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Sorting Nexin 6 Interacts With Breast Cancer Metastasis Suppressor-1 and Promotes Transcriptional Repression

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ABSTRACT

Sorting nexin 6 (SNX6), a predominantly cytoplasmic protein involved in intracellular trafficking of membrane receptors, was identified as a TGF-β family interactor. However, apart from being a component of the Retromer, little is known about SNX6 cellular functions. Pim-1-dependent SNX6 nuclear translocation has been reported suggesting a putative nuclear role for SNX6. Here, we describe a previously non-reported association of SNX6 with breast cancer metastasis suppressor 1 (BRMS1) protein detected by a yeast two-hybrid screening. The interaction can be reconstituted in vitro and further FRET analysis confirmed the novel interaction. Additionally, we identified their coiled-coil domains as the minimal binding motives required for interaction. Since BRMS1 has been shown to repress transcription, we sought the ability of SNX6 to interfere with this nuclear activity. Using a standard gene reporter assay, we observed that SNX6 increases BRMS1-dependent transcriptional repression. Moreover, over-expression of SNX6 was capable of diminishing trans-activation in a dose-dependent manner. J. Cell. Biochem. 111: 1464–1472, 2010.

KEY WORDS: PROTEIN–PROTEIN INTERACTION; YEAST TWO-HYBRID ASSAY; BINDING DOMAIN; FRET; BRMS1; SNX6

Sorting nexins (SNXs) are a family of cytoplasmic proteins involved in intracellular trafficking of membrane receptors and characterized by the presence of a Phox-homology domain (PX) which binds phosphoinositides [Worby and Dixon, 2002] and several proteins [Ishibashi et al., 2001; Parks et al., 2001; Vollert and Uetz, 2004] upstream of a coiled-coil domain [Parks et al., 2001]. Later, the adjacent C-terminal region of PX was defined as a BAR (Bin/Amphiphysin/Rvs) domain [Habermann, 2004]. SNX6 which was identified as an interactor of the TGF-β family of receptor serine–threonine kinases [Parks et al., 2001], is part of the Retromer complex mediating retrograde transport of trans-membrane cargo from endosomes to the trans-Golgi. Moreover, over-expression of SNX6 interferes TGF-β signaling [Parks et al., 2001], a receptor largely involved in metastasis.

Interaction with Pim-1 oncogene phosphorylates SNX6 leading to its nuclear translocation [Ishibashi et al., 2001]. However, the precise functions of SNX6 within the nuclear compartment have not been reported.

Breast cancer metastasis suppressor 1 (BRMS1), a member of a growing metastasis suppressors family, significantly reduce breast [Seraj et al., 2000] and melanoma [Shevde et al., 2002] metastasis without affecting primary tumor growth. Mechanism of action includes restoration of cell–cell communication [Saunders et al., 2001], phosphoinositides signaling reduction [DeWald et al., 2005], and gene repression [Meehan et al., 2004; Cicek et al., 2005; Rivera et al., 2007b; Yang et al., 2008].

In a search for proteins binding to BRMS1, we identify SNX6, as a novel BRMS1 binding partner. GST pull-down experiments and

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fluorescence resonance energy transfer (FRET) analysis validated this interaction both in vitro and in vivo. We studied the interaction in more detail and found that the minimal segment of SNX6 sufficient for BRMS1 binding consists of amino acids 300–406, a segment predicted to form a coiled-coil. BRMS1 sequence contains two predicted coiled-coil (CC) motifs and we show that exclusively the N-terminal CC1, recently crystallized [Spinola-Amilibia et al., 2008], is implicated in the interaction. In addition, our data suggest that combined over-expression of the newly detected interactors further enhances transcriptional repression in a luciferase reporter assay. Furthermore, the interaction could indicate a potential mechanism by which BRMS1 might regulate intracellular membrane receptor trafficking of TGF-β receptor family members and thus affecting TGF-β signaling pathway which has largely been involved in metastasis.

MATERIALS AND METHODS

CELL CULTURE AND TRANSIENT TRANSFECTION EXPERIMENTS

HeLa, U2OS, T47D, and HEK293T cells were cultured in DMEM containing 10% foetal bovine serum, 2 mM glutamine and 1% penicillin/streptomycin. Melanoma cells were maintained as described [Rivera et al., 2007b]. Transfections were performed using Lipofectamine2000 (Invitrogen) following manufacturer's instructions.

PLASMIDS

Human BRMS1 cDNA [Rivera et al., 2007b] was used as a template for PCR and inserted into pAS2.1 (Clontech) to generate pAS2.1-BRMS1 bait vector. Deletion mutants were also created containing: the first putative BRMS1 coiled-coil domain (pAS2.1/CC1; residues 1–88); a BRMS1-CC1 deletion mutant (pAS2.1/ΔCC1; 89–246); a construct lacking both putative CCs domains (pAS2.1/ΔCCs; 181–246); and a construct spanning both CCs (pAS2.1/CCs; residues 1–180). Subcloning into pET-28 (Novagen), pCMV-HA, and pEYFP-N1 (Clontech) create pET28-BRMS1, pCMV-HA-BRMS1, and pEYFP-N1-BRMS1 vectors. Entire SNX6 sequence was cloned into pGEX-6-P2 (GE-Healthcare), pECFP-N1, pACT2, and pCMV-Tag2 (Stratagene) rendering pGEX-6-P2/SNX6, pECFP-N1-SNX6, pACT2/SNX6, and pCMV-Tag2-SNX6 plasmids. Deletion mutants of SNX6 for yeast system were also created: pACT2/PX (residues 1–180); pACT2/BAR (300–406); and pACT2/CC (181–406). Detailed information of specific primers and restriction sites are available upon request.

