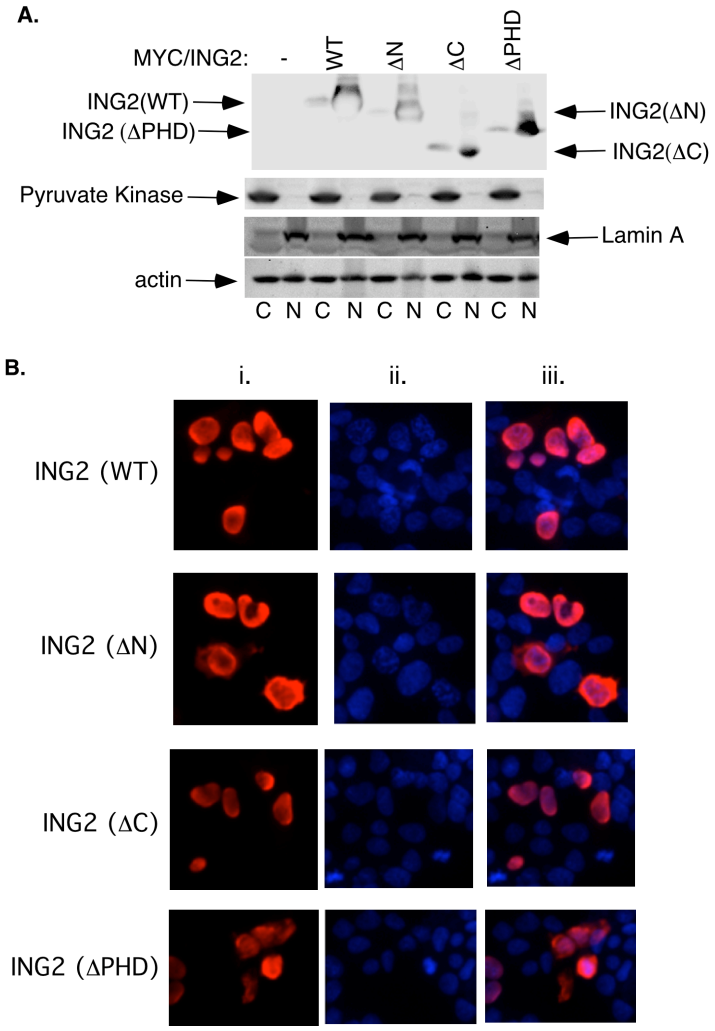
Supplementary Material

Subcellular Fractionation

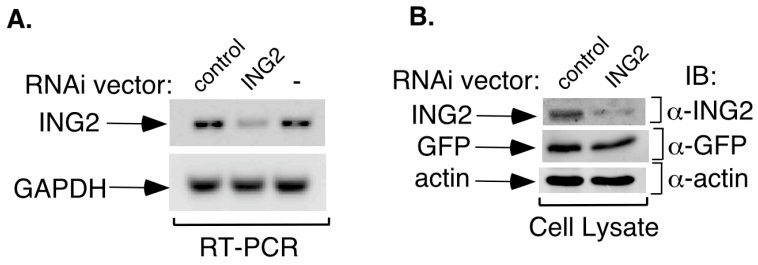
Transfected 293T cells were harvested with phosphate buffered saline (PBS) and centrifuged at 2000 rpm (500g) for 3 min. The pellet was washed, re-centrifuged at 2000 rpm (500g) for 3 min, and resuspended in 500 μL PBS containing 0.1% NP-40 (Roche). Cells were lysed by trituration with an 18.5 gauge needle and centrifugation at 10,000 rpm (9,000g) for 2 min. The supernatant was carefully separated from the pellet and mixed with Laemmli protein sample buffer containing Dithiothreitol (DTT) and this represented the cytosolic fraction. The pellet was washed with PBS containing 0.1% NP-40, triturated, and centrifuged at 13000 rpm (18,000g) for 5 min. Following removal of the supernatant the pellet was resuspended in Laemmli protein sample buffer containing DTT to obtain the nuclear fraction. Cytosoloic and nuclear fractions were subjected to ING2 immunoblotting. Nuclear or cytosolic enrichment of each fraction was confirmed by immunoblotting with antibodies to the nuclear marker lamin A (H-102, Santa Cruz), and the cytosolic marker pyruvate kinase (ab6191, Abcam), respectively. Equivalent protein loading was confirmed by actin immunoblotting.

