Supplemental Materials and Methods

Generation of chimeras. *Smad2*^{m1Mag} homozygous mutant ES cells with the *Rosa26-lacZ* transgene were injected into wild-type blastocysts via standard procedures (1). Chimeric embryos were isolated at E9.5 and stained in whole mount with X-gal staining solution to detect mutant cells (2). Stained embryos were dehydrated in ethanol and embedded in paraffin for histological analysis. Sections were counterstained with Nuclear Fast Red.

Protein Immunoprecipation. PCR-based insertion of a hemagglutinin (HA) tag was performed using pCS2-Flag-Smad2 template (3; Addgene plasmid 14042) to generate pCS2-HA-Smad2. Site directed mutagenesis (Genscript) was performed on these constructs to convert serine 276 to a leucine residue (codon change from TCA to TTA). pCS2-HA-Smad2 or pCS2-HA-Smad2 S276L were transfected into ES cells along with either pCS2-Flag-Smad2 or pCS2-Flag-Smad2 S276L. Cells were harvested 24 hours after transfection in 150 mM NaCl, 10 mM Tris pH 8, 0.2% NP40, and phosphatase and protease inhibitor cocktails (Pierce). Equal amounts of cell lysates were incubated with anti-HA agar (Pierce) overnight at 4°C. Columns were washed three times with 150 mM NaCl, 10 mM Tris pH 8, and 0.5% triton. Pull-down of the Flag-tagged Smad2 proteins was analyzed through western blotting. Whole cell lysate was also analyzed to assure equivalent transfection efficiencies.

Smad4 overexpression. E14 ES cells were transfected with pCMV5 DPC4-HA (4; Addgene plasmid 14038) using Lipofectamine 2000. Five hours after transfection, the media was changed to defined media $\pm$ SB431542. RNA and protein lysates were harvested after 24 hours. Transfection efficiency was assessed by co-transfection with pEGFP-C3.

RNA Knockdown. Dharmacon siRNAs targeting Follistatin were transfected into E14 ES cells following manufacturer's instructions. Forty-eight hours after transfection, the media was changed to defined media $\pm$ SB431542 (SB). Changes in gene expression were compared to a non-targeting siRNA after 24 hours of SB treatment.

Supplemental References

1. Nagy, A., Gertsenstein, M., Vintersten, K., and Behringer, R. (2003) *Manipulating the Mouse Embryo 3rd edition*, Cold Spring Harbor Laboratory Press
2. Vivian, J. L., Klein, W. H., and Hasty, P. (1999) *Biotechniques* **27**, 154-162
3. Hata, A., Lo, R. S., Wotton, D., Lagna, G., and Massague, J. (1997) *Nature* **388**, 82-87
4. Lagna, G., Hata, A., Hemmati-Brivanlou, A., and Massague, J. (1996) *Nature* **383**, 832-836

Supplemental Figure Legends

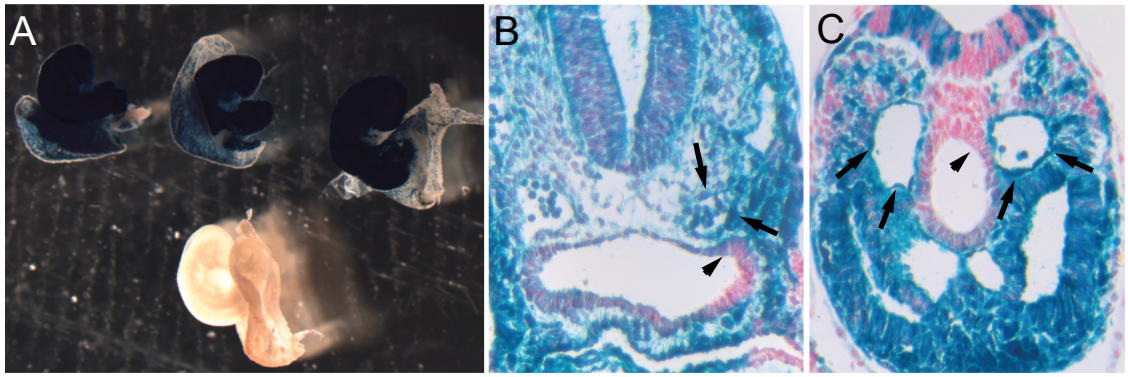
Supplemental Figure 1. *Smad2^{m1Mag}* homozygous ES cells. *Smad2^{m1Mag}* homozygous ES cells tagged with ubiquitous Rosa26-lacZ were injected into wild-type blastocysts. A) LacZ staining indicates high percentage chimeras. B, C) Mutant cells did not contribute to definitive endoderm (arrowhead) of the foregut or hindgut, notochord, or floorplate. Mutant cells did contribute to a morphologically normal endothelial vasculature of the dorsal aorta (arrows). D) Immunoblot analysis between control and mutant ES cells demonstrated that *Smad2^{m1Mag}* mutant protein was stable and activated via phosphorylation. Decreased expression of *Lefty1* and *Smad7* may induce higher pSmad2 levels in the mutant ES cells. E) HA-Smad2 or HA-Smad2 S276L (ENU-induced mutation screen produced an amino acid substitution resulting in a change from a serine to leucine in amino acid 276 in *Smad2^{m1Mag}*) immunoprecipations revealed a decreased ability of the mutant protein to form protein complexes with wild-type and mutant Smad2.