YEAST TWO-HYBRID

A human mammary gland cDNA-library (Clontech) was screened with pAS2.1-BRMS1 vector as described [Rivera et al., 2007a]. For sub-mapping interactions, constructs encoding distinct portions of SNX6 and BRMS1 were cloned into pACT2 and pAS2.1 vectors, respectively. Sequential transformations by lithium acetate into AH109 yeast strain [James et al., 1996] produced moderate–fast growing colonies on high-stringency selection media lacking adenine, tryptophan, leucine, and histidine after 5–7 days according to the Matchmaker protocol (Clontech). Grown colonies were subjected to a colony-lift filter assay for β-galactosidase activity. Inserts were identified by DNA sequencing. Chemicals were from Sigma–Aldrich (St. Louis, MO) unless specified.

GLUTATHIONE *S*-TRANSFERASE PULL-DOWN

GST, GST-SNX6, and 6xHis-BRMS1 proteins were expressed in *Escherichia coli* BL21 (DE3) cells and proteins were expressed as described [Rivera et al., 2007a]. Purifications of GST or GST-SNX6 were performed using Glutathione Sepharose4B (GE-Healthcare) beads according to the manufacturer's protocols. Equal amounts of GST-fusion proteins, assessed by Coomasie blue staining, were incubated with bacterial lysate containing over-expressed His6-BRMS1 protein in 50 mM HEPES, pH 7.5, 50 mM NaCl, 0.1% NP-40 with proteases inhibitors (Roche) binding buffer. After overnight incubation at 4°C glutathione sepharose beads were collected by centrifugation and extensively washed with binding buffer. Proteins were eluted in 2× SDS buffer fractionated onto 12% SDS–PAGE and then subjected to Western blot analysis. Nitrocellulose membranes were probed with anti-6xHis antibody (Clontech), before ECL detection (GE-Healthcare).

CONFOCAL MICROSCOPY AND FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET) ANALYSIS

Cells grown on glass cover-slips were transfected for 48 h, then fixed for 15 min with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 for 30 min and blocked in TNB buffer (100 mM Tris–HCl; 150 mM NaCl, and 0.5% Blocking reagent (Boehringer)) for 1 h at room temperature. Next, cover-slips were sequentially incubated with α-HA (BAbCO; HA.11) and α-SNX6 (Santa Cruz; K-18) antibodies before incubation with secondary antibodies Alexa-Fluor488 and Cy3-coupled, respectively (Jackson Laboratories, West Grove, PA). Images were collected on a Leica-TCS-SP5 laser scanning confocal microscope equipped with a HCX PL APO 63x immersion oil objective (1.4NA). SNX6-ECFP and BRMS1-YFP co-transfected cells were examined for FRET efficiency ($FRET_{eff}$) [Sekar and Periasamy, 2003] by acceptor photobleaching method [Gu et al., 2004]. Energy transfer was detected as an increase in donor fluorescence (ECFP) after photobleaching of the acceptor molecules (EYFP). Images were background-corrected and $FRET_{eff}$ calculated as: $FRET_{eff} = (D_{post} - D_{pre})/D_{post}$; for all $D_{post} > D_{pre}$, where D_{pre} and D_{post} are donor intensities before and after photobleaching, respectively. Polyclonal anti-HDAC2 antibody was from Abcam (ab7029).

LUCIFERASE REPORTER ASSAY

Trans-activation activity assays were performed similarly to previous reports [Meehan et al., 2004; Hurst et al., 2008]. Briefly, HEK293 and T47D cells were plated in 24-well plates and allowed to attach. After 24 h cells were co-transfected, using Lipofectamine2000 in Opti-MEM medium, with various combinations of the following plasmids: pCMV-HA/BRMS1 (200 ng), pCMV-Tag2/SNX6 (100; 200 or 250 ng) and pGL-G5 luciferase reporter plasmid, containing five GAL4 binding sites upstream to the E1B promoter driving the expression of Firefly luciferase [Zurcher et al., 1996]. Assays were conducted in triplicate wells and total DNA amounts were uniformly adjusted with pcDNA3 vector to 0.5 μg/well. After transfection of 48 h, cells were lysed, and 20 μl of cell lysate were

used to measure trans-activation activity, using the Dual-Luciferase Reporter system (Promega), in a Glomax luminometer. To allow the normalization of Firefly luciferase readings based on transfection efficiencies, a co-reporter vector expressing Renilla luciferase (pRLSV40) was used as a transfection control at a ratio of 1:20 in the transfection mixture. Relative luciferase activities were calculated as the light units relative to the reporter plasmid controls and plotted. Data are expressed as percent luciferase activity relative to empty plasmid transfectants set as 100%.

RESULTS

IDENTIFICATION OF SNX6 AS A BRMS1-INTERACTING PROTEIN SECTION

Identification of new partners can provide useful information about protein functions. A human mammary gland cDNA library was screened by a yeast two-hybrid screen using a bait plasmid encompassing the entire human BRMS1-coding sequence that did not show trans-activation activity by itself upon over-expression and served as a negative control (Table I). The protein, which is 246 amino acids long, includes two predicted coiled-coil regions, which could account for protein–protein interactions (CC1 and CC2), and recently characterized localization signals, for nuclear export (NES) and nuclear localization (NLS1 and NLS2) (Fig. 1A) [Rivera et al., 2009].

Screening more than 10^6 yeast transformant colonies for activation of both the *His* and *LacZ* reporter genes yielded several positive clones that grew up on highly selective medium. Two independent clones expressed higher levels of β-galactosidase (β-gal) upon a lift-colony filter assay (Fig. 1B) compared to the remaining clones. After isolation and further plasmid purification of the selected clones, both inserts were different in size, but subsequent sequence analysis revealed that both encoded for human tumor necrosis factor receptor-associated factor 4 (TRAF4)-associated factor 2, also known as sorting nexin 6 (SNX6) [Parks et al., 2001]. One of them encoded the full-length mRNA and was therefore used in subsequent cloning experiments.

