that the free groove of the covalently bound SUMO1 to SMN or that the SIM-like sequence mediate other molecular interactions.

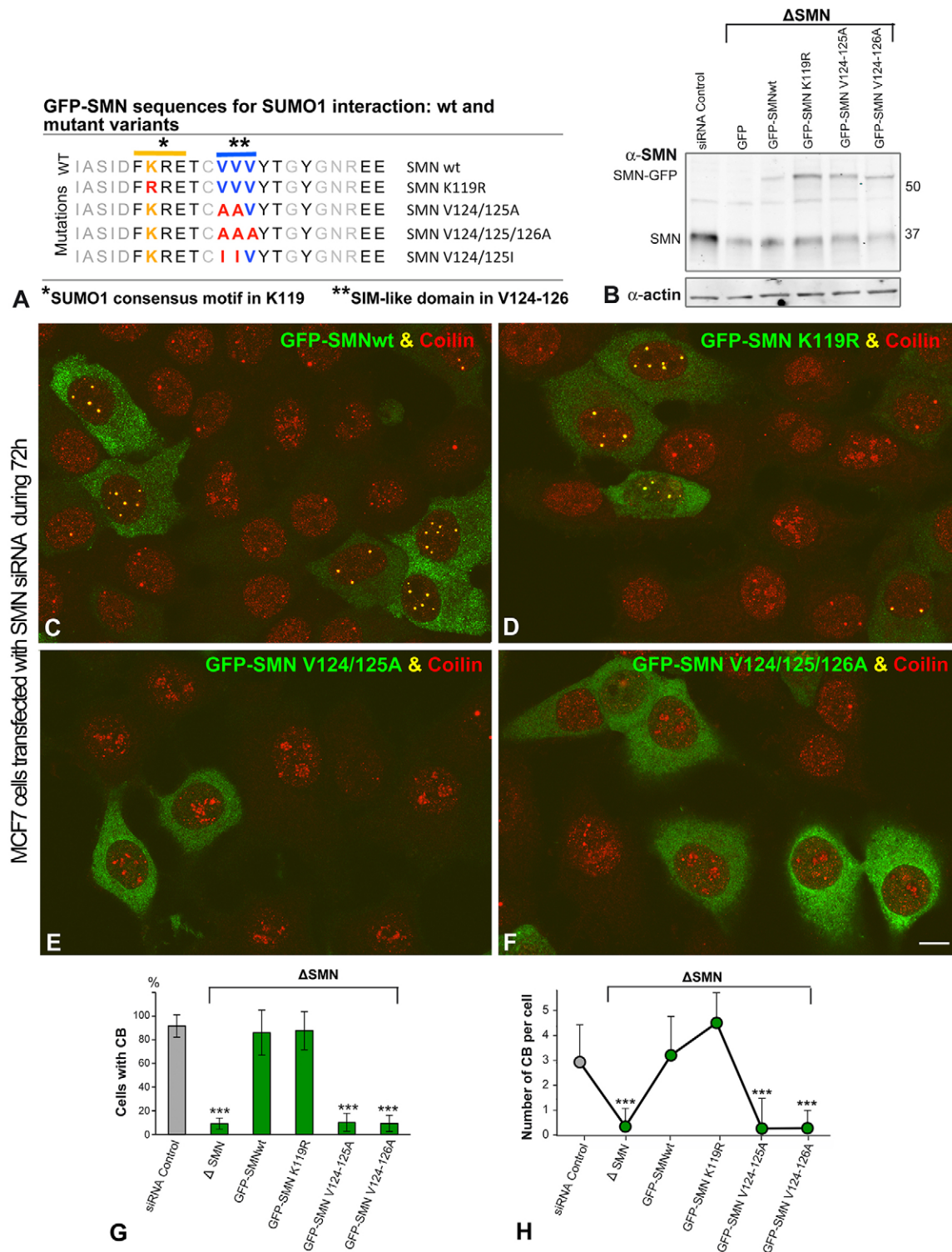


Fig. 2. The SMN SIM-like sequence regulates CB assembly. (A) Table of GFP-SMN mutant variants used in this study. (B) Reduction of endogenous SMN protein levels in SMN-depleted MCF7 cells transfected with the GFP-SMN constructs used here. (C–F) Coilin immunostaining in SMN-depleted cells transfected with GFP-SMNwt, GFP-SMN-K119R, GFP-SMN-V124/125A and GFP-SMN-V124/125/126A constructs. Scale bar: 10 μ m. (G,H) Distribution of CBs in SMN-depleted MCF7 cells expressing SMNwt and SIM-like mutant proteins. Means \pm s.d. from three independent experiments (at least 70 cells per experiment were assayed; *** P <0.001 as compared with control siRNA).