Immunofluorescence Analysis

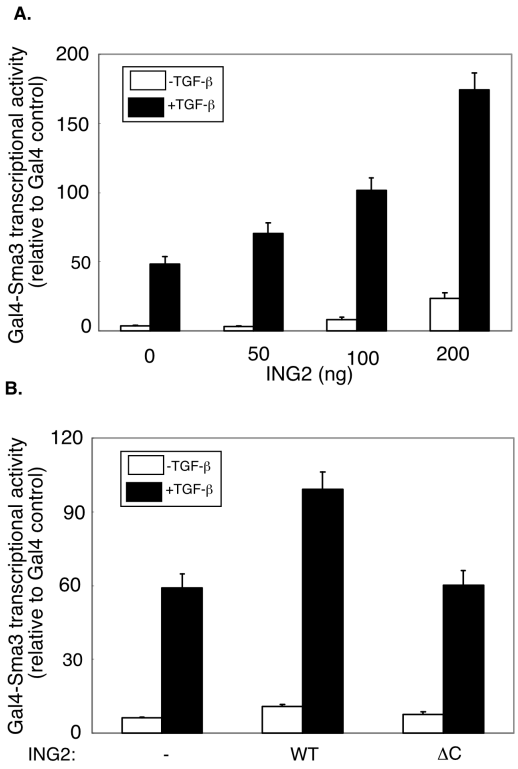
293T cells were seeded on poly-L-lysine coated chamber slides and transfected with mammalian expression vectors expressing different proteins as indicated in Supplementary Fig. 1B and Supplementary Fig.6. 36 h post-transfection, cells were fixed with 4% formaldehyde and permeablized with 0.2% Triton-X100 for 5 min on ice. Following several washes, cells were incubated at 37ºC with a blocking solution consisting of 5% BSA and 5% calf serum in PBS for 1 h. Cells were then switched to a 0.5% BSA-PBS solution containing primary antibodies, as outlined in the legends of Supplementary Fig. 1B and Supplementary Fig. 6, for 1hr at 37ºC. After several washes, cells were incubated in a solution containing fluorescent-conjugated secondary antibodies and the DNA stain Hoechst 33342 for 1hr at 37ºC. Images were acquired using the Cellomics Kinetic Scan Reader that is equipped with Carl Zeiss optics.



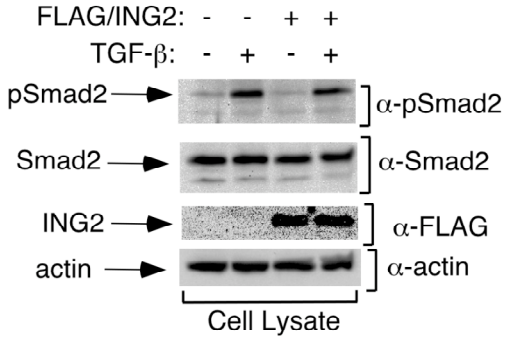
Supplementary Fig. 1. Subcellular localization of ING2. A) Immunoblotting of ING2, puruvate kinase, lamin A, and actin in cytosolic (C) and nuclear (N) fractions of 293T cells transfected with an empty expression vector (-), or one containing cDNA encoding wild type, ΔN, ΔC, or ΔPHD ING2. Pyruvate kinase and lamin A were used as cytosolic and nuclear markers, respectively. B) Immunofluorescence of full length (WT) ING2 protein, or ΔN, ΔC, or ΔPHD deletion mutant ING2 protein expressed in 293T cells. ING2 protein was detected using mouse anti-MYC as primary and cy3-anti-mouse as secondary antibodies (i) and cells' nuclei were visualized using Hoechst staining (ii) as described in Supplementary Materials. Images displayed in iii represent overlay of respective images shown in i and ii. The results shown in A and B indicate that the full length ING2 and deletion mutant ING2 proteins localize mainly in the nucleus.