SNX6 is composed of a BAR domain at the carboxy-terminal end [Habermann, 2004] and a Phox-homology (PX) domain at its N-terminal end (Fig. 1A). Interestingly, the second one of the two identified clones encoded for a shorter form lacking the 180 N-terminal residues spanning the PX domain. This domain binds phosphoinositides and has been described also as a protein–protein interaction domain [Ishibashi et al., 2001; Parks et al., 2001; Vollert and Uetz, 2004]. Thus, our finding suggests that the PX domain of SNX6 is not involved in BRMS1–SNX6 interaction.

BRMS1 INTERACTS WITH SNX6 IN VITRO AND CO-LOCALIZE IN HUMAN CELLS

In order to validate the significance of this interaction and determine whether the interaction between SNX6 and BRMS1 was direct or involved additional (yeast) proteins, we tested binding in an in vitro assay system. Given that commercial antibodies against BRMS1 showed non-reproducible results in our hands for co-immunoprecipating endogenous proteins, we decided to perform more robust approaches over-expressing in a prokaryotic system both interacting proteins, as glutathione-*S*-transferase (GST) or histidine-tagged fusion proteins, respectively, in a prokaryotic system. GST pull-down experiments using GST/SNX6 fusion protein was mixed with over-expressed 6xHis/BRMS1 full-length protein. The GST-SNX6 fusion protein was purified from the mixture using Glutathione Sepharose4B beads and the pulled down proteins were tested for co-precipitation of the BRMS1 protein by Western blotting using an antibody raise against 6xhistidine-tag. As shown in Figure 1C (lane

TABLE I. Minimal Binding Domain Involved in the Interaction of SNX6 With BRMS1

	Gal4-AD (SNX6)	Gal4-BD (BRMS1)	SD/-T/-L/-H	β-Galactosidase assay
Control −ve	pACT2	FL	−	−
Control +ve	Ref 14	Ref 14	+ (+)	+++
	FL (1–406)	FL (1–246)	+ (+)	++
		CC1 (1–88)	+ (+)	+++
		ΔCC1 (89–246)	− (+)	−
		ΔCCs (181–246)	− (+)	−
		CCs (1–180)	+ (+)	+++
	CC (300–406)	FL (1–246)	+ (+)	+
		CC1 (1–88)	+ (+)	++
		ΔCC1 (89–246)	− (+)	−
		ΔCCs (181–246)	− (+)	−
		CCs (1–180)	+ (+)	++
	BAR (181–406)	FL (1–246)	+ (+)	+
		CC1 (1–88)	+ (+)	++
		ΔCC1 (89–246)	− (+)	−
		ΔCCs (181–246)	− (+)	−
		CCs (1–180)	+ (+)	++
	PX (1–180)	FL (1–246)	− (+)	−
		CC1 (1–88)	− (+)	−
		ΔCC1 (89–246)	− (+)	−
		ΔCCs (181–246)	− (+)	−
		CCs (1–180)	− (+)	−

Different domains of BRMS1 were fused to the Gal4-DNA-binding domain (Gal4-BD). SNX6 fragments were fused to the Gal4 transcription activation domain (Gal4-AD). Protein–protein interaction results in activation of reporter genes allowing yeast cells to grow in synthetic drop-out medium lacking, tryptophan, leucine, and histidine (SD/-T/-L/-H). The AH109 yeast cell co-transformed with: pACT2 empty vector and BRMS1; and a pair of interacting proteins previously reported [Rivera et al., 2007a] served as negative and positive controls, respectively. β-Galactosidase activity was evaluated by lift-colony filter assay. Growth of co-transformed yeast cells onto SD/-T/-L is shown in brackets. Numbers shown in brackets within the constructs columns represent the spanning residues according to the full-length protein as shown in Figure 3A,B. Intensity of the blue color is a measure for the interaction strength that was defined as: +++ strong, ++ moderate, + weak, and − not detectable.

