Supplemental Materials and Methods

Creation of *Smurf2*-deficient mice

Mouse Smurf2 genomic DNA was isolated from a BAC clone identified in the 129/SvJ mouse genomic library (Incyte Genomics, Palo Alto, CA). A 2.37 kb Xba1-HpaI and an 8.63 kb XbaI-digested fragments of *Smurf2* genomic DNA were cloned into the plasmid *pLoxP*^{neo} (Yang et al, 1998) at 5′ and 3′ ends of a *neomycin* resistance gene, respectively, to generate the targeting vector (Figure 1A). After linearization with NotI, the targeting construct was electroporated into the TC1 line of embryonic stem (ES) cells and subjected to selection (Deng et al, 1996). ES cell colonies that were resistant to both G418 and FIAU were chosen, and genomic DNA from these clones were digested with BamHI and analyzed by Southern blot using a 5′ external probe (shown in Figure 1A). Homologous recombination in ES cells removed exons 9 and 10, and forced exon 8 to be spliced directly to exon 11 (Figure 1A-D). The targeted ES clone was injected into C57BL/6 blastocysts and male chimeras were mated with either C57BL/6 or NIH Black Swiss females to obtain germ-line transmission of a target inactivated *Smurf2* allele. To obtain *Smurf2*$^{-/-}$ mice in a pure C57BL/6 background, *Smurf2*$^{+/-}$ mice from a 129/SvJ x C57BL/6 mixed background were backcrossed with C57BL/6 mice for more than 10 generations before interbreeding to obtain homozygosity. Routine genotyping was carried out by PCR using primers mSF2I8F3 (5′-ataaactgagattgggagatt-3′) and mSF2E9R (5′-cctaggcactcgtggatcatg-3′) for wild type, and mSF2Xba20215R (5′-acctctgagttcaaagtcag-3′) and TWB-13 (5′-cagctcattcctcccactcatg-3′) for mutated *Smurf2* alleles (Figure 1D). All mice were maintained and handled according to protocols approved by the Animal Care and Use Committee of the National Cancer Institute.

Antibodies and reagents

Anti-Smad3 (Epitomics), anti-Smad3-pS423/pS425 (Rockland), anti-Smad3-pT179 (provided by Dr. F. Liu), anti-Smad2-pS465/pS467 (Cell Signaling Technology), anti-

Smad2/3 (BD Biosciences), anti-Myc (Santa Cruz), anti-Smad4 (Epitomics) and anti-Smurf2 (Santa Cruz) were used for Western blotting. Cycloheximide was purchased from Merck Bioscience. Agarose-conjugated anti-Flag and anti-HA as well as Flag and HA peptides were obtained from Sigma. His_6-Smurf2 and His_6-Smurf2CG proteins were produced in baculovirus exression system after infecting Hi5 cells (Invitrogen), and purified using immobilized metal-ion affinity chromatography followed by elution with imidazole and dialyzing against a solution containing 20mM Hepes, pH 7.3, 300 mM NaCl, 2 mM β-mecaptoethanol. Flag-Smurf2 stably-transfected *Smurf2*$^{-/-}$ MEFs were provided by Dr. M. Blank (to be published elsewhere). All other antibodies and reagents used in this study have been described previously (Yamashita et al, 2005; Millet et al, 2009).