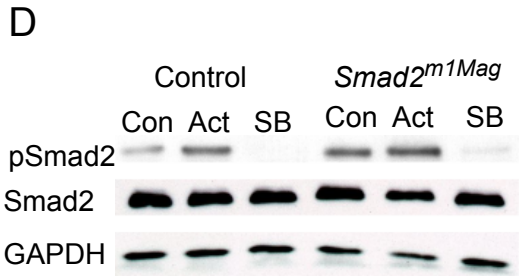
Supplemental Figure 2. Nodal-Smad2 inhibition. A) Immunoblot analysis of pSmad1/5 and pSmad2 of cells cultured in defined media with LIF and BMP4. ES cells were treated with SB431542 (SB), A-83-01, or Activin for the indicated times. To determine the effects of exogenous BMP4, recombinant BMP4 was removed from the media. The doublet band detected by pSmad1/5 is dependant on the resolution quality of the gels, and the presence of two bands has been shown to be potential post-translation modification of the proteins. B) rtPCR analysis of longterm (9 day) treatment of ES cells. *$p<0.05$.

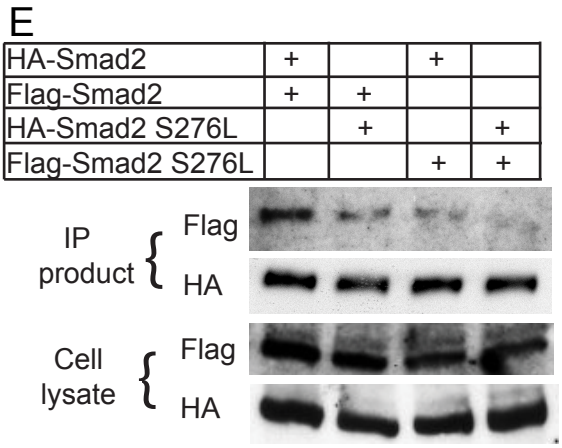
Supplemental Figure 3. SB431542-mediated activation of the BMP pathway in the presence of Smad4 overexpression. ES cells were transfected with pCMV5 DPC4-HA (4) and treated with SB431542. Co-transfection with a GFP reporter demonstrated approximately 50% transfection efficiency. In the presence of excess Smad4, SB431542 (SB) treatment still retains the ability to phosphorylate Smad1/5 (A) and to induce expression of *Id1*(B). Thus, limiting amounts of Smad4 do not contribute to the SB mediated affects.

Supplemental Figure 4. Follistatin siRNA fails to affect SB431542-mediated *Id1* induction. ES cells were transfected with a non-targeting or Follistatin siRNA and were treated DMSO or SB431542 (SB) for 24 hours. Basal expression of *Id1* and SB-mediated expression of *Id1* were not affected by Follistatin knockdown.