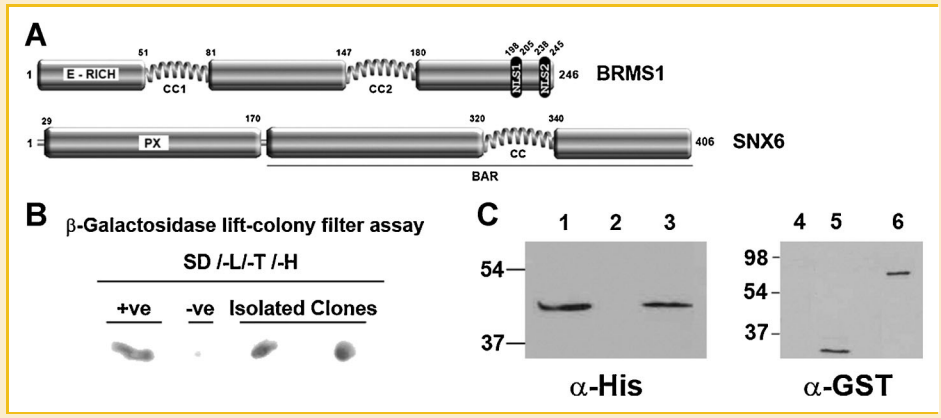

Fig. 1. In vitro association between SNX6 and BRMS1. A: Schematic representation of BRMS1 and SNX6 structures displaying boundaries of different domains: CC; coiled-coil, PX; Phox-homology. B: β-Galactosidase activity of grown co-transformant yeast colonies encoding SNX6 (isolated clones) after two-hybrid assay using BRMS1 bait vector to screen a human breast cDNA library. Positive (+ve) and negative (−ve) controls are also shown as described in the main text. C: GST or GST-SNX6 proteins were mixed with bacterial cell lysates containing 6xHis-tagged BRMS1 (see Materials and Methods Section) and pulled-down with Glutathione Sepharose4B beads. Co-immunoprecipitated proteins were eluted with 2× Laemli buffer, analyzed on 12% SDS-PAGE gels and blotted with specific antibodies raised against 6xHis (lanes 1–3) or GST (lanes 4–6) antibodies. BRMS1 was elicited as a pulled-down protein complex with a GST-SNX6 beads (lanes 3 and 6) but not in the control assays using GST alone (lanes 2 and 5). 6xHis-BRMS1 (lanes 1 and 4) containing samples were shown using α-His antibody but not detected with α-GST antibody. Protein markers are shown in kDa.

3), the full-length BRMS1-His tagged protein bound to GST-SNX6 in vitro. A single protein band was specifically recovered in the GST/SNX6 eluate (Fig. 1C, lane 3). This protein, running slower than the expected molecular mass (32.3 kDa), reacted with the anti-6xHis antibody on Western blots. Thus, we found that BRMS1 was specifically eluted from the sepharose beads containing GST/SNX6. GST protein used as a control in the assay instead of GST-SNX6, showed no co-precipitation of BRMS1 protein (Fig. 1C, lane 2), indicating that the observed binding is specific for SNX6. The presence of GST and GST/SNX6 was specifically confirmed in the same assay using anti-GST antibody (Fig. 1C, lanes 5 and 6). Thus, we conclude that SNX6 and BRMS1 bind each other in vitro and interact in vivo in a yeast assay.

The reported subcellular localization of SNX6 is mainly cytoplasmic [Parks et al., 2001; Worby and Dixon, 2002] although nuclear localization has also been shown [Ishibashi et al., 2001]. However, BRMS1 is mostly nuclear although it has also been detected in the cytosol [Rivera et al., 2007b; Stafford et al., 2008]. We reasoned that SNX6 and BRMS1 have to exhibit at least partial co-localization if their interaction is physiologically relevant. Therefore, we performed a co-localization analysis. Since we could not reliably detect endogenous BRMS1 protein by commercially available antibodies, we assessed the subcellular localization of HA-tagged BRMS1 transiently over-expressed in human cells followed by indirect immunofluorescence detection. We observed a predominantly nuclear localization pattern of the recombinant BRMS1 proteins upon transient transfection (Fig. 2A,B), in agreement with previous reports [Ishibashi et al., 2001; Worby and Dixon, 2002; Rivera et al., 2007b; Stafford et al., 2008]. As observed in transfection experiments, endogenous (Fig. 2A) and over-expressed SNX6 (Fig. 2B) partly co-localized with differently tagged BRMS1. This co-localization was detected more intensively in the nuclei (Fig. 2A cytofluorogram) of different human cell types (Fig. 2A,B and Supplementary Figs. S1 and S2).

DIRECT SNX6–BRMS1 PROTEINS INTERACTION DETECTED BY FRET

To complement co-localization studies and further examine the SNX6–BRMS1 interaction, we performed a FRET analysis. To achieve this goal, full-length BRSM1 and SNX6 proteins were tagged to the N-terminus of enhanced versions of yellow (EYFP) and cyan (ECFP) fluorescence proteins, respectively. Both constructs were co-transfected into human cells and FRET efficiency ($FRET_{eff}$) was measured by acceptor photobleaching method as previously reported [Gu et al., 2004]. This method calculates $FRET_{eff}$ as increments in the emission of the ECFP donor after photobleaching of the EYFP acceptor, which take place exclusively once both fluorophores are in close vicinity within a distance of a few nanometers and it is indicative of protein–protein interaction. Several plasmid pair-wise combinations were co-transfected into mammalian cells as negative controls, including among others co-transfection of pEYFP-N1-BRMS1 or pECFP-N1-SNX6 with a construct encoding for the full length of an unrelated molecule (pECFP-N1-p27 [Fuster et al., 2010] and pEYFP-N1-NMI [Rivera et al., unpublished data], respectively) as shown in Supplementary Figure S1. After photobleaching of randomly selected areas with the YFP-light, the ECFP channel increased considerably both in the nucleus and cytoplasm of co-transfected cells (Fig. 2B,C) while no increase was observed in cells co-transfected with the described unrelated molecules, even though some of them (p27-ECFP and BRMS1-EYFP) showed a perfect co-localization into the nuclear compartment (Supplementary Fig. S1). Moreover, the $FRET_{eff}$ values, measured and averaged from at least 10 cells along different experiments, consistently demonstrated, a higher $FRET_{eff}$ values within the nuclear compartment ($44 \pm 11.5\%$) compare to those observed for the cytosol ($27.5 \pm 7.5\%$). Interestingly, these differences showed statistical significance ($P < 0.05$), although we do not know the biological significance yet (Fig. 2C).

