Supplemental Materials

Molecular Biology of the Cell

Wilkes et al.

Supplementary Figure Legends

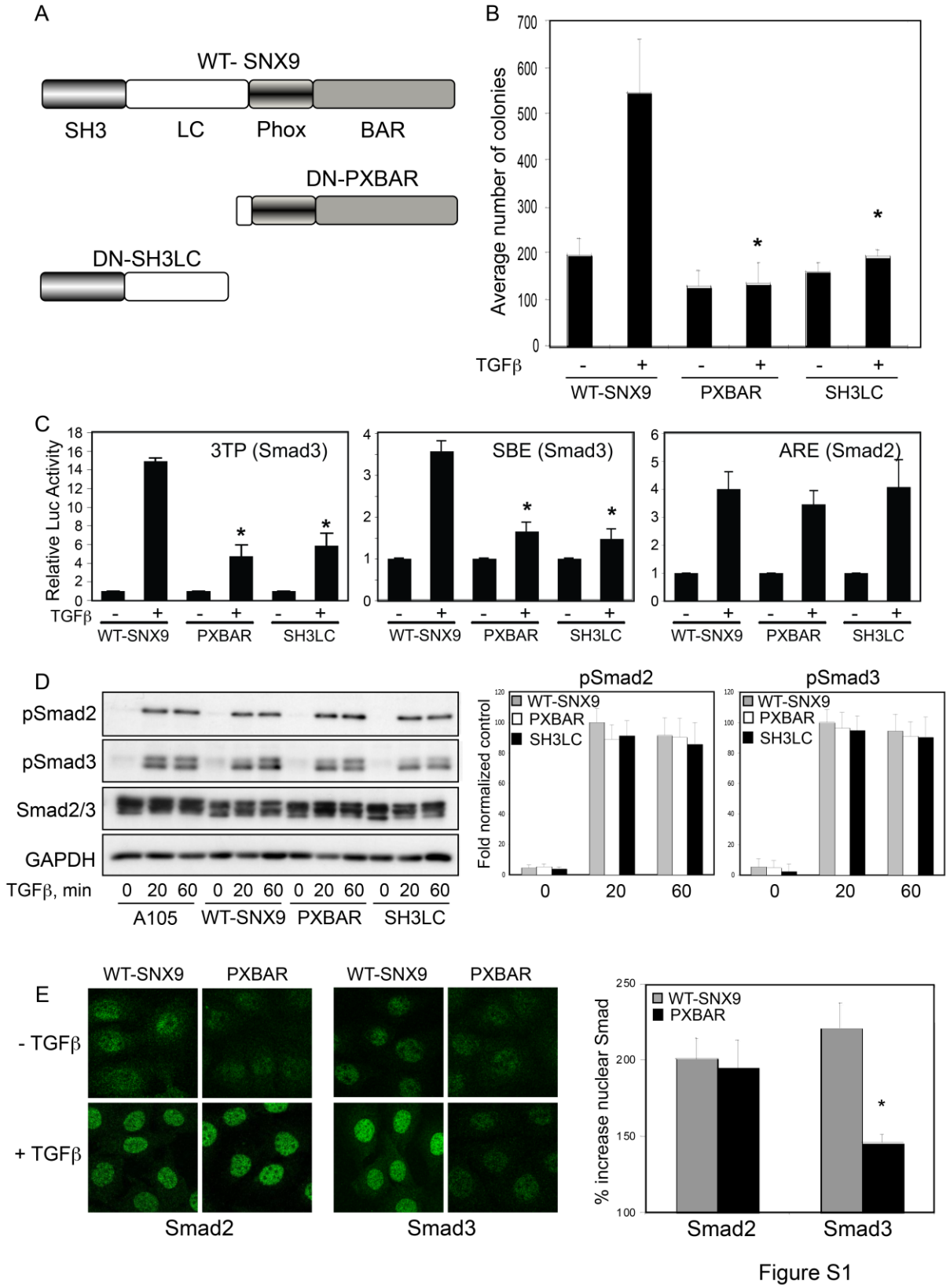

Figure S1

Figure S1. SNX9 knockdown and dominant negatives distinguish Smad3 from Smad2 responses. (A) Cartoon depicting the domain structure of wild-type (WT) and dominant negative (DN) SNX9 constructs. SNX9 consists of a SH3 domain (known to bind proline rich sequences such as in dynamin2, WASp, and Cdc42-associated kinase 2), a low complexity region (LC; binds clathrin and AP2), a Phox homology domain (PX; binds phosphatidyl inositide-P's), and a Bin/Amphiphysin/Rvs domain (BAR; dimerization and membrane binding module able to sense membrane curvature) (Carlton et al., 2005; Worby and Dixon, 2002). (B) Wild-type (WT-SNX9, clone SNX9.1) and dominant negative SNX9 (SH3LC, clone SH3LC.2; and PXBAR, clone PXBAR.6) expressing A105 cells (AKR-2B cells with chimeric and native TGFβ receptors; Anders and Leof, 1996) were seeded for soft agar in the absence (-) or presence (+) of 10 ng/ml of TGFβ. Each bar represents the mean number of colonies >100 □m ± sd on day 10 from three independent experiments. * denotes statistical significance (defined as $p<0.05$) between stimulated WT and PXBAR and SH3LC clones. (C) A105 cells stably expressing WT or the indicated DN SNX9 constructs were transiently transfected with the indicated luciferase reporters and processed as in figure 