Regarding the snRNP biogenesis pathway, it is well known that SMN is involved in the following steps (Fig. 4A): (1) SMN oligomerization (Lorson et al., 1998; Martin et al., 2012), (2) SMN interaction with Gemin proteins (Zhang et al., 2011), (3)

SMN binding to the Sm complex proteins of snRNPs, which are key constituents of spliceosomes (Pellizzoni et al., 2002), and (4) SMN-coilin interaction in CBs (Hebert et al., 2001) (Fig. 4A).

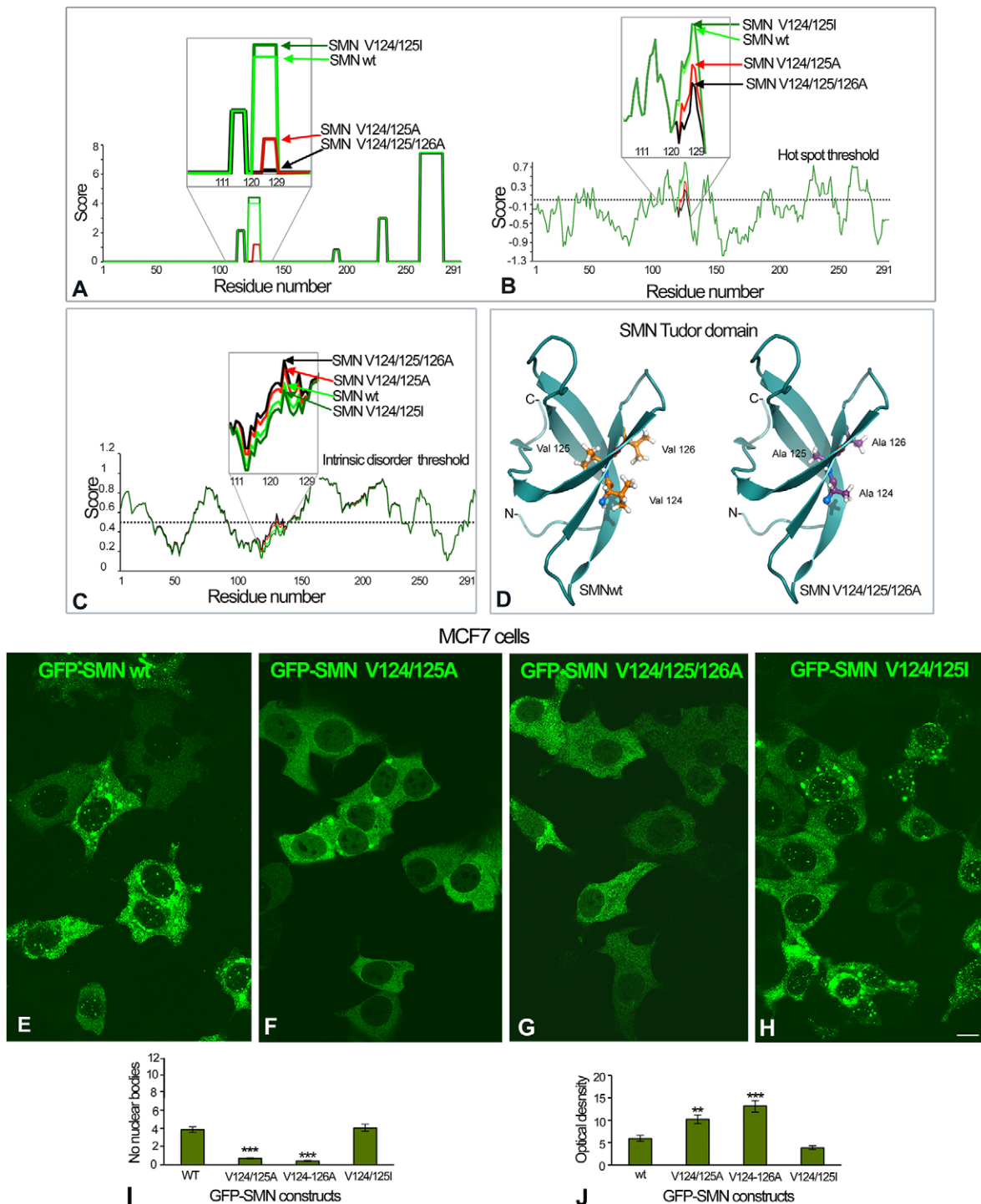


Fig. 3. The hydrophobicity in SMN SIM-like motif correlates with the aggregation propensity. (A,B) The AGGRESCAN server predicts that both V124A/V125A and the V124A/V125A/V126A mutations reduce the hydrophobicity and aggregation propensity of the SIM-like domain. (C) The IUPred server predicts that there is a tendency for disorder of SIM-like mutants with decreased hydrophobicity. (D) A PyMOL ribbon representation for the SMN Tudor domain. On the left, hydrophobic residues of the SIM-like domain are highlighted by a stick representation in orange. In the right panel, V124, V125 and V126 were replaced by alanine residues (purple sticks) to create the mutant model. (E–H) SIM-like mutants V124A/V125A or V124A/V125A/V126A fail to assemble GFP–SMN-positive nuclear bodies. Scale bar: 15 μm. (I) Distribution of the nuclear bodies in MCF7 cells transfected with GFP–SMN constructs ($n=200$ cells per condition; means \pm s.d.; *** $P<0.001$ as compared with controls). (J) Fluorescence intensity analysis of nuclear GFP–SMNwt and mutant variants in MCF7 cells. ($n=100$ per condition; means \pm s.d.; ** $P<0.01$, *** $P<0.001$ as compared with wild-type or the V124I/V124I mutant).

First, we want to determine whether the SIM-like domain is involved in SMN homo-oligomerization. We performed co-immunoprecipitation experiments in 293T cells transfected with

GFP, GFP–SMNwt or GFP–SMN-V124/125A. Immunoblotting for GFP immunoprecipitates with the anti-SMN antibody demonstrated that wild-type and mutant GFP–SMN proteins

