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SUMO Interaction Motifs in Sizn1 Are Required for Promyelocytic Leukemia Protein Nuclear Body Localization and for Transcriptional Activation*[S]

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Mutations in Sizn1 (Zcchc12), a novel transcriptional co-activator in the BMP signaling pathway, are associated with X-linked mental retardation. Previously, we demonstrated that Sizn1 positively modulates the BMP signal by interacting with Smad family members and cAMP-responsive element-binding protein-binding protein. To further define the molecular basis of Sizn1 function, we have explored its subcellular localization and generated various deletion mutants to carry out domain analyses. Here, we report that Sizn1 localizes to promyelocytic leukemia protein nuclear bodies (PML-NBs). Sizn1 deletion mutants that disrupt the MA homologous domain or the middle region fail to target to the PML-NB. We show that two SUMO interaction motifs (SIMs) in Sizn1 can bind to SUMO and govern SUMO conjugation to Sizn1 in the absence of the consensus motif for SUMO attachment. Interestingly, the SIM mutant Sizn1 localizes to nuclear bodies, but not to PML-NBs. Thus, SIMs mediate the localization of Sizn1 to PML-NB. Interestingly, mutations in SIM sequences and deletion of the MA homologous domain also affected the transcriptional co-activation function of a Sizn1. Taken together, our data indicate that the SIMs in Sizn1 are required for its PML-NB localization and for the full transcriptional co-activation function in BMP signaling.

Normal brain development requires a highly orchestrated gene expression network that is modulated by an array of transcription factors and cofactors including histone modification enzymes, chromatin remodeling enzymes, and related factors. Working as a balance between positive and negative regulators, these various factors play key roles in defining the spatial and temporal pattern of gene expression necessary for normal development (1–3).

Sizn1 (Zcchc12) is a recently identified, novel transcription co-activator that positively modulates BMP signaling through its interaction with Smad family members and recruitment of CREB-binding protein (CBP)[3] to the transcription complex (4). Our previous data indicate that Sizn1 is expressed in a subset of ventral forebrain septal neurons where it contributes to BMP-dependent cholinergic neuron specific gene expression (4, 5). Furthermore, mutations in *Sizn1* have been associated with X-linked mental retardation (6). Its association with human disease and our limited understanding of its cellular function prompted us to further define the cellular localization of Sizn1 and to identify the roles played by its various structural domains.

PML nuclear bodies (NB) are found in the nucleus as large ring-shaped protein complexes (7). They are ~0.3–1 μm in diameter, with the main component being PML protein (8, 9). PML-NBs are implicated in diverse nuclear functions including transcription, DNA repair, apoptosis, tumor suppression, proteolysis, and anti-viral activity (10). They are very close to chromatin but are not known to be localized at transcriptionally active sites (7, 11, 12).

SUMOylation refers to a post-translational conjugation of SUMO to a cellular protein. SUMOylation has been implicated in cell cycle progression, intracellular trafficking, transcription, and DNA repair (13). SUMO is covalently conjugated to target proteins via an isopeptide bond by a mechanism that involves E1 (ubiquitin-activating enzyme; SAE1/2), E2 (ubiquitin carrier protein; Ubc9), and E3 (ubiquitin-protein isopeptide ligase) enzymes (13). SUMO is removed by isopeptidase (SENP/Supr-1). A recent model for PML-NB formation proposes that PML-SUMO conjugation and noncovalent interaction of PML to SUMOylated PML via a SUMO interaction motif (SIM) are necessary to form PML-NB and for the subsequent recruitment of PML-NB accessory protein, such as SUMOylated proteins and/or proteins containing SIMs (14, 15). Supporting this model, BLM, CBP, Daxx, HIPK2, p53, and Sp100 are known to require SUMOylation to be incorporated in PML-NB (8, 16–20).

Our previous data predict that Sizn1 should be expressed in the nucleus where it would interact with Smad proteins to modulate BMP signaling (4). Herein we report that Sizn1 proteins

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–6 and data.

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[3] The abbreviations used are: CBP, CREB-binding protein; GST, glutathione *S*-transferase; CREB, cAMP-responsive element-binding protein; NB, nuclear body; GFP, green fluorescent protein; NLS, nuclear localization signal; PNMA, paraneoplastic MA antigen; shRNA, short hairpin RNA; BMP, bone morphogenic protein; PML, promyelocytic leukemia protein; SIM, SUMO interacting motif; mSIM, mutant SIM.

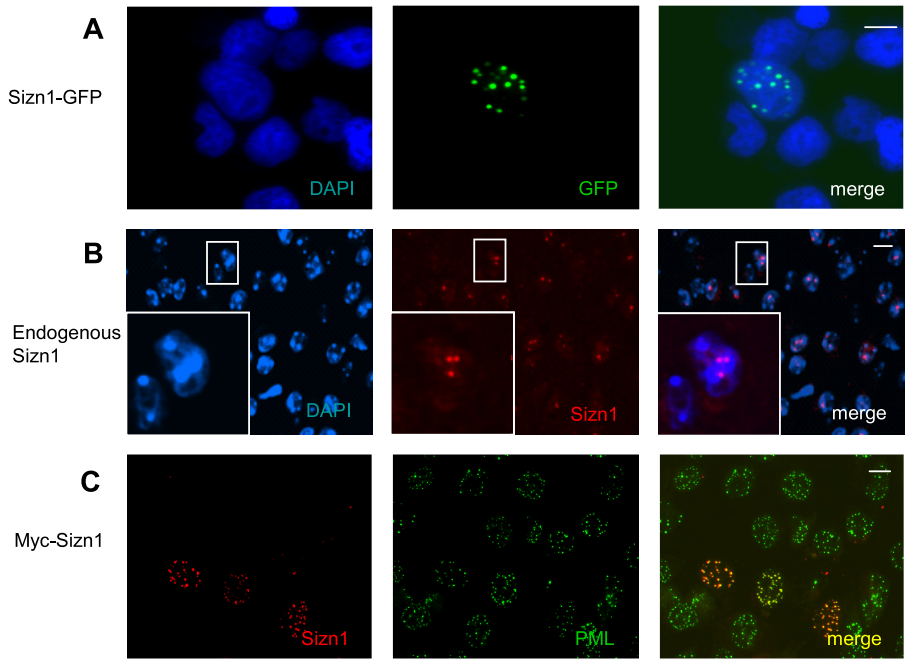

FIGURE 1. **Sizn1 is a nuclear protein localized in PML bodies.** *A*, the GFP-Sizn1 fusion protein is localized in a speckled pattern within the nucleus of HEK293T cells. *B*, endogenous Sizn1, detected by immunofluorescence using an anti-Sizn1 antibody, is expressed in a nuclear speckle pattern in the septal nucleus of the P3 neonatal brain. (*Lower left box* is a high power image corresponding to the *smaller box* in each image.) *C*, Sizn1 co-localizes with nuclear PML bodies in C2C12 cells that were transfected with pMIWIII/Myc-Sizn1. *Scale bars* indicate 10 μm.

are localized on PML-NBs. We have identified three peptide domains in Sizn1 that code for the localization of PML-NB: two SIMs and the MA homology domain that consists of a highly conserved amino acid sequence found in paraneoplastic MA antigen (PNMA) proteins. Mutations of the SIMs or deletion of the MA homologous domain perturbs PML-NB localization of Sizn1 and interferes with BMP signaling co-activation.