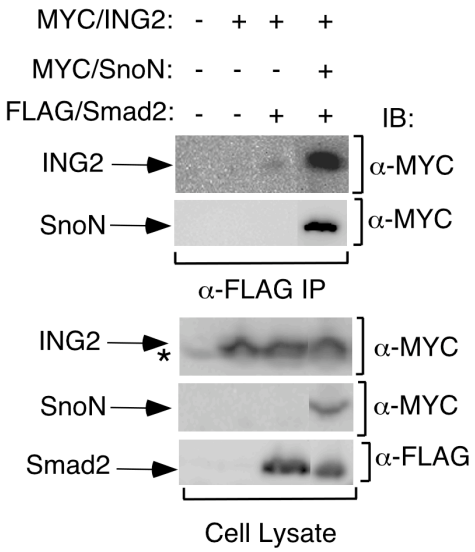
Supplemetary Fig. 2. ING2 RNAi triggers endogenous ING2 knockdown in Mv1Lu cells. A) ING2 mRNA analysis by RT-PCR of RNA extracted from Mv1Lu cells that were transfected with a control or an ING2 RNAi plasmid, or untransfected (-) (see "RT-PCR" section in Experimental Procedures for details). B) ING2, GFP, and actin immunoblotting of lysates of Mv1Lu cells transfected as in A. Quantitative analysis indicated that the ING2 RNAi produces approximately 40 to 60% reduction in endogenous ING2 in Mv1Lu cells.



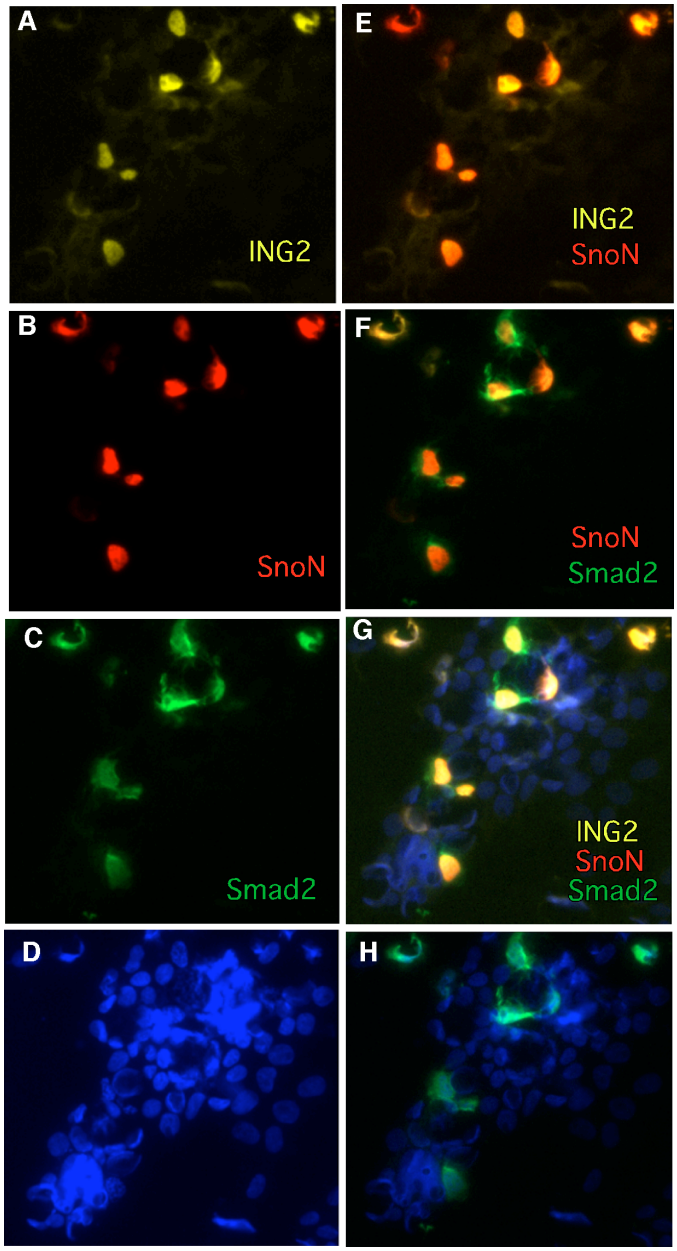
Supplementary Fig. 3. ING2 enhances Smad3 transcriptional activity. A) Lysates of untreated or TGF-β-treated Mv1Lu cells transfected with the pG5-E1b-luciferase and β-galactosidase reporter constructs together with the Gal4 alone or Gal4-Smad3 fusion expressing vectors in the absence or presence of different amounts of ING2 expressing plasmids, were subjected to luciferase and β-galactosidase assays and data were analyzed as described in Experimental Procedures. The bar graph represents the mean (± SD) of triplicate measurement of the Gal4-Smad3 activity relative to the respective Gal4 control. B) Gal4-Smad3 transcriptional activity was determined in lysates of untreated or TGF-β-treated Mv1Lu cells transfected with the reporter constructs as described in A together with an empty expression vector, one expressing a full length (WT) or C-terminally deleted (ΔC) ING2 protein. These data show that the PHD-containing C-terminal region is critical for the ability of ING2 to induce Smad3 transcriptional activity.