1C. Each bar represents the mean fold induction relative to untreated ± standard deviation (sd) of three clones (WT-SNX9.1, WT-SNX9.4, WT-SNX9.6; DN-PXBAR.6, DN-PXBAR.28, DN-PXBAR.22; DN-SH3LC.2, DN-SH3LC.24, DN-SH3LC.34) from two independent experiments. (D; left panel) Parental A105 cells or A105 clones stably transfected with wild-type (WT-SNX9.1) or dominant negative (DN-PXBAR.6 and DN-SH3LC.2) SNX9 were left untreated (0) or stimulated with 5 ng/ml TGFβ for 20 or 60 min. Western analysis was performed for the indicated phospho (p) or total protein. (D; right panels) Quantitation of pSmad2 or pSmad3 levels normalized to GAPDH. No statistically significant effect on R-Smad phosphorylation was observed with either DN-SNX9 construct. (E; left panels) Wild-type (WT-SNX9.1) or dominant negative (DN-PXBAR.6) SNX9 clones were grown to confluence on coverslips and either left untreated or stimulated for 45 minutes with 5 ng/ml TGFβ. Cells were fixed and stained with primary antibody to total Smad2 or Smad3 and AF488-conjugated goat anti-rabbit secondary antibody. (E; right panel) Each bar represents the mean fold increase in nuclear fluorescent intensity stimulated by TGFβ ± sd from three independent experiments in WT-SNX9.1 or DN-PXBAR.6 cells. * denotes statistical significance of Smad3 nuclear inhibition for DN-PXBAR compared to WT-SNX9. Analogous results were observed with DN-SH3LC (unpublished observations).