EXPERIMENTAL PROCEDURES

Plasmids—pCMV/Sizn1-green fluorescent protein (GFP) was generated by subcloning the mouse Sizn1 coding region into pcDNA3-CTGFP (Invitrogen). pMIWIII/Myc-Sizn1 (4) mutants were subcloned by the PCR product containing *Sizn1* deletion or point mutants into HindIII and EcoRV of pMIWIII/Myc as Myc fusion protein (supplemental data). The Sizn1-GFP fusion protein (mutant) expression constructs were cloned by serially adding Sizn1 and GFP PCR products to pMIWIII, (supplemental data). For *in vitro* translation of Sizn1 mutants, the plasmid pcDNA3.1-topo (Invitrogen) was used to subclone the PCR product containing the Sizn1 coding sequence but without a Myc tag. pGST-Smad1 MH1, MH2, and pGST-*SUMO* were constructed by subcloning *Smad1 MH1*, *MH2*, and *SUMO* PCR product into EcoRI and XhoI of pGEX-5T (Amersham Biosciences), respectively (supplemental data). A *SUMO* DNA fragment containing BamI and NotI sites was cloned into pEBG to make a mammalian GST-SUMO expression vector (supplemental data). The PML knockdown constructs were generated by subcloning the double strand sequence (5′-CAATCATGTTACCAGTGAC-3′) into the pLL3.7 vector. CBP mutant expression constructs were kindly provided by Dr. Hsiu-Ming Shih (Institute of Biomedical Sciences, Academia Sinica, Taipei, Republic of China).

Cell Culture, Transfection, and Luciferase—HEK293T and C2C12 cell lines were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Hyclone; 15% fetal bovine serum for C2C12 cells) at 37 °C and 5% CO_2. C2C12 cells were transfected with various combinations of the following plasmids using FuGENE 6 (Roche Applied Science): reporter constructs (*SBEx4*-luciferase) (0.1 μg), β-galactosidase expression vector driven by the cytomegalovirus promoter (cytomegalovirus-β-galactosidase) (0.05 μg), constitutively active BMPR1a (QD) (0.2 μg) and pMIWIII/Myc-Sizn1 (each 0.5 μg) as described previously (4). Empty vectors for each construct were used to ensure transfection of equal amounts of DNA. Cell extracts were prepared with a Promega lysis buffer followed by centrifugation. Luciferase activity was measured by a Promega luciferase assay system as described previously (4). Transfection efficiency was standardized with β-galactosidase activity. All assays were performed in duplicate. HEK293T cell was used for GST pulldown assay to carry out *in vivo* SUMOylation and SUMO binding assay.

GST Pulldown Assay—GST pulldown assays were performed as described previously (4). After transfection of pEBG-SUMO and Sizn1 mutant expression constructs, cells were lysed in TNE buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 5% glycerol) containing a protease inhibitor mixture (Roche Applied Science) and 10 mM *N*-ethylmaleimide (Sigma). After centrifugation, supernatants were mixed with glutathione-sepharose 4B (Amersham Biosciences) and incubated for 45 min on ice. After washing the unbound protein with TNE buffer five times, the eluate was analyzed by Western blot. GST-SUMO, GST-Smad1, GST-Smad1 MH1, GST-Smad1 MH2, and GST-Sizn1 protein were produced in *Escherichia coli* and purified with glutathione beads (Amersham Biosciences) according to the manufacturer's protocol. *In vitro*-translated products were generated using a TnT kit (Promega) in the presence of [^{35}S]methionine. GST pulldown assays were performed as described above after mixing GST fusion protein and ^{35}S-labeled protein.

Immunocytochemistry—C2C12 cells were grown in slide chambers and transfected with Sizn1 or its mutants using the FuGene 6 transfection reagent (Invitrogen) according to the manufacturer's protocol. After 24–36 h, the cells were washed with phosphate-buffered saline and fixed in 4% paraformaldehyde at room temperature (18 °C) for 5 min. The cells were then permeabilized in phosphate-buffered saline containing 1% bovine serum albumin and 0.2% Triton X-100. Mouse anti-PML (Clone 36.1–104, Millipore; 1:100) or mouse anti-Myc