RT-PCR and real-time PCR

RNA was prepared with Trizol (Invitrogen) according to the manufacturer's instructions. Total RNA was converted to cDNA by using a Reverse Transcription Kit (Biorad). Smad7 (QT00124607), c-Jun (QT00296541), and CTGF (QT00096131) primers were ordered from Qiagen. Primers for Smurf2: E6F: 5'-TAAGTCTTCAGTCCAGAGACC-3', E7R: 5'-CGTGTTGGGCGTTCCCACTGT-3', E11R: 5'-AATCTCTTCCCTAGACACCTC-3'; PAI-1: 5'-ACAGTGGGAAGAGACGCCTTC-3' and 5'-GGTGGGCAAGGTGGACATTTC-3'; Itch: 5'-ACAGCTTGATTCCATGGGTA-3' and 5'-TTCCACTTGGGACTGTTTGT-3'; WWP1: 5'-AACACAGAAGCTTTGCCATC-3' and 5'-TGAGACTGCCACTGTTCAAA-3'; WWP2: 5'-CACACAACTGACCCTGAACC-3' and 5'-TTCTCTTGAAGGTGGCTGTG-3'; NEDD4L: 5'-TGAGCAAGCTCACCTTCCA-3' and 5'-CCCGTGACAGTTGACGAAC-3'. Hprt and Smurf1 primers were previously described (Yamashita et al, 2005)). Real-time PCR was performed in ABI 7900HT Fast Real-Time PCR System using SYBR® Green PCR Master Mix (ABI). RNA transcript in each sample was assayed 3 times and expression level was normalized to that of the Hprt transcript as an internal control.

Supplemental Figure Legends

Figure S1: The mutated Smurf2 allele was not functional. **A**. To determine whether the targeted disruption of exon 9 and 10 of the *Smurf2* gene generated a null mutation, we examined expression and function of the mutant allele. RT-PCR analysis using primers located in exon 6 and 11 revealed a fragment of 562 bp in *Smurf2*$^{-/-}$ and 806 bp in wild type mice. Sequencing of the mutant fragment indicated that the 562 bp fragment was generated by directly splicing from exon 8 to exon 11 as predicted (Figure 1B). **B**. Real-time PCR analysis using primers located in exon 6 and 7 indicated that the mutated *Smurf2* transcript is less stable than its wild type counterpart as it accumulated to a much lower lever. In contrast, expression of other related HECT E3 ligases was not changed comparing to that in wild type (WT) and *Smurf2*$^{-/-}$ (KO) MEFs. **C**. Mutated Smurf2 protein does not interact with Smad3. To determine whether this putative product was functional, we cloned the mutant cDNA into a Myc-tagged expression vector. After transfected in *Smurf2*$^{-/-}$ MEFs, we found that wild type Smurf2 was able to pulled down co-transfected Flag-tagged Smad3 protein while this mutated Smurf2 protein could not. WT: wild type Smurf2; M: mutated Smurf2. **D**. The mutated Smurf2 protein was not capable to suppress TGF-β-induced PAI-1-Luc reporter activity after transfected in *Smurf2*$^{-/-}$ MEFs. Smurf2^{M}: mutated Smurf2.

Figure S2: Similar Smad protein level and phosphorylation in stably-transfected *Smurf2*$^{-/-}$ MEFs and Smurf1 and/or Smurf2-deficient embryos. **A**. Reconstitution of Smurf2 in *Smurf2*$^{-/-}$ MEFs does not affect TGF-β-induced Smad3 phosphorylation. Stably-transfected *Smurf2*$^{-/-}$ MEFs were treated with TGF-β for different time, and Smad3 phosphorylation was detected by Smad3 pS423/pS425 antibody. **B**. Loss of Smurf1 and/or Smurf2 did not affect Smad2/3 protein level and their C-terminal phosphorylation. Protein extracts from E9.5 embryos of *Smurf1*$^{-/-}$ (SF1KO), *Smurf2*$^{-/-}$ (SF2KO) or *Smurf1*$^{-/-}$ *Smurf2*$^{-/-}$ (DKO) were subjected to SDS-PAGE and Western blot

with specific antibodies. C. Relative ratio of pSmad2/Smad2 and pSmad3/Smad3 in B. The optical densities of protein bands in Western blot of the above were quantitated using ImageJ.