Supplemental Figure 5. SB431542 further reduces *Smad7* expression in ES cells with Smad7 knockdown. ES cells were transfected with pLKO.1-non-targeting shRNA or pLKO.1-Smad7 shRNA, subcultured, and selected with puromycin for 48 hours. During the last 24 hours, cells were treated with SB431542 (SB). Treatment with SB lowered the expression of *Smad7* in cells with non-targeting shRNA and Smad7 shRNA. *$p<0.05$.







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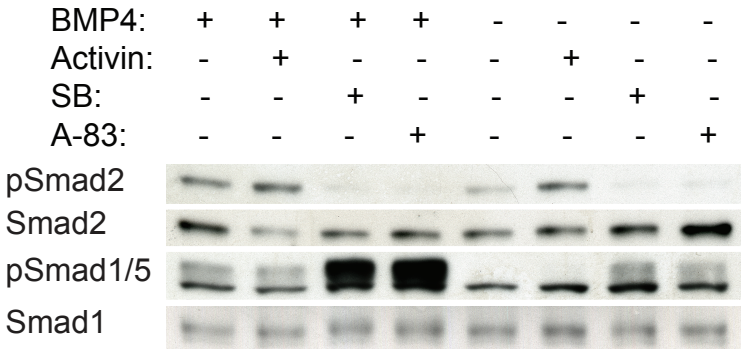
A



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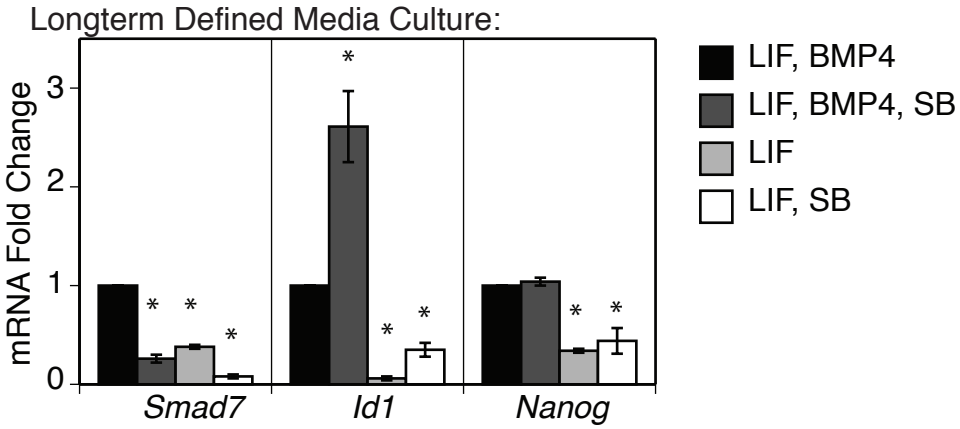