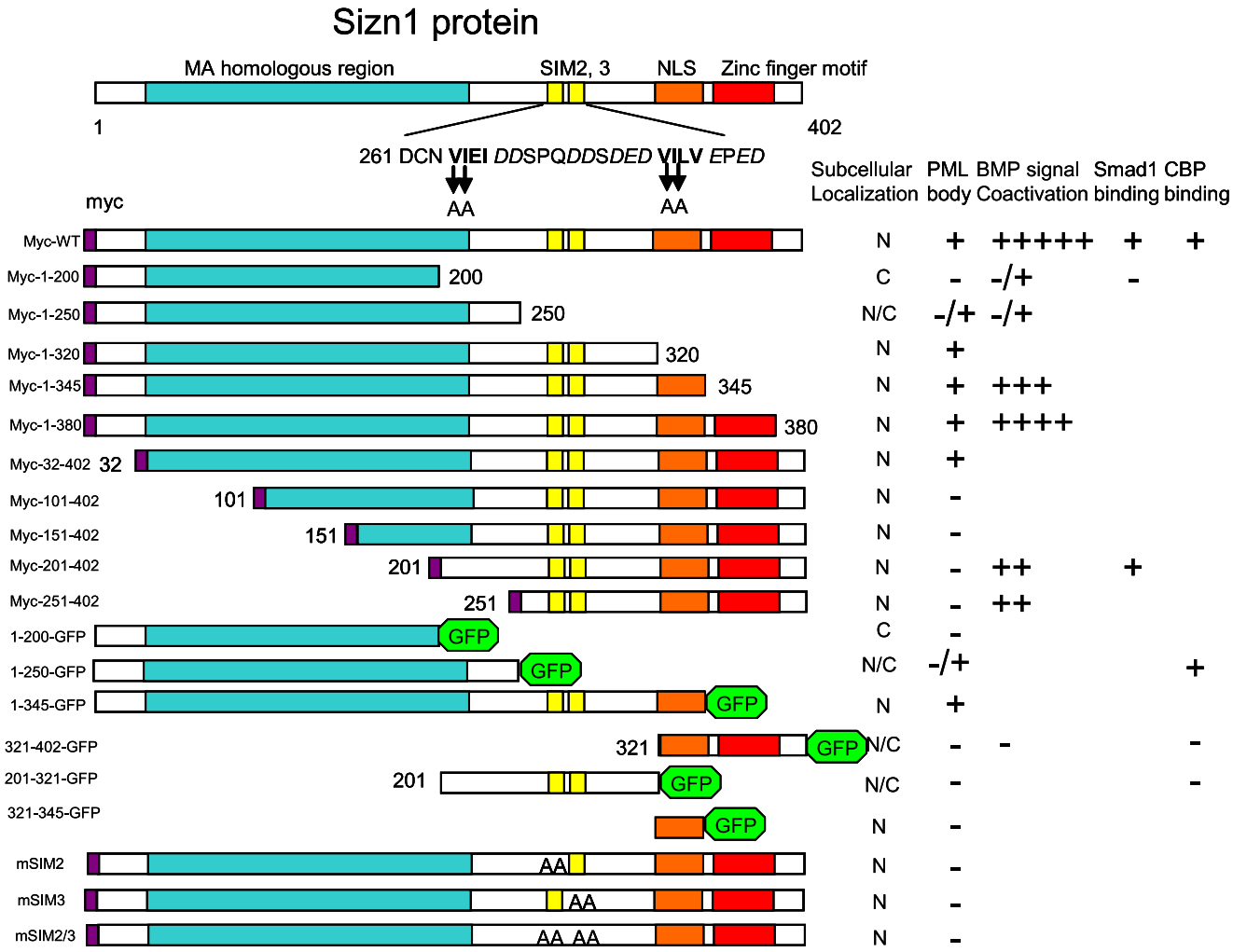

FIGURE 2. **Schematic representation of Sizn1 functional domains and deletion mutants used in this study.** Sizn1 has an MA homologous domain at the N terminus, two SIMs, and a putative NLS. The Myc tag is illustrated with a *purple box*, and the GFP tag is illustrated with a *green box*. *N*, nucleus; *C*, cytoplasm. *Boldface* and *italic type* on SIM represent hydrophobic and positively charged amino acid residues, respectively. *Arrows* indicate mutated amino acids in SIM2 and SIM3 (Val, Ile to Ala, Ala). The putative NLS region (200–250) identified in this study is not depicted in the diagram. The constructs used for *in vitro* translation did not contain the Myc tag. *WT*, wild type.

(9E10; 1:200), and rabbit anti-Sizn1 (1:100) antibodies were added and incubated in the same buffer at room temperature for 1 h, followed by incubation with goat anti-rabbit IgG or anti-mouse IgG conjugated with Alexa Fluor 568 or 488 (1:500; Molecular Probes), together with 4′,6-diamidino-2-phenylindole (0.1 mg/ml) at room temperature for 30 min. Images were captured on a Leica DM6000B microscope equipped with epifluorescence and a DFC360FX camera. The images were acquired and processed in LAS-AF software (Leica).

RESULTS

Sizn1 Localizes onto PML-NB—The subcellular localization of Sizn1 was first defined by expressing a Sizn1-GFP fusion protein in HEK293T cells. The Sizn1-GFP fusion protein exhibited a speckled pattern of nuclear expression (Fig. 1*A*). Endogenous Sizn1, labeled with an anti-Sizn1 antibody, was also detected in nuclear speckles in the septal nucleus of the basal forebrain (Fig. 1*B*). These data indicate that Sizn1 is expressed in the same pattern *in vitro* and *in vivo*. The nuclear speckled pattern of expression suggested that Sizn1 might localize to one of many possible known intranuclear bodies including PML-NB, Gems/Cajal body, and/or heterochromatin (21). Using double-label immunostaining, we found Sizn1 to co-localize with PML-NBs, but not to Gems, Cajal bodies, or heterochromatin. (Fig. 1*C*) (data not shown). Based on these data, we conclude that Sizn1 is an accessory protein of PML-NB.

The MA Homologous Domain (32–230) and Middle Region (250–320) of Sizn1 Are Necessary for PML-NB Localization—To understand the molecular basis of how Sizn1 is targeted to PML-NB, we analyzed the protein sequence *in silico* and identified a MA homology domain (32–230), four putative SUMO interacting motifs (SIM1 (147–150), SIM2 (264–267), SIM3 (278–281), and SIM4 (306–309)), a single nuclear localization signal (NLS; 321–345), and a classic zinc finger domain (CHCC; zinc-knuckle domain, 348–370) (Fig. 2 and supplemental data) (4, 22).

To identify the sequences responsible for PML-NB localization of Sizn1, a series of Myc-tagged (N-terminal) or GFP-tagged (C-terminal) deletion mutants were generated (Fig. 2). All of the deletion mutants, except for sequence 1–200, local-