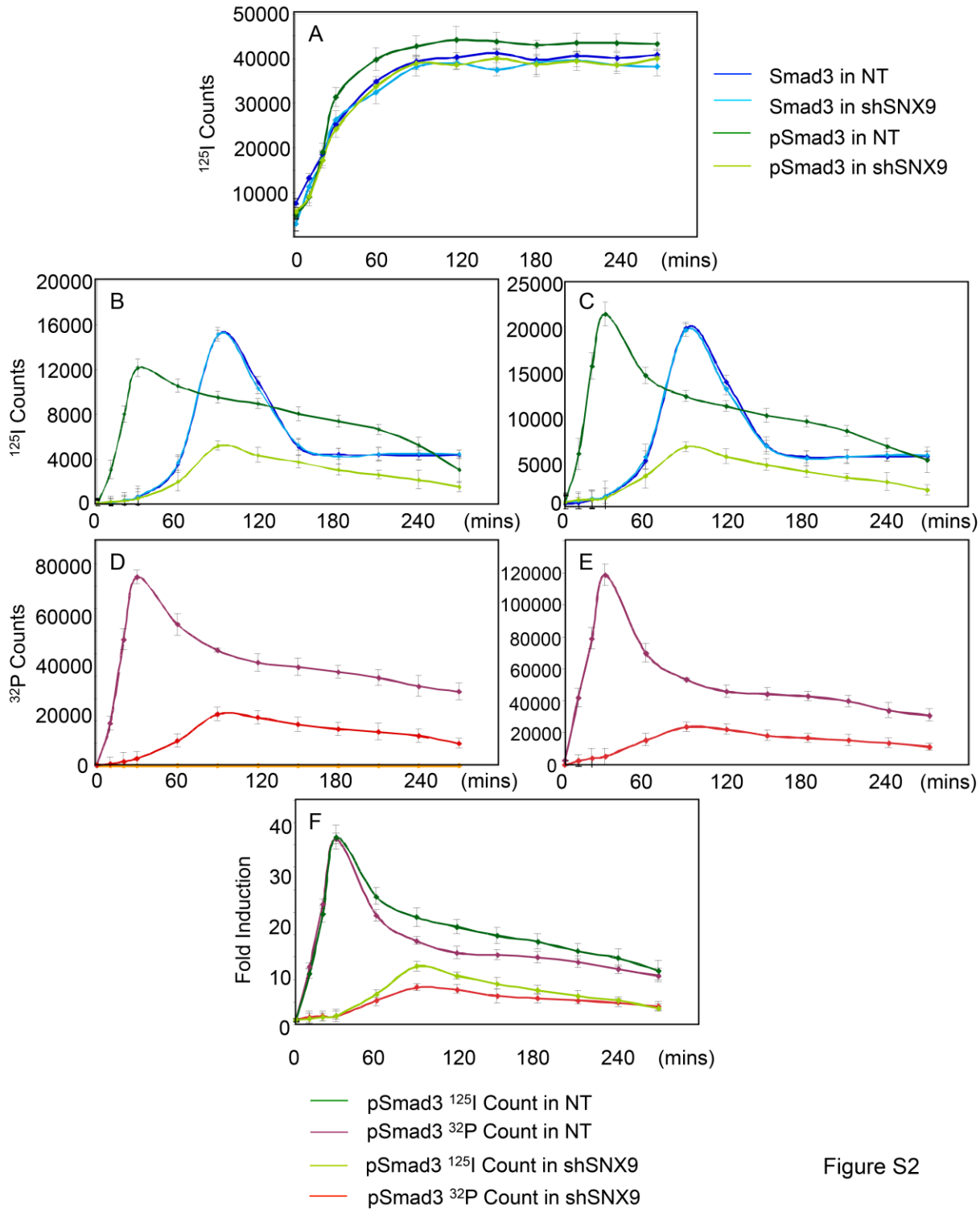
A
B
C
D
E
F
125I Counts
32P Counts
Fold Induction
(mins)
Smad3 in NT
Smad3 in shSNX9
pSmad3 in NT
pSmad3 in shSNX9
pSmad3 125I Count in NT
pSmad3 32P Count in NT
pSmad3 125I Count in shSNX9
pSmad3 32P Count in shSNX9