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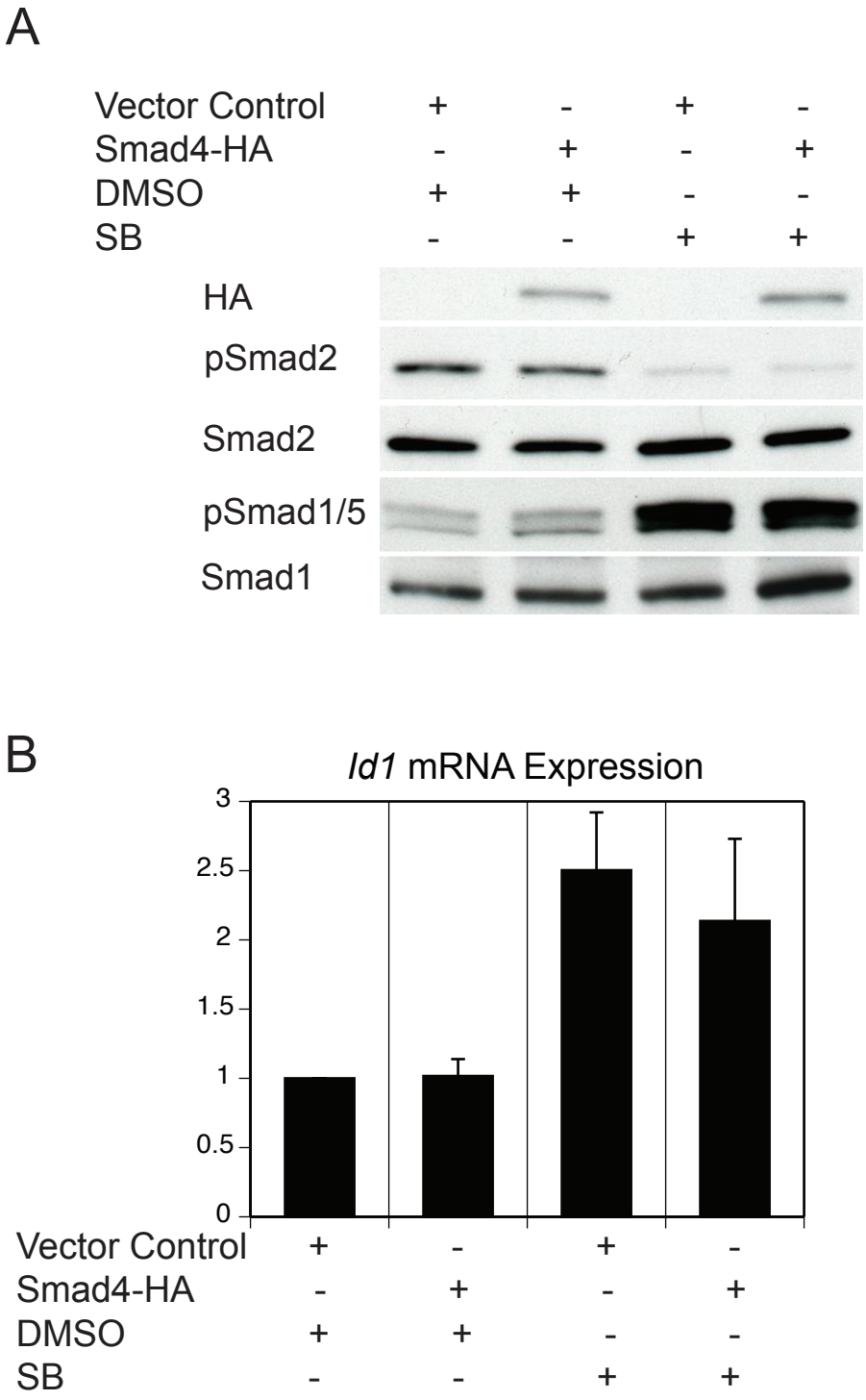

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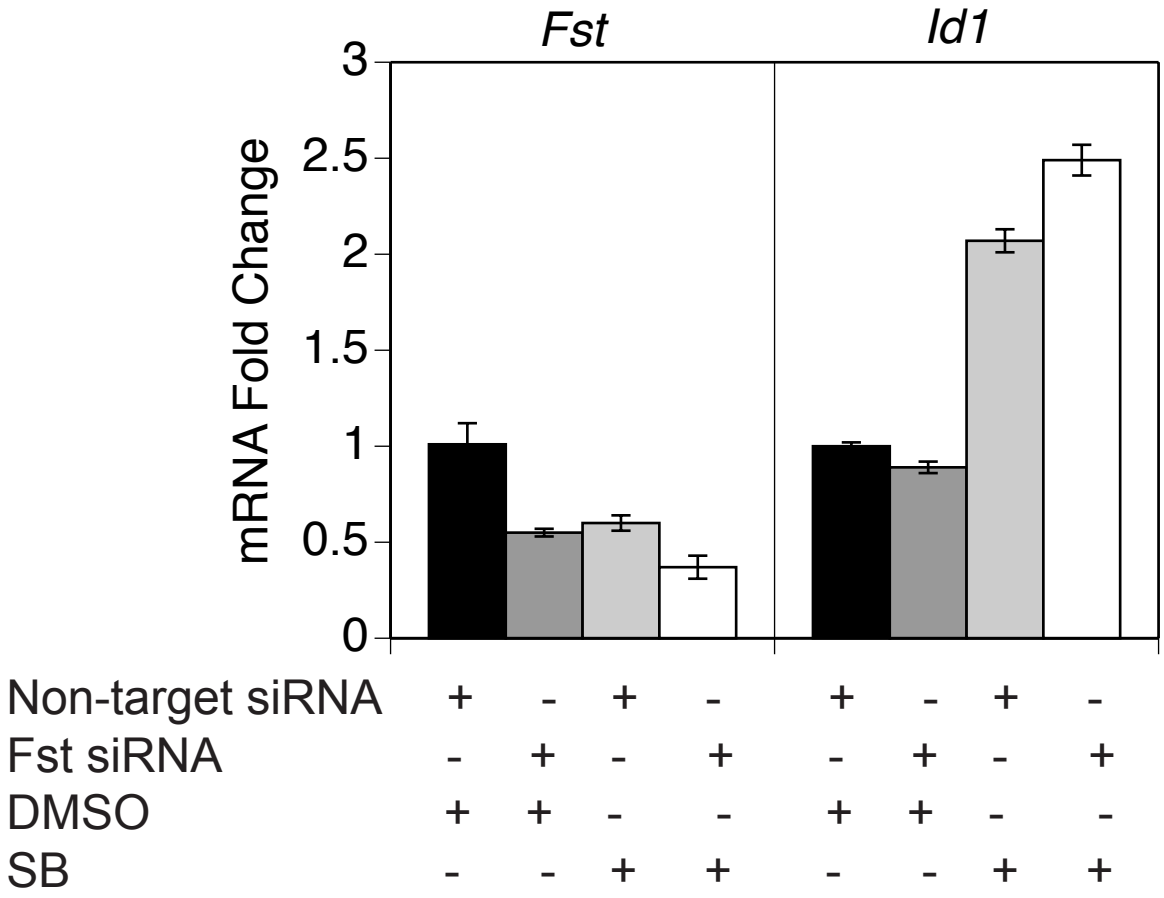