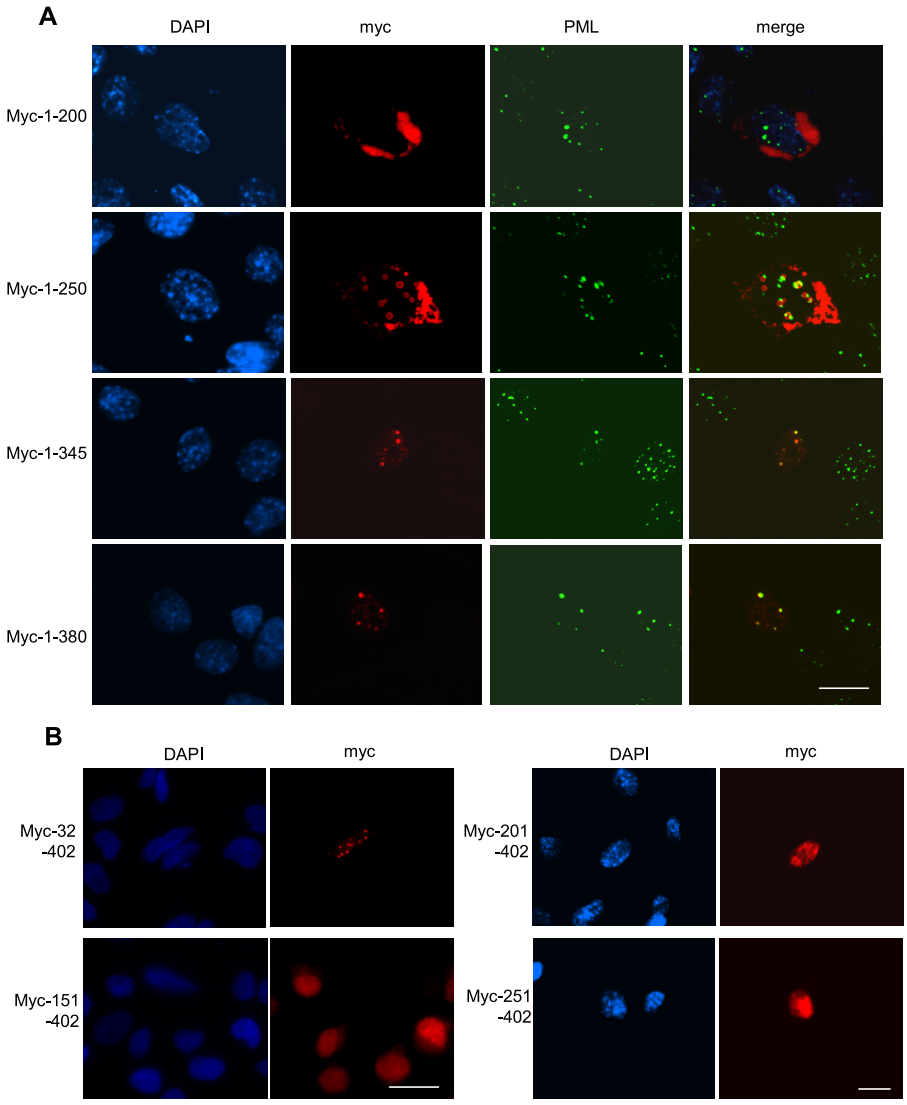

FIGURE 3. **Subcellular localization of Sizn1 deletion mutants tagged with Myc.** C2C12 cells were transfected with the indicated deletion mutants and immunostained with anti-PML and anti-Myc. *A*, C-terminal deletion mutants. Only Myc-1–345, Sizn1 mutants containing the NLS. *B*, *N*-terminal deletion mutants. Only the Myc-32–402 mutant showed proper PML-NB localization, whereas the others showed diffused nuclear distribution. In all figures, 4′,6-diamidino-2-phenylindole (*DAPI*) is used to stain the nucleus. *Scale bars* indicate 10 μm.

ized to the nucleus, although the 1–250 sequence was also detected in the cytoplasm (Fig. 3*A*). Among the C-terminal deletion mutants, only the 1–320, 1–345, and 1–380 sequences showed correct targeting to PML-NBs (Fig. 3*A* and supplemental data). In the case of the 1–250 sequence, although a speckled pattern of its expression was observed in the nucleus, it did not co-localize with PML-NBs. Thus, based on the C-terminal deletion mutants, proper targeting to the PML-NBs appears to require sequences between 251–320.

Analysis of the N-terminal deletion mutants identified another domain required for PML-NB localization. The N-terminal deletion mutants, with the exception of the 32–402 sequence, did not localize to PML-NBs, although their targeting to the nucleus was normal (Fig. 3 and supplemental data). This suggested that the MA homology domain (32–230) is essential for the correct targeting of Sizn1 to the PML-NB.

To confirm our data, we tested the 1–200, 1–250, and 1–345 truncated proteins fused with GFP. The 1–345 GFP proteins localized to PML-NB, whereas the 1–200 GFP protein did not enter the nucleus as seen with Myc-1–200 construct (Fig. 4*A*). Furthermore, the 1–250 GFP construct did not localize to PML-NB but showed a speckled pattern of expression in the nucleus where it exists near PML-NB (Fig. 4*A*). These data are consistent with the results we observed with the Myc-tagged constructs.

The *in silico* analysis predicted an NLS at position 321–345 in Sizn1. The 251–402 sequence contains this putative NLS and localizes to the nucleus as predicted (Fig. 3). To verify that this domain alone is sufficient to act as an NLS, we fused GFP to the 321–345 sequence and tested its localization. This sequence targeted protein to the nucleus correctly, but not to nuclear speckles (Fig. 4*B*). These data indicate that the 321–345 sequence is sufficient for nuclear localization, but not for PML-NB targeting. Interestingly, the 1–250 and 1–320 mutants, which do not contain this NLS domain, showed nuclear expression, whereas the 1–200 mutant did not. These data suggest that the sequence in the 200–250 region may contain another NLS, which was not predicted by sequence analysis. To confirm this, we examined the expression of the 200–320 GFP fusion construct and found this protein to be predominantly localized to the nucleus with slight cytoplasmic expression (Fig. 4*B*). Taken together, these results reveal that Sizn1 has two NLSs (200–250 and 321–345), and either one is sufficient for its nuclear localization.

Sizn1 Can Be SUMOylated in a SIM-dependent Manner—PML protein is known to undergo SUMOylation (covalent attachment) and to interact noncovalently with SUMO. Both the SUMOylation modification and the noncovalent interaction with SUMO are associated with proper PML-NB formation (14, 15). In addition to PML SUMOylation, many proteins such as Daxx and CBP that localize to PML-NBs are SUMOylated (8, 16–20). Given that Sizn1 localizes to PML-NBs, we hypothesized that Sizn1 would be SUMOylated. To test this hypothesis, we co-transfected GST-SUMO and Myc-Sizn1 expression constructs in the HEK293T cells and carried out GST pulldown assays. We detected multiple Sizn1 bands from GST pulldown products when immunoblotted with Myc antibody. SUMO-conjugated forms of Sizn1 were observed at a higher molecular mass (*white arrow* in Fig. 5*A*; at least two