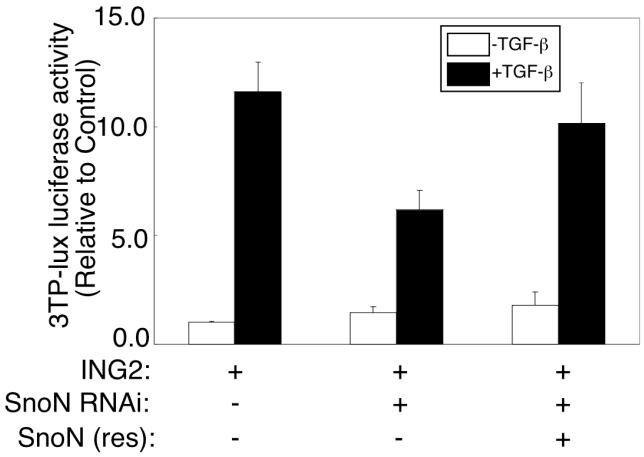
Supplementary Fig. 4. ING2 expression does not enhance TGF-β-dependent Smad2 phosphorylation. Lysates of untreated or TGF-β -stimulated Mv1Lu cells transfected with an empty expression vector (-) or one expressing ING2, were immunoblotted with antibodies to recognize serine 465 and 467 phosphorylated-Smad2 (α-pSmad2), total Smad2 (α-Smad2), ING2 (α-FLAG), and actin (α-actin).



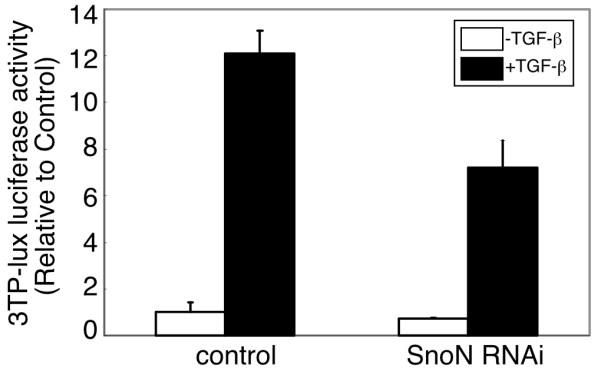
Supplementary Fig. 5. SnoN promotes ING2-Smad2 interaction. Lysates of 293T cells expressing MYC tagged ING2, alone or with FLAG tagged Smad2, alone or together with MYC tagged SnoN were subjected to Smad2 immunoprecipitation (α-FLAG IP). ING2 and SnoN proteins coimmunoprecipitating with Smad2 were visualized by anti-MYC immunoblotting (α-MYC IB). Expression of ING2, SnoN, and Smad2 were confirmed by immunoblotting of lysates with the appropriate antibodies. The data show that SnoN expression dramatically enhances the association between ING2 and Smad2. * indicates non-specific MYC-immunoreactive protein band.