$FRET_{eff}$ was also measured by the alternative acceptor sensitized emission method (see Supplementary Materials and Methods). As

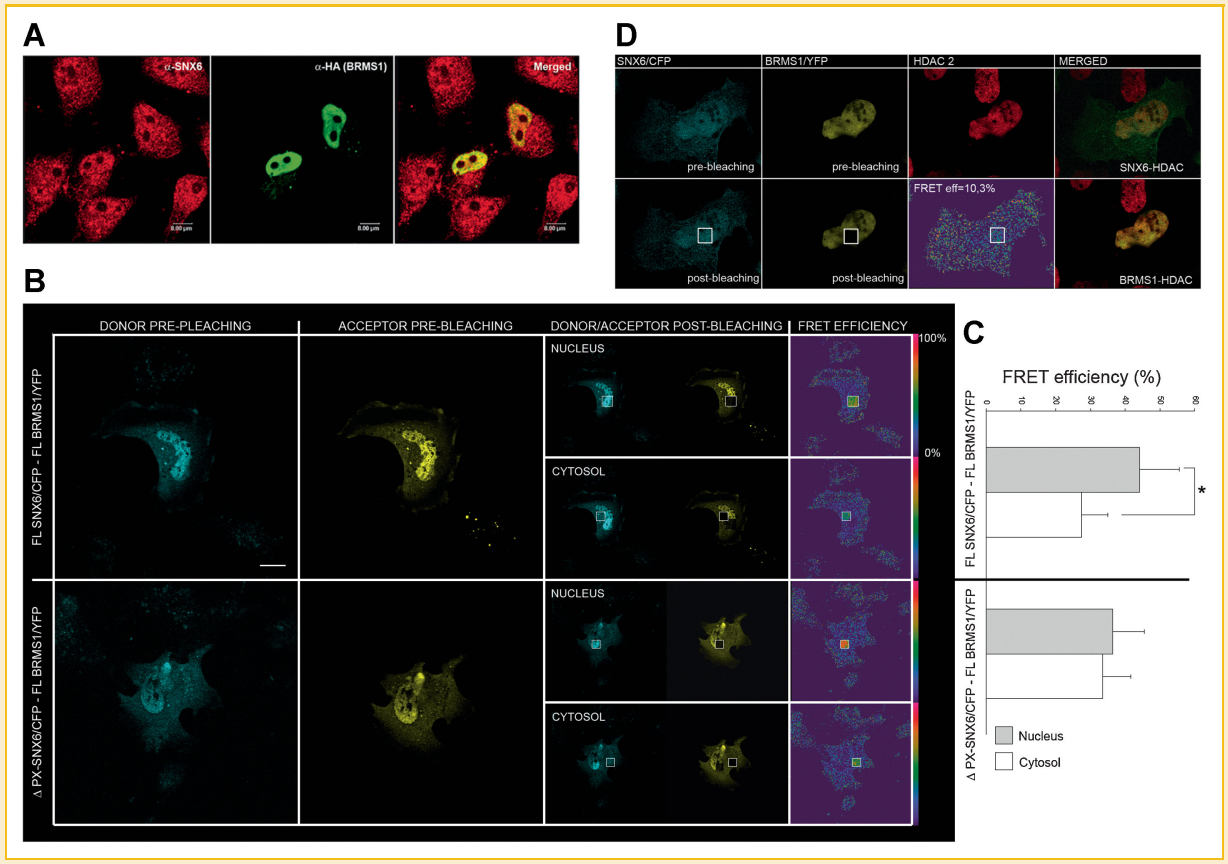

Fig. 2. Co-localization and FRET detection of SNX6–BRMS1 interaction. A: Partial co-localization of SNX6 and BRMS1. HeLa cells were stained using α-HA (green color, middle panel) and anti-SNX6 (red color, left panel). Right panel shows co-localization represented by a merge of adjacent spots populations overlapping red and green signals. Staining was analyzed by confocal microscopy and images show scanning of a single plane. B: Representative images of donor (ECFP) and acceptor (EYFP) fluorescence intensity before and after EYFP photobleaching. Images of co-transfected cells with full-length BRMS1 acceptor molecule and full-length SNX6 (top panels) or a PX-deletion mutants (bottom panels) donor molecule are shown. Scale bar, 20 μm. $FRET_{eff}$ (%) measured in randomly selected areas (white boxes) is shown at the right-hand panel as pseudo-colored images relatives to amplitude of $FRET_{eff}$ as shown in the scale bar. C: Averaged $FRET_{eff}$ (%) measured both in nuclei and cytosol of fixed melanoma cells ($n \geq 20$ cells for each experiment) as the increase of acceptor molecules following acceptor (BRMS1-EYFP) photobleaching. Note the significant reduction ($^{*}P < 0.05$) of $FRET_{eff}$ in cytosol compare to the nucleus in full-length transfected proteins. Error bars represent mean ± SEM from four different independent experiments carried out in duplicates. D: Representative images of co-transfected cells as described for subpanel (B), where endogenous HDAC-2 was detected by a polyclonal antibody. Pair-wise combinations of merged images are shown in the right-hand panel as stated.

shown in Supplementary Figure S2, cells co-transfected with constructs encoding ECFP-SNX6 and EYFP-BRMS1 fusion proteins confirmed the acceptor photobleaching $FRET_{eff}$ values reported above. Negative controls were also run in parallel (Supplementary Fig. S2).

Thus, FRET analysis confirms that interaction occurs, providing strong evidence for the association of SNX6 with BRMS1 in both cytoplasmic and nuclear compartments and thus corroborating the results of in vivo yeast two-hybrid, pull-down assays, and co-localization studies. Our findings suggest that SNX6 may play a role in the nucleus. Similarly, cytosolic presence of BRMS1 does not seem to be an artifact and might play a functional role in that compartment.

The PX motif was previously described as a binding site for diverse protein partners [Ishibashi et al., 2001; Xu et al., 2001; Worby and Dixon, 2002]. Since this motif is absent in one of the two fished out clones upon yeast screen, we hypothesized that this motif is not essential for the interaction. To test this hypothesis, we engineered and over-expressed a deletion mutant of SNX6 where the PX domain was not present (ECFP/ΔPX-SNX6). $FRET_{eff}$, measured by acceptor photobleaching, within the nucleus and cytoplasm showed values of 36.5 ± 7.5% and 33.6 ± 8%, respectively (Fig. 2C), which were indeed similar to those obtained upon co-transfection of full-length proteins, confirming that PX domain from SNX6 is dispensable for its interaction with BRMS1.