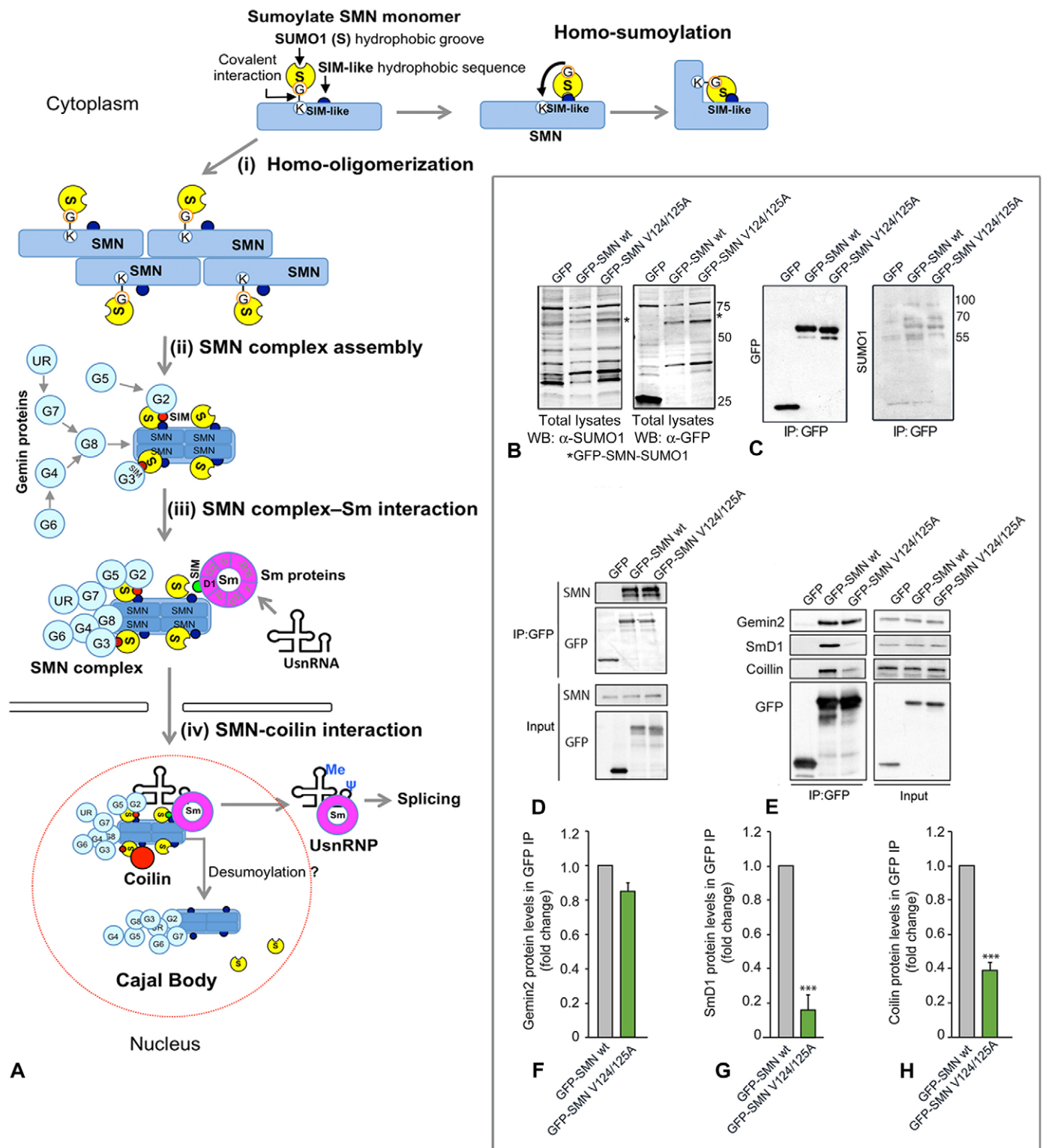


Fig. 4. The SIM-like domain regulates SMN interactions with Smd1 and coilin. (A) Schematic representation of the putative functions of the SMN SIM-like domain in snRNP biogenesis and CB assembly. (B,C) Covalent interaction of GFP–SMNwt and GFP–SMN-V124/125A with SUMO1 was detected by western blotting (B, asterisk) or coimmunoprecipitation (C). (D) 293T cells were transfected with GFP, GFP–SMNwt or GFP–SMN-V124/125A. After GFP immunoprecipitation from cell lysates, SMN enrichment was monitored by anti-SMN in western blot analysis. (E) 293T cells were transfected with GFP, GFP–SMNwt or GFP–SMN-V124/125A. After GFP immunoprecipitation from cell lysates, endogenous Gemin2, Smd1 or coilin protein levels were detected by western blotting. (F–H) Graphs show the average of three independent experiments. ****P*<0.001.

interact with similar affinity to endogenous SMN (Fig. 4D), indicating that the integrity of the SIM-like domain is not necessary for SMN self-oligomerization.

Second, we investigated whether the SIM-like domain participates in the assembly of the SMN complex, in which the SMN oligomer conjugates to eight proteins, Gemin2–Gemin8 and Unrip. The SMN–Gemin2 interaction is of particular importance because both proteins form a