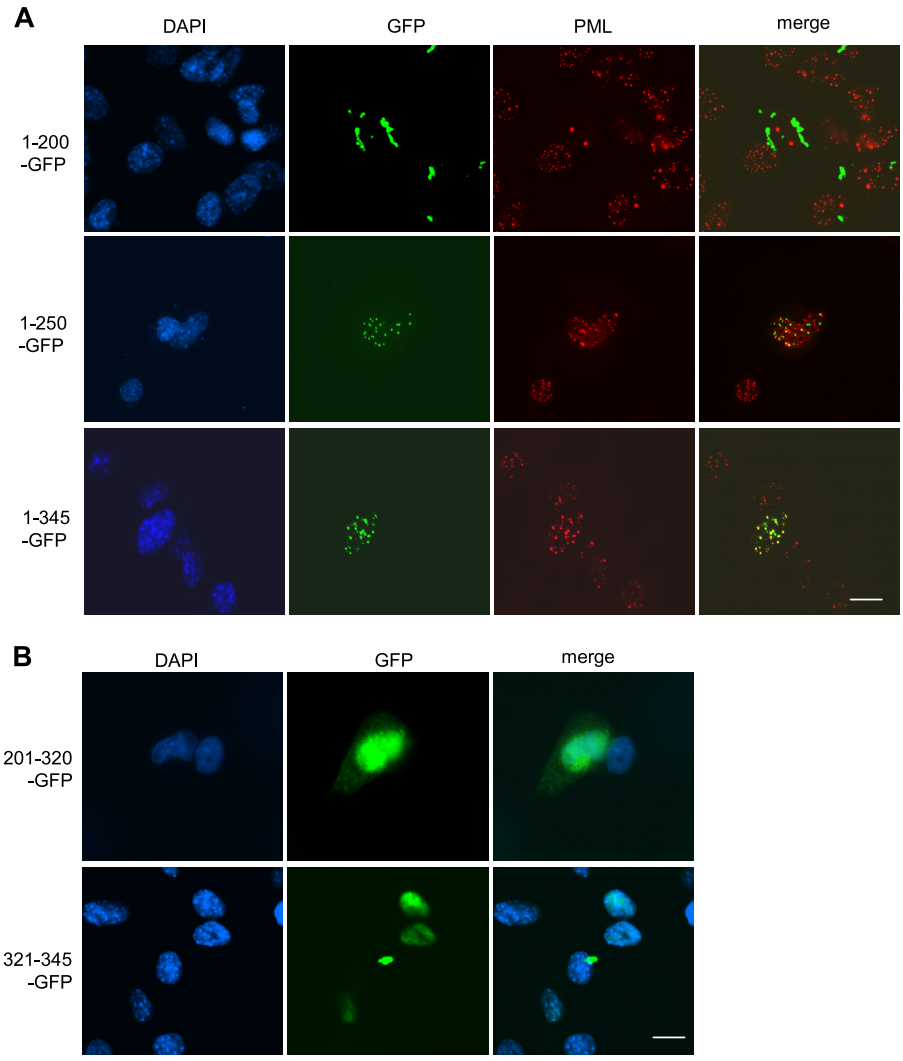

FIGURE 4. **Subcellular localization of GFP-tagged Sizn1 deletion mutants.** Immunostaining of GFP-tagged mutants in transfected C2C12 cells using anti-PML. *A*, C-terminal deletion mutants. GFP fluorescence merged with PML antibody staining shows that only the 1–345-GFP is correctly targeted to the PML-NB. *B*, deletion mutants containing an NLS (predicted and unpredicted). 321–345 (predicted NLS) can target GFP to the nucleus but not to the PML-NB. 200–320 (without predicted NLS) can target GFP to the nucleus with weak cytoplasmic distribution. *Scale bar* indicates 10 μm. *DAPI*, 4′,6-diamidino-2-phenylindole.

higher molecular mass bands). The unshifted band corresponds to unSUMOylated Sizn1 that is nonconvalently interacting with SUMO (*black arrow* in Fig. 5*A*). These data suggest that Sizn1 can be SUMOylated and can also noncovalently interact with SUMO.

To map the domains responsible for SUMOylation and SUMO interaction, we co-transfected GST-SUMO and deletion mutants of Sizn1 into HEK293T cells and carried out GST pulldown assays. All of the mutants, except for the 1–250 sequence, showed both forms of interaction with SUMO, one with SUMOylation (Fig. 5*A*, see *white arrow*) and the other with noncovalent interaction (Fig. 5*A*, see *black arrow*). These data indicate that the 251–345 sequence is required for SUMOylation and SUMO interaction. For SUMOylation, the lysine residue in the context of ΨK*X*(D/E)sequence (where Ψ is a hydrophobic residue, K is lysine, *X* is a any amino acid, and D/E is an acidic residue) is required for SUMO-covalent attachment (13). However, in one case, even without this consensus sequence flanking lysine, SUMOylation can still occur (23). SUMOylation of Sizn1 appears to be another example because none of the lysine residues in Sizn1 follow this sequence rule. These data indicate that the sequence 251–345 is also involved in SUMOylation and is sufficient to make SUMOylation possible when added to 1–250, which is not capable of being SUMOylated on its own.

The known SIMs have a hydrophobic core ((V/I)*X*(V/I)(V/I)) followed or preceded by a negatively charged cluster of amino acids (22, 24). Based on sequence analysis, we found four candidate regions in Sizn1 (Fig. 2 and supplemental data) (22). Two candidate regions, SIM2 and -3, exist in the 251–320 region that we identified in the GST pulldown assay (Fig. 5*A*; SIM1 and SIM4 fall outside of the predicated SUMO interacting motif region). To confirm a direct interaction between SIMs on Sizn1 and SUMO, we mutated these SIMs by replacing valine and isoleucine with two alanine residues in consecutive hydrophobic cores and carried out GST pulldown assay with *in vitro*-translated mutant protein (see Fig. 2 for sequence information). As predicted, mSIM2 and mSIM3 showed lower binding activity to SUMO, and the double mutants did not bind SUMO (Fig. 5*B*). These data verify that SIM2 and -3 can mediate direct interaction with SUMO.

This led us to hypothesize that Sizn1 SUMOylation would occur by a nonconventional mechanism (23). We postulated that this would be dependent on its ability to interact noncovalently with the SUMO moiety of charged Ubc9 through a SIM. To test this hypothesis, we examined whether mutations in SIM2 or SIM3 affect SUMOylation of the Sizn1. After transfecting GST-SUMO and Sizn1 expression constructs harboring mutations in SIM to HEK293T cells, we performed a GST pulldown assay. If SUMOylation is dependent on the SIM sequence as we hypothesized, mutation in SIM domains would disrupt SUMOylation of Sizn1. As predicted, SUMOylation was not detected in SIM3 and SIM2/3 double mutants, and reduced SUMOylation was detected with the SIM2 (Fig. 5*C*). Thus, SIM2 and SIM3 can direct Sizn1 SUMOylation.