Figure S2

Figure S2. Differing kinetics of nuclear entry between phosphorylated and unphosphorylated Smad3. ^{125}I labeled TAT-Smad3 and phosphorylated (^{32}P) TAT-Smad3 proteins were generated as described in Materials and methods. (A) AKR-2B cells expressing non-targeting shRNA (NT; A-NT.8) or shRNA against SNX9 (shSNX9; A-77.7) were transduced and ^{125}I incorporation in total cell lysates determined at the indicated times. (B) ^{125}I incorporation in nuclear lysates from cultures as in (A). (C) Nuclear counts in (B) at each time point normalized to maximal cell transduction observed in (A). (D) Nuclear ^{32}P counts from NT and shSNX9 cells transduced with TAT-pSmad3. (E) Normalized nuclear ^{32}P incorporation to maximal cell transduction observed in (A). (F) To document that ^{125}I and ^{32}P were assessing the same populations of TAT-Smad3 peptides (i.e., the delayed nuclear uptake of ^{125}I pSmad3 in shSNX9 cells observed in (B) was not due to a sub-population of nonphosphorylated TAT-pSmad3 molecules), the ^{125}I and ^{32}P counts shown in (C) and (E), respectively, were normalized to their fold induction to allow direct comparison. Data reflect the mean $\pm$ sd from 3 experiments for the 30-240 min time points and 2 experiments for the 10, 20, and 270 time points.

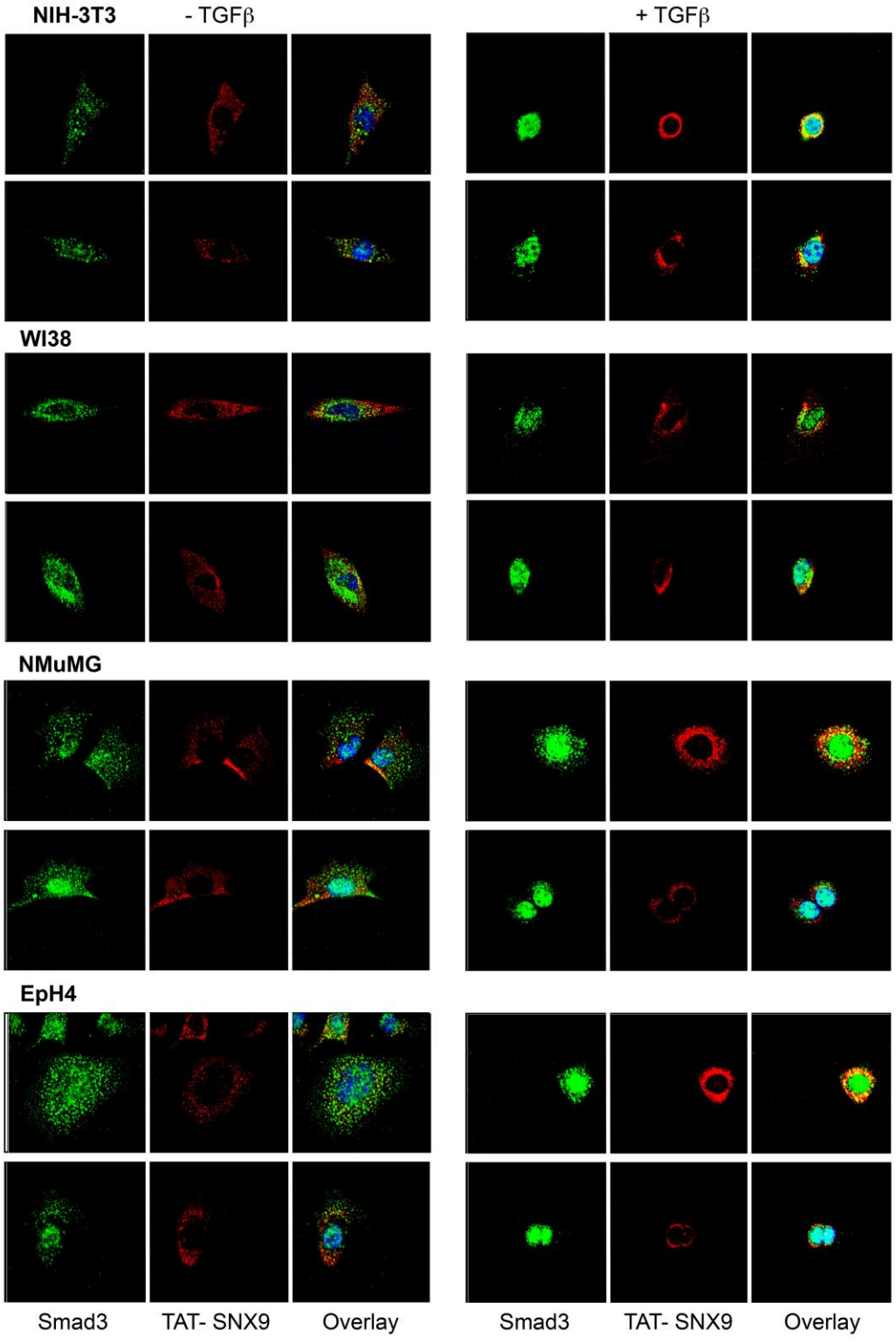
NIH-3T3
- TGFβ
+ TGFβ
WI38
NMuMG
EpH4
Smad3
TAT- SNX9
Overlay
Smad3
TAT- SNX9
Overlay