heterodimer in the core of the SMN complex, which recognizes spliceosomal Sm proteins (Paushkin et al., 2002). Co-immunoprecipitation experiments with GFP–SMNwt and GFP–SMN-V124/125A proteins demonstrated that although the SIM-like mutation slightly reduced the interaction of SMN with endogenous Gemin2, this change is not statistically significant (Fig. 4E,F). These results are consistent with previous reports suggesting that the SMN motifs responsible for SMN oligomerization and Gemin2 binding are far from the Tudor domain (Martin et al., 2012).

Next, we investigated the interaction of the SMN SIM-like domain with the Smd1 protein of the Sm complex. This complex contains seven proteins encircled in a single-stranded domain of snRNA (Fischer et al., 2011). Interestingly, snRNP assembly requires a direct binding of SMN to the Smd1 protein (Grimm et al., 2013). Previous studies have demonstrated that the point mutations Y130C and E134K, which are in residues flanking the SIM-like cause SMA. Particularly, E134 is essential for the SMN–Smd1 interaction (Bühler et al., 1999; Selenko et al., 2001). In this context, our co-immunoprecipitation experiments showed that the binding of GFP–SMN-V124/125A with the endogenous Smd1 is reduced more than 5-fold compared with GFP–SMN-wt (Fig. 4E,G).