IDENTIFICATION OF MINIMAL DOMAINS INVOLVED IN BRMS1–SNX6 INTERACTION

In order to further map the regions of SNX6 and BRMS1 proteins that are relevant for its interaction, we created a series of truncated constructs (Fig. 3A,B) and perform yeast two-hybrid experiments. Engineered SNX6 constructs-domains were back-transformed into yeast together with the various BRMS1 constructs. After assessing self-activation inability for all the designed constructs (data not shown), all possible pair-wise combinations were tested for positive interactions by the activation of both reporter genes.

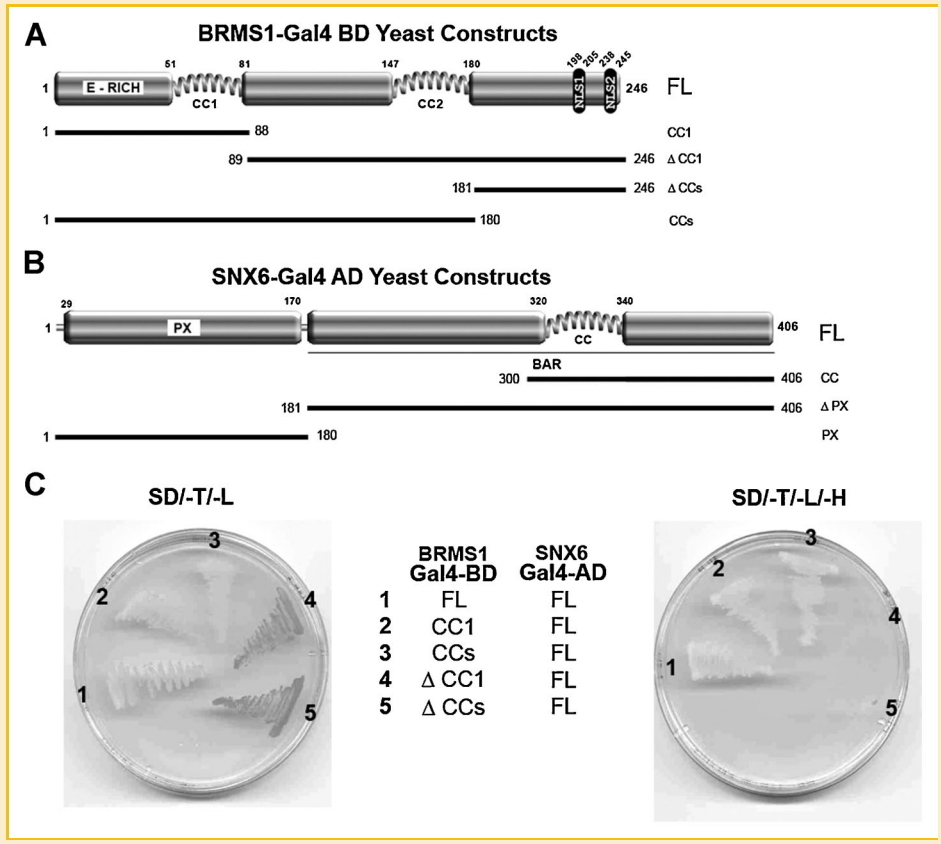

Fig. 3. Minimal domains responsible for SNX6–BRMS1 interaction. Diagram of BRMS1 (A) and SNX6 (B) deletion mutants are schematically depicted and referenced to the full-length protein sequences. C: Representative images of yeast cells co-transformed with a prey vector encoding the entire SNX6 and a combination of bait plasmids encoding the complete sequence of BRMS1 (1); 1–88 N-terminal residues containing the first putative coiled-coil domain (2), or both coiled-coil domains (3), or mutants lacking the first (4), or both coiled-coils (5). Co-transformed yeasts were grown on selective medium (SD-T-L) to assess co-transformation and further screened for a histidine reporter gene (SD-T-L-H).

We did not detect any binding of deletion mutant expressing solely the SNX6-PX domain (PX 1–180) with any of the BRMS1 constructs assayed, as judged by the inability of yeast cells to grow on deficient media (Table I). This result reinforces the hypothesis that the PX domain is dispensable for the interaction with BRMS1. Instead, SNX6 full-length construct strongly activated both reporter genes in yeast cells co-expressing all three constructs where the first 88 N-terminal residues of BRMS1 were present (FL, CC1, and CCs). A series of further truncated constructs mapped the minimal SNX6-binding site within BRMS1 protein to amino acids 1–88, containing the predicted coiled-coil domain (Fig. 3C and Table I), thus highlighting its crucial role. We therefore suggest that the N-terminal fragment of BRMS1, harboring a predicted coiled-coil domain, is necessary and sufficient for binding to SNX6.

Results in Table I show that a construct encompassing the entire BAR domain of SNX6 achieved interaction to the same extent that a shorter construct including a coiled-coil region, concluding that the C-terminal end of SNX6, shorter than the BAR domain, is necessary and sufficient for binding to BRMS1.

Of note is the observation that BRMS1-CC1 construct showed the strongest β-gal activity. This result suggests that a negative regulatory effect might exist in the context of full-length BRMS1 (e.g., the CC1 region might have been partially shielded in the presence of BRMS1 C-terminal end).