Figure S3

Figure S3. Increased co-localization of SNX9 and Smad3 upon TGFβ stimulation in different cell types. NIH3T3, WI38, NMuMG, and EpH4 cells were grown on glass coverslips and transduced with TAT-SNX9 protein (0.8 μM) for 90 min. After transduction cells were left untreated (left 3 panels) or stimulated with 5 ng/ml TGFβ for 60 min (right 3 panels). Cells were then fixed/permeabilized and incubated with anti mouse-HA (to visualize TAT-HA-SNX9 peptide) or anti rabbit -Smad3. TAT-SNX9 and Smad3 were detected by immunofluorescence analysis using anti mouse Alexa Fluor 594 or anti rabbit- Alexa Fluor 488 coupled secondary antibody, whereas nuclei of cells were stained with DAPI. Two fields of each cell type are shown.

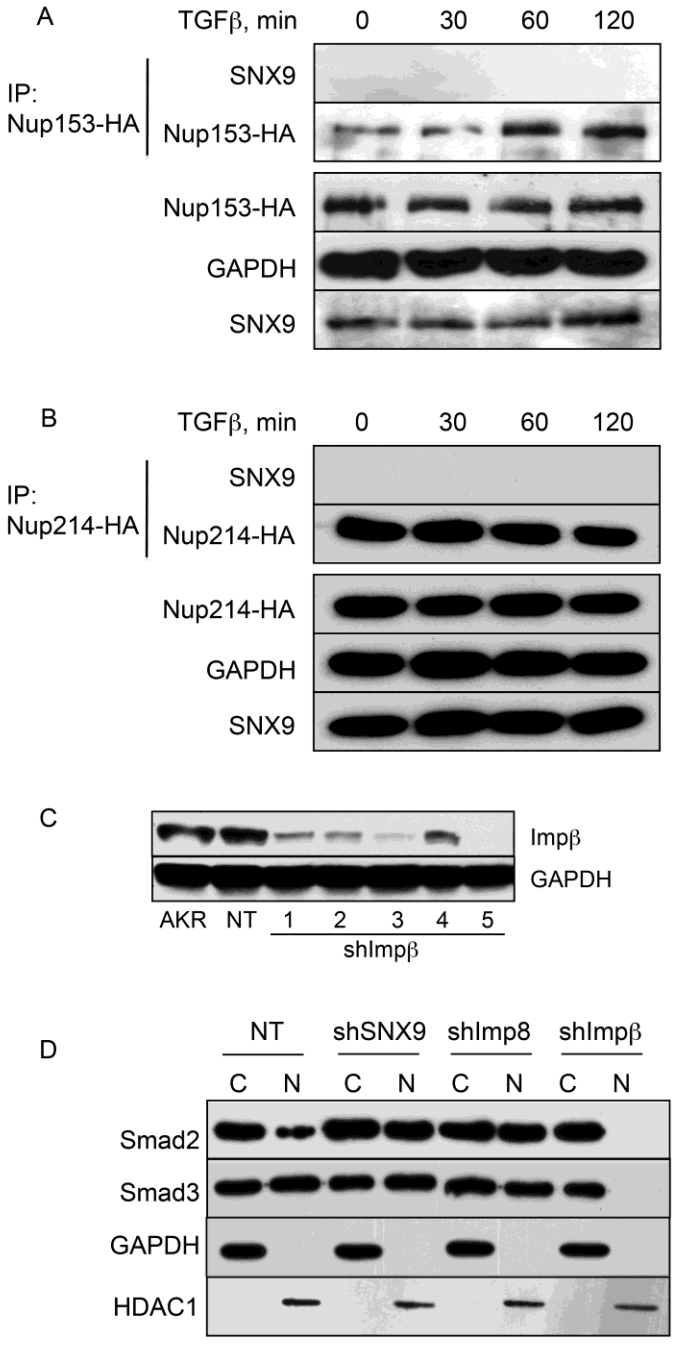
A
TGFβ, min
0
30
60
120
IP:
Nup153-HA
SNX9
Nup153-HA
Nup153-HA
GAPDH
SNX9
B
TGFβ, min
0
30
60
120
IP:
Nup214-HA
SNX9
Nup214-HA
Nup214-HA
GAPDH
SNX9
C
Impβ
GAPDH
AKR
NT
1
2
3
4
5
shImpβ
D
NT
shSNX9
shImp8
shImpβ
C
N
C
N
C
N
C
N
Smad2
Smad3
GAPDH
HDAC1