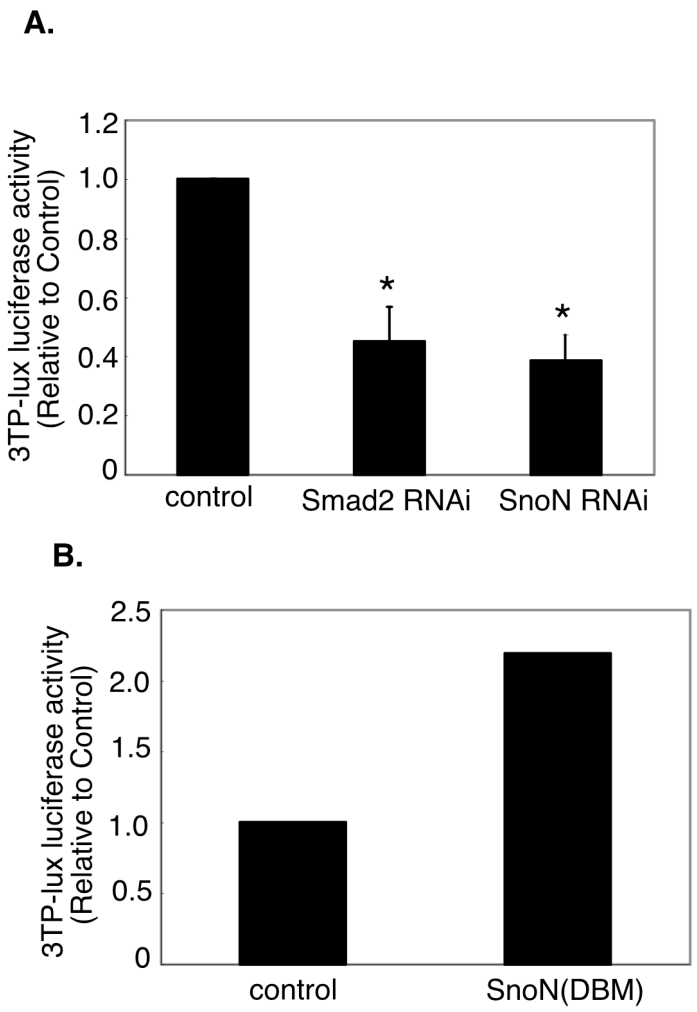
Supplementary Fig. 6. ING2 colocalizes with SnoN and Smad2 in the nucleus. 293T cells coexpressing MYC/ING2, HA/SnoN, and FLAG/Smad2 were subjected to ING2, SnoN and Smad immunoflourescence. Cells were incubated with chicken a-ING2, rabbit □-SnoN, and mouse □-Smad2 antibodies followed by cy5-□-chicken (yellow-ING2, A), cy3-□-rabbit (red-SnoN, B), and cy2-□-mouse (green-Smad2, C) antibodies, and imaged as described in Supplementary Material. Nuclei were visualized using Hoechst 33342 DNA dye (cyan, D). Images shown in the right hand column depict overlay of ING2 and SnoN (E), SnoN and Smad2 (F), ING2, SnoN, Smad2, and nuclei (G), and Smad2 and nuclei (H). The data show that ING2, SnoN and Smad2 colocalize in the nucleus.



Supplementary Fig. 7. The SnoN rescue construct reverses SnoN RNAi effect on ING2 enhancement of TGF-β-dependent transcription. Lysates of Mv1Lu cells were transfected and treated in the absence or presence of TGF-β as indicated and described in Experimental Procedures. Low concentrations of SnoN rescue expressing vector was used. The values represent the mean (±SD) of triplicate measurements from a representative experiment that was repeated three times.



Supplementary Fig. 8. SnoN RNAi suppresses TGF-β-induced transcription in MDCK cells. MDCK cells transfected with 3TP-luciferase and CMV-β-galactosidase reporter plasmids together with the control or the SnoN RNAi vector were incubated overnight in the absence or presence of TGF-β. Luciferase activity in the lysates of cells was determined as described in Fig 1D. The data shown represent the mean (± SD) of triplicate determinations from a representative experiment that was repeated four times. The data show that SnoN RNAi inhibits TGF-β-induced 3TP-luciferase activity supporting the idea that endogenous SnoN positively regulates TGFβ-induced transcription in these cells.



Supplementary Fig. 9. SnoN activates the 3TP-lux reporter gene in rat cerebellar granule neurons. A) Primary granule neurons were prepared from postnatal day 6 rat pups and transfected with the 3TP-luciferase reporter gene and pRL-TK expression plasmid, the latter to serve as internal control for transfection efficiency, together with the control, Smad2, or SnoN RNAi vector. Neurons were harvested three days later and subjected to a Dual Luciferase Assay. Knockdown of Smad2 and SnoN significantly reduced 3TP-luciferase activity compared to control ($P < 0.005$, ANOVA; n=3; values indicate mean ± SEM). B) Lysates of granule neurons transfected with the 3TP-lux reporter gene and pRL-TK expression plasmid together with an expression plasmid encoding a constitutively active form of SnoN owing to a mutation that stabilizes SnoN protein levels (SnoN (DBM)) or its control vector, were analyzed as in A. SnoN DBM enhanced the 3TP-luciferase activity (average of two independent experiments shown).