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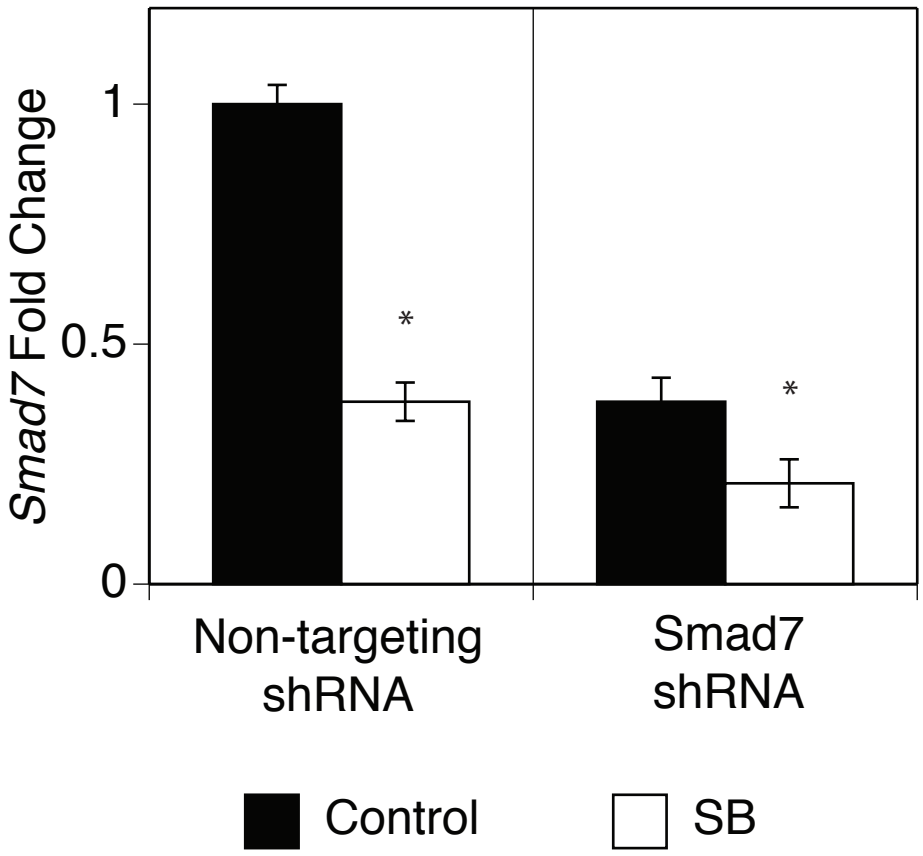

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