SIMs in Sizn1 Are Essential to Localize to PML-NB—We showed that the MA homologous domains and the middle region of Sizn1 are required for PML-NB localization. This middle region contains the SIM2 and SIM3 sequences. Thus,

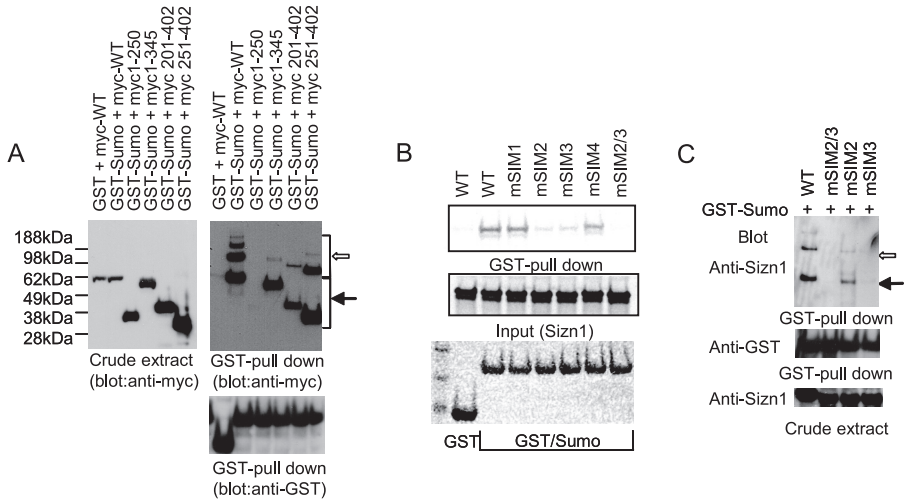

FIGURE 5. **Sizn1 is a** SUMO**ylated protein, and its SUMOylation is dependent on SIMs.** *A*, GST/SUMO pull-down assay with HEK293T cells transfected with GST-SUMO and Myc-Sizn1 plasmids (full-length and deletion mutants). Both SUMOylated (covalent conjugation, *white arrow*) and nonSUMOylated forms of Sizn1 (noncovalent interaction with SUMO, *black arrow*) are detected when the wild type (*WT*) and Sizn1 mutants were co-transfected with the exception of the Myc-1–250 mutant. Note that the C-terminal deletion mutants migrate slowly in SDS-PAGE for their size. *B*, autoradiography of the GST/SUMO pulldown assay with *in vitro*-translated and ^{35}S-labeled Sizn1. The mSIM1, mSIM2, mSIM3, and mSIM4 contain two amino acid mutations in a single SIM sequence, whereas mSIM2/3 harbors mutations in both SIM2 and -3. *C*, GST pulldown assay with HEK293T cells transfected with GST-SUMO and Sizn1 (the wild type and mutants contain modified SIMs). The level of both SUMOylated and nonSUMOylated forms of Sizn1 are pulled down, but reduced levels are found for mSIM2; none are detected with the mSIM3 and mSIM2/3 mutants.

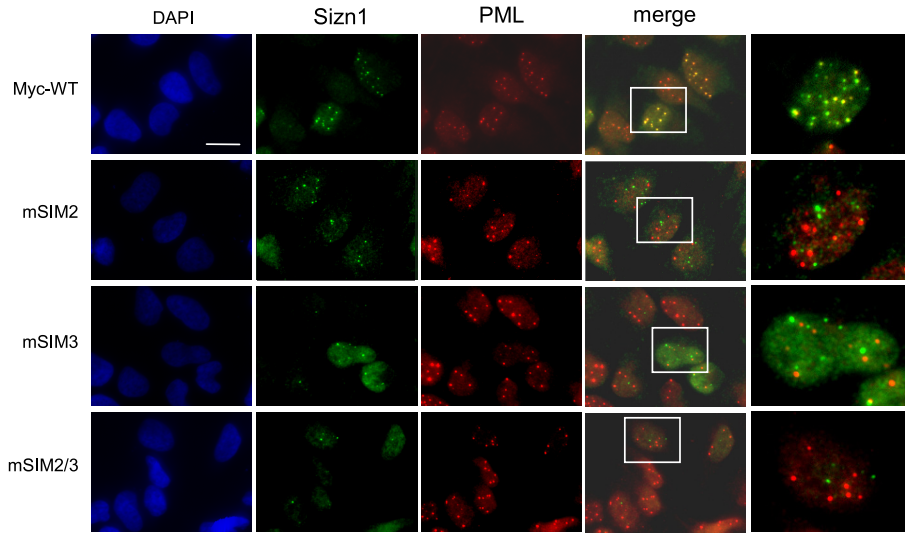

FIGURE 6. **Mutations in SIM sequences cause Sizn1 to localize in distinct nuclear puncta that do not overlap with PML-NB.** C2C12 cells were transfected with the Sizn1 wild type (*WT*) and mutant forms followed by immunostaining with anti-PML and anti-Sizn1 antibodies. In mutants, speckled staining of the Sizn1 does not overlap with PML-NB, unlike in the wild type. The *right column* identifies the enlarged images in the merged image. *Scale bar* indicates 10 μm. *DAPI*, 4′,6-diamidino-2-phenylindole.

we tested if these SIMs would be necessary for PML-NB localization. We examined the subcellular localization of SIM mutants in cells. Surprisingly, the SIM2, SIM3, and SIM2/3 mutants did not localize to PML-NB, although they showed a speckled pattern in the nucleus (Fig. 6). These data indicate that SIM2 and -3 are necessary for PML-NB localization. Interestingly, the 200–402 and 250–402 sequences, which contain these two SIMs, but not a MA homologous domain, also did not localize properly to PML-NB (Fig. 3). Taken together, these results suggest that Sizn1 requires both the MA homologous domain and SIMs for correct PML-NB localization.

Sizn1 Is Expressed in a Speckled Pattern in Nucleus in Absence of PML Protein—PML-NB accessory proteins are known to distribute evenly throughout the nucleus in *PML*$^{-/-}$ cells (8). The 1–250 mutant containing the MA homologous domain showed large dot structures in the nucleus of transfected cells. In addition, SIM mutants make a nuclear body that is not in PML-NB. Therefore, we asked whether Sizn1 distributes evenly in the nucleus in the absence of PML protein. We designed a short hairpin RNA (shRNA) target vector against nuclear PML protein. After co-transfection of the Sizn1 expression construct and the PML knockdown construct to C2C12 cell lines, we examined the Sizn1 subcellular localization by immunostaining. We first verified that shRNA expression knocked down PML protein in the nucleus (*arrows* in Fig. 7 and supplemental data). Surprisingly, Sizn1 was detected in a speckled pattern even in the absence of PML protein (Fig. 7). This suggests that Sizn1 may be able to form nuclear bodies independent of PML, consistent with our previous data showing that SIM2 or SIM3 mutants can make a nuclear body, but it does not co-localize onto PML-NB.