Figure S4

Figure S4. Nucleoporins and Impβ have distinct roles in R-Smad nuclear accumulation. (A) AKR-2B cells were transiently transfected with HA epitope tagged Nucleoporin153 (Nup153-HA), stimulated with 5 ng/ml TGFβ, and lysates prepared as in figure 7A. Following HA immunoprecipitation (IP), associated SNX9 (top panel) or transfected Nup153-HA (2^{nd} panel) were detected by Western blotting. The bottom 3 panels reflect expression of Nup153-HA, GAPDH, and SNX9, respectively, in total cell lysate. HA-tagged Nup153 was used as we were unable to find a commercially available antibody which reproducibly immunoprecipitated endogenous Nup153 (unpublished observations). (B) Same as (A) except HA-epitope tagged Nup214 was used for the same reason. (C) AKR-2B cells were infected with lentivirus expressing non-targeting (NT) or 5 distinct shRNAs (1-5) to Impβ. Following 2 weeks selection in puromycin, lysates were prepared and processed for Western blotting. Cultures expressing Impβ shRNA 5 were used for all studies where shImpβ is indicated. (D) AKR-2B cells stably expressing non-targeting shRNA (NT; A-NT.8) or shRNA to SNX9 (shSNX9; A-77.7), Imp8 (shImp8; A-89.4) or Impβ (shImpβ; non-clonal population) were fractionated into cytoplasmic (C) and nuclear (N) pools. The indicated proteins within each fraction were detected by Western blotting. GAPDH (cytoplasmic marker) and HDAC1 (nuclear marker) reflect fraction purity.