Figure S3: Loss of Smurf1 has no effect on Smad3 ubiquitination although Smad3 can be ubiquitinated by over-expressed Smurf1. **A**. Loss of Smurf1 has no effect on Smad3 ubiquitination. *Wild type* and *Smurf1*$^{-/-}$ MEFs were transfected with Flag-Smad3 and HA-ubiquitin. After 24 h transfection and overnight starvation, cells were treated ± TGF-β for 1 h. Cell lysates were subjected to Flag immunoprecipitation (IP). Ubiqutinated Smad3 was detected by anti-Smad3 (Top). Expression levels of transfected proteins in whole cell lysate (WCL) are shown on the bottom. **B**. Over-expressed Smurf1 induces Smad3 ubiquitination. *Smurf2*$^{-/-}$ MEFs were transfected with the indicated expression vectors. After 24 h transfection and overnight starvation, cells were treated ± TGF-β for 1 h. Cell lysates were subjected to Flag IP. Ubiqutinated Smad3 was detected by anti-Flag. Expression levels of transfected proteins in WCL are shown at the bottom.

Figure S4: Smurf2 has no effect on ubiquitination of TβRI, Smad7 or Smad4. *Smurf2*$^{-/-}$ MEFs were transfected with the indicated expression vectors. After 24 h transfection and overnight starvation, cells were treated ± TGF-β for 1 h. Cell lysates were subjected to Flag IP. Ubiqutinated protein was detected by anti-HA. Expression levels of transfected proteins in WCL are shown at the bottom. Asterisks in the bottom panel indicate specific Flag-tagged proteins.

Figure S5: Smurf2 is localized in both cytoplasm and nucleus. Wild type MEFs were subjected to subcellular fractionation after TGF-β treatment. The presence of Smurf2 in the nuclear and cytoplasmic fractions was determined by Western blot analysis. Lamin B1 and α-tubulin were used as nuclear and cytoplasmic specific protein markers, respectively.

Figure S6: Smad3 Q mutant can be poly-ubiquitinated as wild type Smad3. Wild type MEFs were transfected with Flag-Smad3 (WT or Q mutant) and HA-ubiquitin. After 24 h transfection, cells were treated with 10 μM MG132 and 10 μM SB431542 overnight. Cell lysates were subjected to Flag IP after lysed in RIPA buffer with 0.1% SDS. Ubiqutinated Smad3 was detected by anti-HA (Top). Expression levels of transfected proteins in WCL are shown on the bottom.