Finally, we investigated whether the SMN SIM-like mutation interferes with the binding of coilin to SMN (Fig. 4A). Previous studies have shown that the recruitment of SMN and snRNPs to CBs depends on the direct interaction of SMN with coilin (Hebert et al., 2001; Tucker et al., 2001). Interestingly, the co-immunoprecipitation experiment also revealed that the SIM-like mutation present in GFP–SMN-V124/125A severely impaired its binding with the endogenous coilin (more than 2-fold compared with the SMNwt) (Fig. 4E,H). Although previous binding assays have shown that the C-terminal of coilin interacts with the SMN Tudor domain (Hebert et al., 2001), our biochemical results demonstrate that the SIM-like sequence also contributes to regulating this interaction.

In conclusion, our data demonstrate that the SIM-like sequence of SMN is essential for CB assembly and also suggest that it is involved in snRNP biogenesis. We demonstrate that the new SMN mutations reported here (V124A/V125A and V124A/V125A/V126A) do not affect the nuclear localization of SMN but disrupt the interaction with coilin and Smd1 proteins. Given that aberrant splicing is a biochemical defect in SMA (Zhang et al., 2011), our data add a significant component to SMA pathophysiology investigation. Further studies on the SIM-like domain might be an important hot spot for screening point mutations in SMA patients with heterozygous deletion of the *SMN1* gene.

MATERIALS AND METHODS

Cell culture and transfection assays

UR61, MCF7 and 293T cell lines were cultured as described previously (Tapia et al., 2012). Transfections with siRNA directed against the human 3'UTR SMN were performed with Lipofectamine-RNAiMAX (Invitrogen), and cells were assayed 48 h after transfection. For rescue experiments, human GFP–SMNwt and mutant constructs were

transfected 48 h after siRNA transfection. The GFP–SMNwt and Myc–SMN constructs used in this study have been described previously (Sleeman et al., 2003; Pellizzoni et al., 1998).

Mutagenesis

GFP–SMN-binding mutants were constructed by site-directed mutagenesis by PCR using the QuikChange II mutagenesis kit (Stratagene).

Confocal microscopy and quantification

Endogenous and ectopically expressed SMN, other CB markers and SUMO were imaged with a Zeiss LSM-510 confocal microscopy system equipped with a 63× oil objective (1.4NA). See antibodies used in supplementary material Fig. S1.

Immunoprecipitation

Immunoprecipitation with Sepharose-coupled GFP binder was as previously described (Rothbauer et al., 2008). For immunoprecipitation of endogenous protein, lysates were first mixed with rabbit IgG or rabbit anti-SMN antibody for 2 h at 4°C before addition of protein-A–agarose for 2 h at 4°C.

Western blotting

Proteins transferred from SDS-PAGE gels to polyvinylidene difluoride membranes were visualized with the Odyssey system (LI-COR Biotechnology).

Sumoylation assay

For the sumoylation assay, Myc-tagged SMN proteins were in vitro synthesized using TnT quick coupled transcription/translation system (Promega) following the manufacturer's instructions. Recombinant Myc–SMN proteins were immunoaffinity purified using anti-Myc beads (Sigma-Aldrich, St. Louis, MO) and verified by SDS-PAGE. For the *in vitro* sumoylation assay, the K007 kit (LEA Biotech International) was used according to the manufacturer's protocol. Protein sumoylation was analyzed by SDS-PAGE and western blotting using anti-Myc (Ab1014) and anti-SUMO1 (21 C7, Zymed) antibodies.

Statistical analysis

Statistical significance was determined by unpaired, two-tailed Student's *t*-tests.

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Competing interests

The authors declare no competing interests.

Author contributions

O.T. and V.L. conceived of experiments and performed co-immunoprecipitation assays, R.B. and A.P. performed mutagenesis and transfection experiments, M.L. and M.T.B. designed experiments and wrote the manuscript.

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Supplementary material

Supplementary material available online at <http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.138537/-DC1>

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