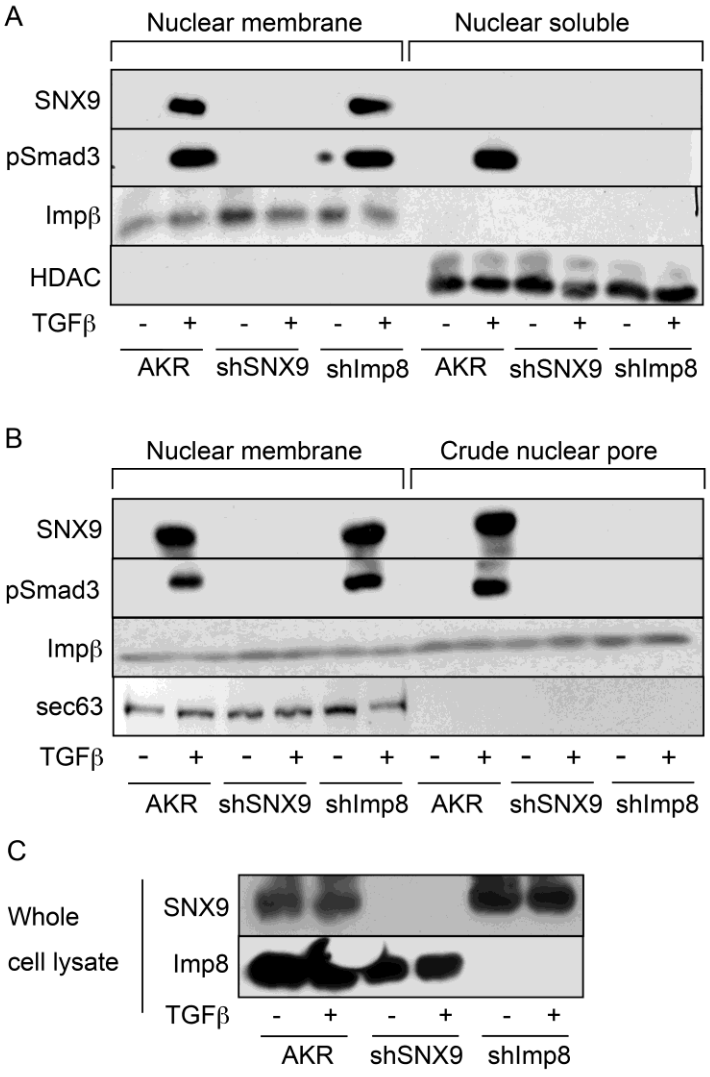
A
Nuclear membrane
Nuclear soluble
SNX9
pSmad3
Impβ
HDAC
TGFβ
- + - + - + - + - + - +
AKR shSNX9 shImp8 AKR shSNX9 shImp8
B
Nuclear membrane
Crude nuclear pore
SNX9
pSmad3
Impβ
sec63
TGFβ
- + - + - + - + - + - +
AKR shSNX9 shImp8 AKR shSNX9 shImp8
C
Whole
cell lysate
SNX9
Imp8
TGFβ
- + - + - +
AKR shSNX9 shImp8

Figure S5

Figure S5. SNX9 does not enter the soluble nuclear fraction but does co-purify with the nuclear pore in an Imp8-dependent manner. (A) AKR-2B (AKR), SNX9 KD (shSNX9; A-77.7) or Imp8 KD (shImp8; A-89.4) cell lines were left untreated (-) or stimulated (+) for 30 min with 5 ng/ml TGFβ. Nuclear membrane (lanes 1-6) and soluble (lanes 7-12) fractions were prepared and expression of the indicated proteins assessed by Western analysis. (B) Analogous to (A) except that a crude nuclear pore fraction (lanes 7-12) was isolated. Sec63 represents a rough endoplasmic reticulum/outer nuclear membrane marker. (C) Documentation of SNX9 and Imp8 knockdown in cells used for (A) and (B).