SNX6 AFFECTS TRANS-ACTIVATION AND CONTRIBUTES TO BRMS1-TRANSCRIPTIONAL REPRESSION

The results shown above indicate that BRMS1 interacts with SNX6 both in the nucleus and the cytoplasm. It has previously been reported that BRMS1 affects transcriptional repression by its interaction with different components of the mSin3–HDAC complex [Meehan et al., 2004]. We sought to investigate a possible functional relationship between BRMS1 and SNX6 by testing whether SNX6 might play a role in the modulation of transcriptional repression. To address this hypothesis, we carried out a conventional trans-activation assay as previously described [Meehan et al., 2004]. We performed a standard luciferase reporter assay in human cells transfected with a construct (pGL-G5) containing five binding sites of Gal4 promoter [Zurcher et al., 1996]. Proliferating HEK 293T cells exhibited a basal reporter activity that we set as 100% activity (Fig. 4A,B). As expected, trans-activation activity was specifically repressed to 65% upon the transient expression of BRMS1 (Fig. 4A). Importantly, co-expression of BRMS1 and SNX6 exhibited an additive effect on transcriptional repression reducing reporter

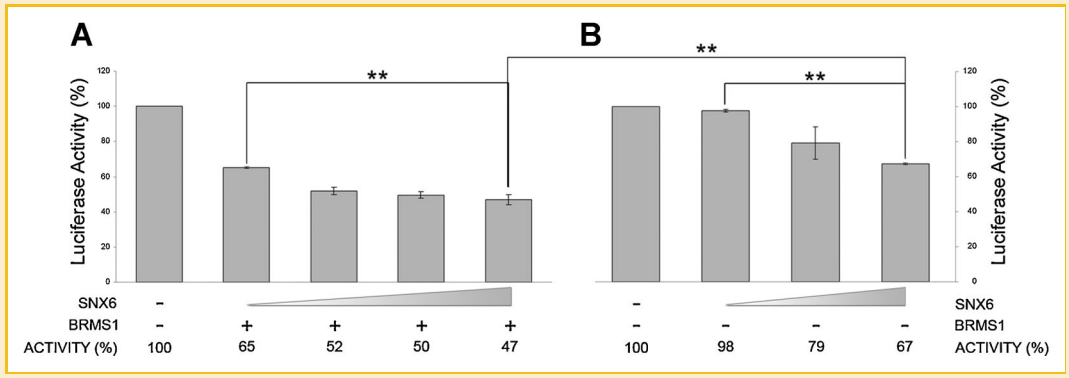

Fig. 4. Modulation of BRMS1-dependent trans-activation by SNX6. T47D transiently transfected cells with 100 ng of pGL-G5 luciferase reporter construct alone (lane 1) or in combination with HA-BRMS1 (0.20 μg) (lanes 5–8) were assayed in a dual-luciferase reporter system. Increasing amounts (0.1; 0.2; or 0.25 μg) of Flag-SNX6 expression plasmids were used as indicated. Equal amounts of plasmid DNA (0.5 μg/well) was accomplished with pcDNA3 vector. Cells in 24-well plates were lysed in 100 μl of passive lysis buffer 48 h post-transfection and 20 μl were used for the assay. Luciferase activity was normalized to Renilla measurements to take into account transfection efficiency variations. Data from five independent experiments assayed in triplicate are presented as the mean percentage ± SD compared to relative luciferase activity of cells transfected only with pcDNA3 vector (lane 1) considered as 100% activity. Symbol * represents statistical significance with $P < 0.05$.

activity up to more than twofold in an SNX6-dose dependent manner (Fig. 4A). The combination of BRMS1 expression with increasing amounts of SNX6 constructs displayed a statistically significant ($P < 0.001$) increase in transcriptional repression capability as compared to that exerted by BRMS1 when transfected alone (Fig. 4A). Moreover, transient expression of increasing amounts of SNX6 transfected plasmid also reduced luciferase reporter activity almost as effectively as BRMS1 when the highest SNX6 plasmid dose was used (Fig. 4B). Interestingly, the latter result suggests that SNX6 might play a role in transcriptional repression, although, no trans-activation domain has been reported for this protein. Whether this effect can be mediated by SNX6 on its own or by acting as a co-repressor is not known yet and should be further analyzed.

Similar and consistent repression of a reporter luciferase activity was also achieved when a different human T47D breast cell line was used (data not shown), suggesting that the observed effect on transcriptional repression might not be cell specific but a general mechanism.

Altogether, these combined results hypothesize a conceivable mechanism by which SNX6 and BRMS1 could modulate gene expression in mammalian cells.

DISCUSSION

In this study, we demonstrate that SNX6 and BRMS1 interact in vivo in a yeast two-hybrid system. Some previously reported BRMS1-interacting proteins were also fished out in our assay (data not shown), assessing the consistency for the performance of our screening.

This interaction can be reconstituted in vitro indicating that no additional yeast proteins are involved. GST pull-down assays demonstrated that an over-expressed construct encompassing the entire sequence of BRMS1 precipitated SNX6, validating yeast results and suggesting direct binding. The protein–protein interaction was further confirmed by FRET analysis. As shown in Figure 2 and Supplementary Figures S1 and S2, mammalian cells over-expressing ECFP-SNX6 and EYFP-BRMS1 showed specific and considerably high $FRET_{eff}$ values, demonstrating that SNX6–BRMS1 interaction occurs in vivo. The identification of this novel interaction of BRMS1 with a bona fide cytosolic protein is in agreement with recent reports demonstrating the nucleo-cytoplasmic shuttling capacity of BRMS1 and its cytosolic presence [Stafford et al., 2008; Rivera et al., 2009].

Moreover, the interaction of BRMS1 with SNX6 reported in this work opens up the possibility of a cytosolic role for this suppressor of breast cancer metastasis.