N Terminus of Sizn1 Associates with CBP-(1–450) and C Terminus of Sizn1 Can Interact with the MH1 Domain of Smad1—The above observations define the domains of Sizn1 required for subcellular localization. However, the domains required for Sizn1 function in BMP signaling are not known. We reported previously that Sizn1 can interact with Smad1 to co-activate *ChAT* and *VaChT* gene expression (4). To find the interaction domain between Sizn1 and Smad1, GST-tagged full-length Smad1 (GST-Smad1) or deletion mutant proteins (GST-MH1 and GST-MH2 only contain the MH1 or MH2 domain of Smad1, respectively) were pulled down with glutathione beads in the presence of *in vitro*-translated Sizn1 protein. GST-Smad1 and GST-MH1 were precipitated with ^{35}S-labeled Sizn1 protein (Fig. 8*A*). These data indicate that the Smad1 MH1 domain can interact with Sizn1 directly. To define the binding domain of Smad1 in Sizn1, we used GST-Smad1 and *in vitro*-translated Sizn1 deletion mutant proteins for a GST pulldown

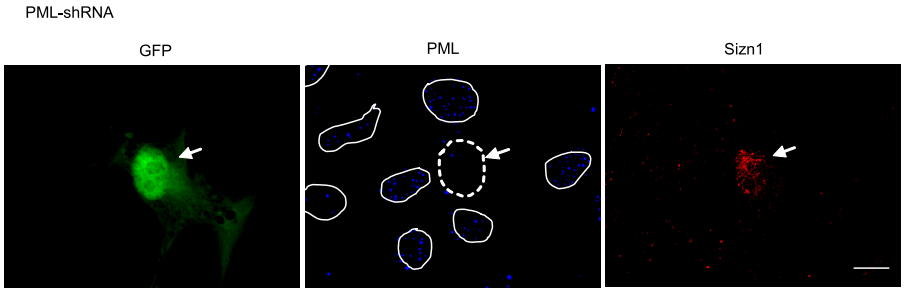

FIGURE 7. **Sizn1 maintains punctate localization in the nucleus in the absence of PML protein.** C2C12 cells were transfected with PML-shRNA (*GFP*) and Sizn1 expression constructs. The cells were immuno-labeled with anti-PML (*blue color*) and anti-Sizn1(*red color*) antibodies. Cells transfected with the PML-shRNA expression construct show GFP (*green*, expressed from vector) but no PML staining. However, punctate labeling for Sizn1 is still present. *Arrows* indicate the PML-shRNA expressing cells. The *solid* and *dashed circles* indicate nontransfected and transfected nuclei, respectively. *Scale bar* indicates 10 μm.

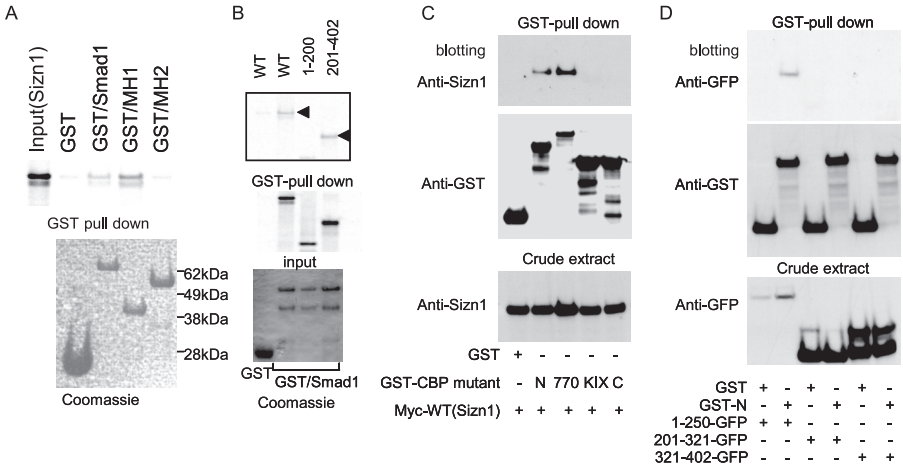

FIGURE 8. **The C terminus of Sizn1 interacts with the MH1 domain of Smad1 and the N terminus of CBP.** *A* and *B*, autoradiography of the *in vitro* GST pulldown assay. *A*, GST-Smad1(full-length), GST-MH1 (only MH1 domain of the Smad1), and GST-MH2 (MH2 domain of the Smad1) were used to pull down *in vitro*-translated ^{35}S-labeled Sizn1 (full-length). *B*, GST-Smad1 was used to pull down *in vitro*-translated ^{35}S-labeled Sizn1 (full-length, 1–200, and 201–402, respectively). Full-length Sizn1 and the 201–402 mutant protein interact with Smad1. The 1–200 mutant protein showed minimal signals very similar to the negative control. *C* and *D*, GST pulldown assay with HEK293T cells. *C*, full-length Sizn1 and various GST-CBP deletion constructs were co-transfected. Only CBP(N) and 1–770 are able to pull down Sizn1. *N*, *770*, *KIX*, and *C* indicate 1–450, 1–770, the KIX domain, and 1891–2441 fused with GST, respectively. *D*, GST-CBP(N) and various Sizn1 deletion mutants were co-transfected. Only 1–250-GFP (Sizn1) is pulled down with GST-CBP(N).

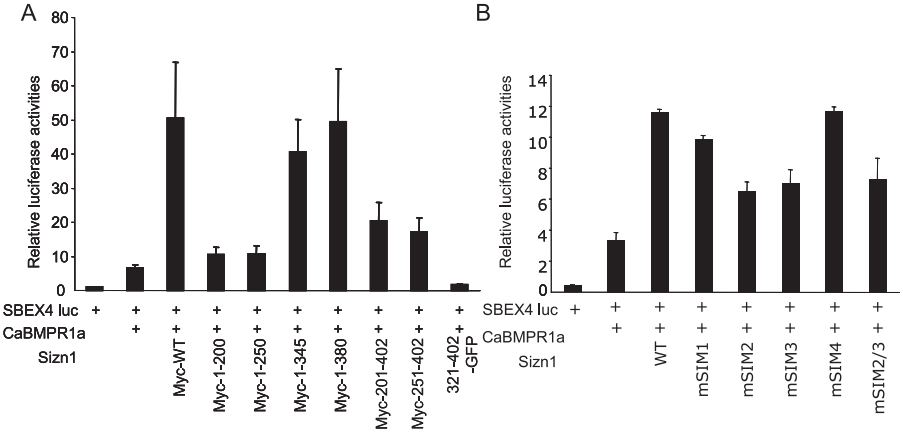

FIGURE 9. **PML-NB localization of Sizn1 is important for its functional co-activator of the BMP signaling pathway.** *SBEx4*-luciferase and *caBMPR1a* were co-transfected with Myc-tagged Sizn1 deletion mutants (*A*) or SIM point mutants (*B*) into C2C12 cells, and luciferase activities were measured ($n = 4$ and $n = 2$, respectively). Activity is diminished in the presence of Sizn1 mutant protein ($p < 0.05$ for 1–200, 1–250, 201–402, 251–402, and 321–402-GFP compared with control (*WT*) (Student's *t* test)). No statistical difference was found for the 1–345 and 1–380 constructs ($p < 0.05$ for mSIM1, mSIM2, mSIM3, and mSIM2/3; compare with control (*WT*)). *Error bars* indicate the S.D.

assay. The 200–402 region of Sizn1 was found to interact with Smad1 (Fig. 8*B*).