Table S1. qPCR and Mutagenesis Primers

Gene	Forward or Reverse	Sequence (5' to 3')
qPCR		
GAPDH - mouse	Forward	CCACCCATGGCAAATTCCATGGCA
	Reverse	TCTAGACGGCAGGTCAGGTCCACC
Serpine - mouse	Forward	GAAAGGGCACGCATTGGTAA
	Reverse	GATGGTGCTGTTCCAAA
Smad7 - mouse	Forward	GACGAAGAGAGTCTCCGAGG
	Reverse	GCTCTCATGAGCTGCTGGC
CTGF - mouse	Forward	GCATCCTCCTACCGCGTC
	Reverse	CAGTCCTGGCCCATAGCAG
Goosecoid - mouse	Forward	GCTGTCCTGGAATTTCCTCTGT
	Reverse	GTGTGTCCACAGCACTTCCC
MixL1 - mouse	Forward	ATCCCCTGGGCCTTCTTACT
	Reverse	GGGAAACTGAGTCAAGCCGA
Furin - mouse	Forward	GAGACTTCTTCTTCTTCGGCG
	Reverse	CCAGGATGTTGTCGATGCTGA

Mutagenesis		
SNX9 BAR Mut (R426E)	Forward	GCAGGAGCACTGGAAGGAATGCACGGGCC CATTAC
	Reverse	GTAATGGGCCCGTGCATTCCTTCCAGTGCT CCTGC
SNX9 BAR Mut (K433E, K437E)	Forward	TTACCCGAGGAATATCAGGAGATAGGAAAG GCCTTGC
	Reverse	TATCTCCTGATATTCCTCGGGTAATGGGCCC GTGC
SNX9 RYK Mut	Forward	CCTACTAACACTAATCGATCTGTAAACCACC AGGCTGCGCACTTTGACTGGTTATATGAGC GTCTCCT
	Reverse	AGGAGACGCTCATATAACCAGTCAAAGTGC GCAGCCTGGTGGTTTACAGATCGATTAGTGT TAGTAGG
SNX9 Δ13C	Forward	TTTTACGAAACGATTGCATAGGCCCCCGGGT
	Reverse	ACCCGGGGGCCTATGCAATCGTTTCGTAAAA
SNX9 esc	Forward	CCATGGAACCAGAGGCACCGGATTTAGACT TAGTAGAAATAGAGCA
	Reverse	TGCTCTATTTCTACTAAGTCTAAATCCGGTG CCTCTGGTTCCATGG

Table S2. Antibodies

Antibody	Company	Catalog #	WB Dilution	Primary
pSmad2	Calbiochem	566415	1/1000	Rabbit
Total Smad2/3	Millipore	07-408	1/1000	Rabbit
Total Smad2	AbCam	ab63576	1/1000	Rabbit
pSmad3	(Wilkes et al., 2003)	N/A	1/3000	Rabbit
pSmad3	Cell Signaling	9520	1/1000	Rabbit
Total Smad3	AbCam	ab28379-100	1/3000	Rabbit
Smad4	Cell Signaling	9515	1/2000	Rabbit
SNX9	Santa Cruz	sc49143	1/1000	Goat
GFP	Roche	11814460001	1/1000	Mouse
GAPDH	Chemicon	MAB374	1/15000	Mouse
HDAC1	Cell Signaling	2062	1/1000	Rabbit
Sec63	AbCam	ab99031	1/800	Rabbit
Myc Tag	Roche	11667149001	1/500	Mouse
Flag Tag	Sigma	F3165-5MG	1/1000	Mouse
HA Tag	Roche	11666606001	1/1000	Mouse
H2B	Millipore	07-371	1/1000	Mouse
Imp8	AbCam	ab72109-100	1/1000	Rabbit
Impβ	AbCam	ab36775-50	1/1500	Rabbit
Imp7	AbCam	ab15840-100	1/1000	Goat
pSer/Thr	BD Bioscience	612548	1/500	Rabbit

Supplementary References

Anders, R.A., and Leof, E.B. (1996). Chimeric granulocyte/macrophage colony-stimulating factor/transforming growth factor-ß (TGF-ß) receptors define a model system for investigating the role of homomeric and heteromeric receptors in TGF-ß signaling. J. Biol. Chem. *271*, 21758-21766.

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Worby, C.A., and Dixon, J.E. (2002). Sorting out the cellular functions of sorting nexins. Nat. Rev. Mol. Cell Biol. *3*, 919-931.