A previous report, using oligonucleotide microarrays coupled to a robust platform of informatic analysis, has shown that in response to BRMS1 re-expression the signature of diverse secretory/trafficking pathway-genes changed significantly. Interestingly, SNX1 and SNX5, two other members of the sorting nexin family, were repressed upon BRMS1 re-expression [Champine et al., 2007].

A common feature of the SNX family is its PX domain. SNX6 contains a predicted coiled-coil region C-terminal to the PX domain. Whereas homo- and heteromeric interactions with other members of the family involve both PX and coiled-coil domains, the majority of the interactions with membrane receptors, as well as with the oncogene Pim-1, take place by the PX motif [Ishibashi et al., 2001; Xu et al., 2001; Worby and Dixon, 2002]. Interestingly, one of the two cDNAs pulled out in our yeast two-hybrid screen lacked this motif, suggesting that, unlike most of the interactions reported for SNX6, the PX domain is dispensable for its interaction with BRMS1. This fact was confirmed in human cells by FRET analysis where protein–protein interaction efficiency, in cells transfected with a PX deletion mutant (ΔPX-SNX6/ECFP), was indistinguishable from cells over-expressing full-length SNX6. In addition, we have precisely mapped the regions mediating the interaction to the predicted coiled-coil motif of SNX6 and the CC1-BRMS1 located at the N-terminal end.

It is worth to mention that we have observed that SNX6 and BRMS1 interact both in the cytoplasm and the nucleus. Despite the

fact that no functional activity had been reported for SNX6 in the nucleus, it has been shown that Pim-1-mediated phosphorylation of SNX6 leads to its translocation from cytoplasm to the nucleus [Ishibashi et al., 2001]. Moreover, several PX domain-containing proteins interact with nuclear proteins [Vollert and Uetz, 2004] relating lipids with nuclear functions [Shi and Gozani, 2005]. BRMS1 has largely been restricted to the cell nucleus [Samant et al., 2000] where it acts as a transcriptional co-repressor by interacting with diverse components within the large mSin3–HDAC complex or in a smaller complex with HDAC1 [Meehan et al., 2004].

We therefore sought to address whether the novel SNX6–BRMS1 interaction co-localize with the HDAC complex. Despite the fact that FRET protein–protein interaction could not be detected between SNX6-ECFP and the endogenous HDAC2, likely due to the large size of the specific antibody anti-HDAC used, co-localization studies by confocal microscopy showed that BRMS1–SNX6 complex (Fig. 2B,C) co-localizes with the endogenous HDAC complex upon transient expression (Fig. 2D). These results raise the hypothesis of BRMS1–SNX6 playing a role in transcription. Furthermore, using a well-established luciferase reporter assay, we show that over-expression of SNX6 in human cells is capable of modulating the transcriptional machinery revealing a previously non-described function for SNX6 within the nuclear compartment and confirming that our findings might be of physiological relevance.

The absence of a trans-activation domain within the SNX6 sequence suggests that it might act as a transcriptional co-factor. In fact, a previous report, using a similar reporter assay, clearly indicated that SNX6 interferes with TGF-β signaling in a dose-dependent manner, although the assay did not provide a direct measure for any single component of the pathway but the ability of TGF-β to induce gene expression, concluding that the increasing amounts of SNX6 modified the TGF-β receptors. Importantly, although the significance of the BRMS1–SNX6 interaction is still not fully understood, the co-expression, in different human cell types, of BRMS1 and SNX6 in a gene reporter assay enhanced the transcriptional repression exerted by BRMS1, as judged by the reduction of luciferase trans-activation (Fig. 4). The transcriptional blockade achieved after co-expression although modest, it was consistently observed along five different experiments performed in triplicate and found statistically significant. Further studies are needed to evaluate the biological significance of the observed transcriptional repression.

We show that SNX6 affects transcriptional repression. This observation might be in agreement with previous data since SNX6 was first identified as a Smad1 protein partner [Kim et al., 2000] and later on reported to interact to a different extent with several members of the TGF-β family of serine–threonine kinase receptors interfering with TGF-β signaling [Parks et al., 2001]. Interestingly, TGF-β signaling have been largely associated to metastasis [Padua and Massague, 2009].

Moreover, it has recently been reported that the interaction of SNX6 with GIT1 enhances degradation of the epidermal growth factor receptor (EGFR) and alters EGFR signaling [Cavet et al., 2008]. EGFR has long been associated to carcinogenesis and it has been proposed that EGFR contributes to intravasation and metastasis [Xue et al., 2006]. The fact that SNX6 directly affects two signaling pathways involved in metastasis provides an interesting functional relationship to our reported molecular interaction between SNX6 and BRMS1.

BRMS1 was first identified as a largely down-regulated gene in highly metastatic breast carcinoma and melanoma cells. Reintroduction of BRMS1 in both human and mouse-derived cells correlated with reduced metastatic potential in in vivo metastasis assays [Samant et al., 2000, 2002; Shevde et al., 2002]. Interestingly, the aggressiveness of the highly metastatic C8161 melanoma cells and MDA-MB-231 and Hs 578T breast carcinoma cell lines has been recently correlated [Topczewska et al., 2006] with the over-expression of a secreted Nodal protein, which is precisely a ligand of the Activin and TGF-β family of receptors which interact with SNX6.

In summary, our study provides evidences of a novel interaction between the intracellular trafficking protein SNX6 and the breast cancer metastasis suppressor BRMS1, which is mediated by their respective coiled-coils motives. Furthermore, our results supply for the first time evidences that SNX6 might play a novel role as a modulator of the transcriptional repression machinery. Whether SNX6 is a component of the previously described BRMS1–HDAC complex or constitutes a novel level of transcription regulation is still unknown and should be the focus of further studies.

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