Our previous data indicate that Sizn1 associates with CBP to co-activate transcription (4). To elucidate the domain permitting Sizn1 to associate with CBP, we carried out GST pulldown assays with cells transfected with deletion mutants (Fig. 8, *C* and *D*). From its N terminus, CBP contains zinc finger motifs, a CREB-binding domain (also known as a KIX domain), a bromodomain, a histone acetyltransferase domain, and a glutamine-rich domain. Each of these domains associate with multiple transcriptional co-regulators (25). The N terminus of Sizn1 (amino acids 1–250) was found to associate with the N terminus of CBP containing the zinc fingers (amino acids 1–450). This domain is also known to interact with PML (Fig. 8, *C* and *D*) (26). In addition, the SIM mutants have similar affinity to Smad1 or CBP (supplemental data). Taken together, we conclude that the N terminus and C terminus of Sizn1 interact with the N terminus of CBP and with the C terminus of Smad1, respectively.

Sizn1 SIM Domains Are Important for Functional Co-activation of BMP Signaling—Next, we tested the hypothesis that the cellular localization of Sizn1 is required for functional modulation of BMP signaling. To understand the functional domain(s) of Sizn1 required for BMP signaling regulation, the *SBEx4*-luciferase reporter assay that we utilized previously (4) was employed with the deletion constructs described above (Fig. 9). The 1–345 and 1–380 mutants retained ~80–90% of wild type activity. However, the 200–402 and 250–402 mutants showed lower co-activation than the wild type (Fig. 9). The activities of the 1–200 and 1–250 mutants were essentially similar to the negative control (Fig. 9). These data are consistent with previous observations that the C terminus of Sizn1 showed binding to Smad1 protein in GST pulldown

assays (Fig. 8*A*). In other words, Sizn1 mutants, unable to bind to Smad1, cannot co-activate BMP signaling (Fig. 9*A*). Mutant 200–402, which does not associate with CBP, shows reduced co-activation activity (Fig. 9*A*). These data suggest that Sizn1 has a CBP-independent mechanism of facilitating pathway activation. The weak activation activity of the 200–402 and 250–402 mutants is potentially a consequence of the loss of CBP binding (see Fig. 8). However, we cannot exclude the possibility that the C terminus may interact with other transcriptional co-activators or co-repressors that have not yet been defined.

Finally, to define the role of the SIMs in the function of Sizn1, the SIM mutant constructs were also tested in the reporter assay (4). SIM2 and SIM3 mutants showed lower co-activation activities when compared with the wild type (Fig. 9*B*). This indicates that the SIMs in Sizn1 are required for full co-activation of BMP signaling pathway. However, these two mutants have a higher activity than the negative control. These data indicate that PML localization, SUMOylation, and/or interactions with other as yet undefined SUMOylated proteins are not sufficient for co-activation of the BMP pathway but are required for the full co-activation.

DISCUSSION

Mutations in *SIZN1*/*Sizn1*, like numerous other genes located on the X-chromosome, result in mental retardation in males (6). Although the roles of most mental retardation genes in brain development and function are poorly understood, we have begun elucidating the mechanisms of action for *Sizn1*. We showed previously that Sizn1 was a modulator of BMP signaling through its activity as a transcriptional co-activator and that it is necessary for normal basal forebrain cholinergic neuron specific gene expression (4). Here, we have gained further understanding of how Sizn1 functions by determining that it localizes to PML-NBs and that this localization is dependent on two SIM domains and the MA homologous domain. Furthermore, we show that the full BMP signaling co-activation function for Sizn1 is dependent on the SIMs. We speculate that, in fact, the localization of Sizn1 to PML-NBs is necessary for full transcriptional co-activation activity.

Many nuclear bodies have been identified, with each defined by its primary component protein. One such example is the PML-NB, which varies in number and size depending on the cell type and tissue (9, 27, 28). Proliferating cells express more PML protein and exhibit more PML-NBs than nonproliferating cells.[4] Although PML-NB is reported to have roles in transcription (10), based on their expression, their reputed roles in DNA replication and repair may be more important (29, 30). Although our data clearly show that Sizn1 co-localizes with PML-NBs, it is likely that Sizn1 has a role independent of PML-NBs that has yet to be characterized. This assertion is based on the fact that the PML protein is primarily localized in a proliferative zone during brain development, whereas Sizn1 is not (4).[5] SUMO-1 nuclear bodies, which are distinct from PML-NB by virtue of their size and absence of PML protein (31), have been recently identified. We have shown that Sizn1 can form nuclear bodies in the absence of PML or SUMOylation. Thus, Sizn1 may function as a unique nuclear body, independent of PML-NB (the main component of SUMO-1 nuclear bodies or in an as yet unidentified nuclear structure).

We also show that the MA homologous domain in Sizn1 is required for the specific cellular localization of this protein. The Sizn1-(250–402) mutant, which has both defined SIMs, but not the MA homologous domain, can be SUMOylated but does not localize to PML-NB. Thus, the MA homologous domain must play a role, directly or indirectly, in PML-NB localization. The MA domain in Sizn1 is ~50–60% similar to the C terminus of the PNMA protein (32–34). PNMAs have no known function, although they have been predicated to interact with RNA (32–34). One PNMA protein, PNMA4 (MOAP1), is known to localize to mitochondria where it interacts with Bax and RASSF1A to modulate apoptosis (35–37). Although proteins with 50% homology or more are generally thought to be structurally and sometimes functionally similar, the differences between Sizn1 and PNMA4 might be explained by their other domains. PNMA4 contains a Bax-interacting motif (36), whereas Sizn1 has the two SIMs that we have defined.

Sizn1 does not have a consensus sequence of SUMO conjugation; however, we have shown that it is SUMOylated. We predict that the two SIMs in Sizn1 can determine a SUMO conjugation, as seen for other SIM-containing protein (23). Thus, it is likely that the SIMs in Sizn1 generate the structure within the protein that increases its efficiency for SUMOylation.

In summary, we have performed a structure-function analysis of Sizn1 that has lead to an understanding as to how Sizn1, a known human mental retardation gene, functions in the cell. The data will not only help guide studies of other candidate mental retardation genes but will also assist with further functional analyses of Sizn1.

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[4] G. Cho and J. A. Golden, unpublished data.
[5] G. Cho, Y. Lim, and J. A. Golden, unpublished data.

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