Tang et al Supplemental Figures

Figure S1

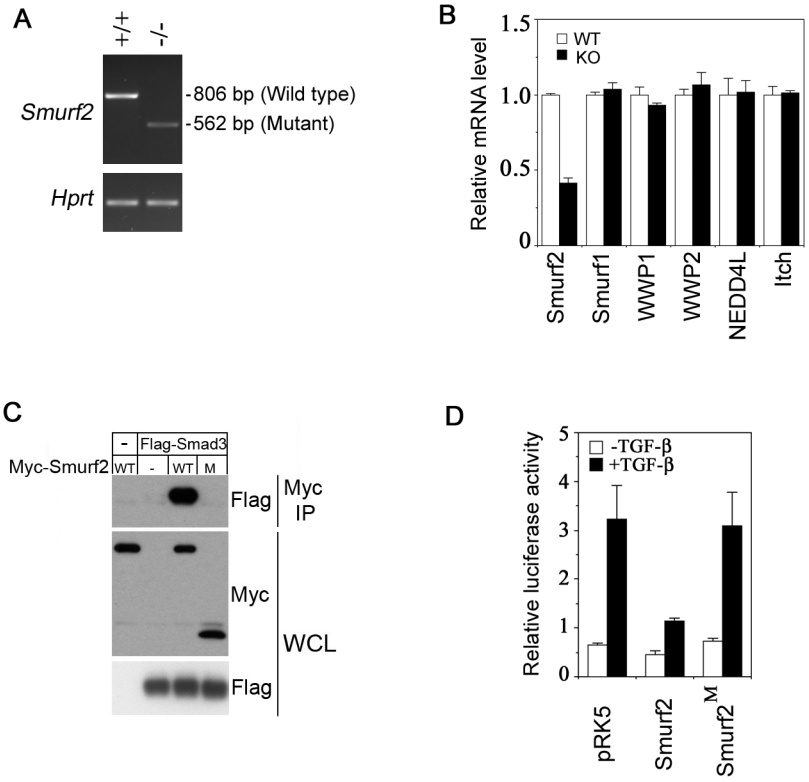

Figure S2

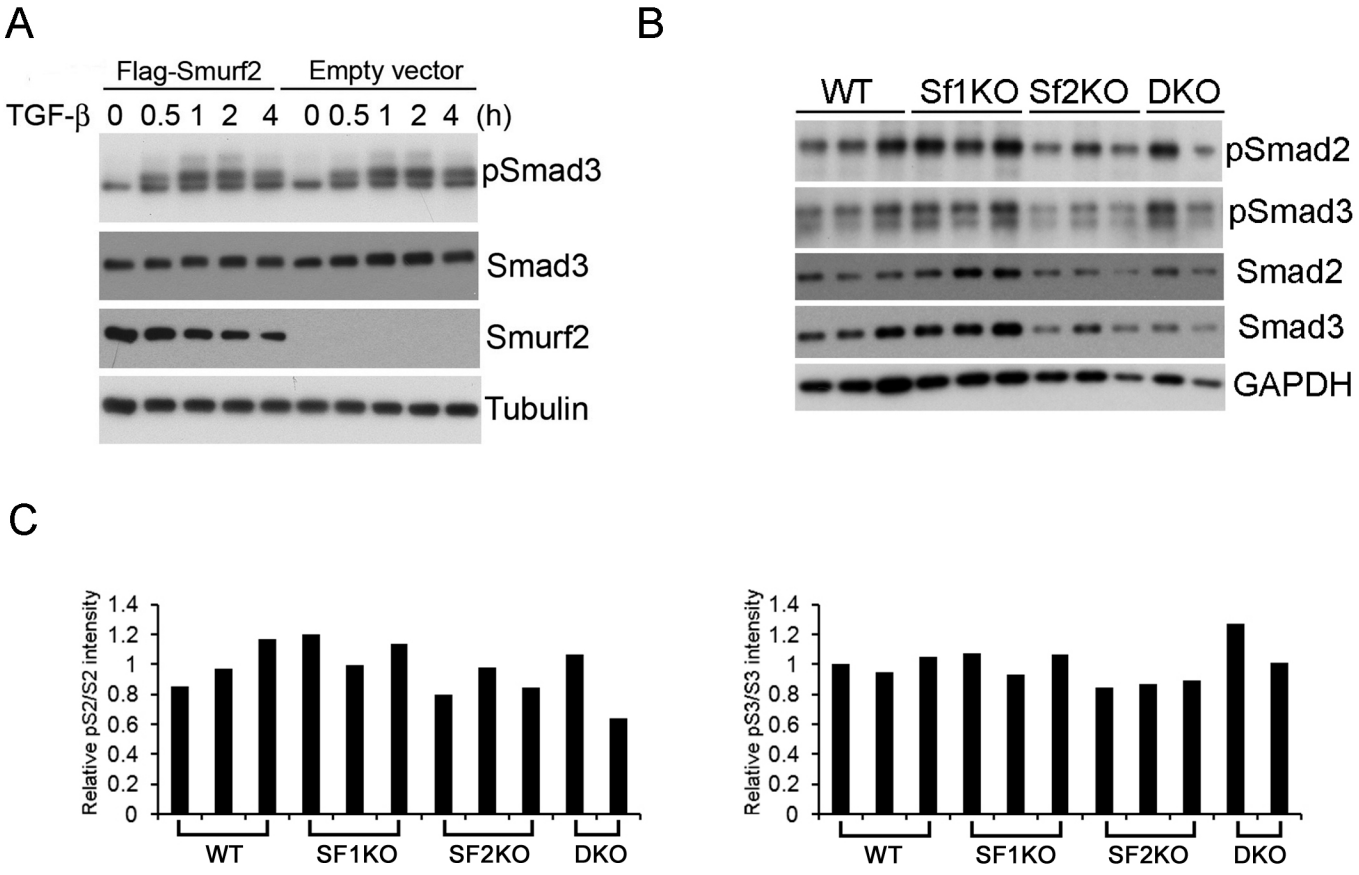

Figure S3

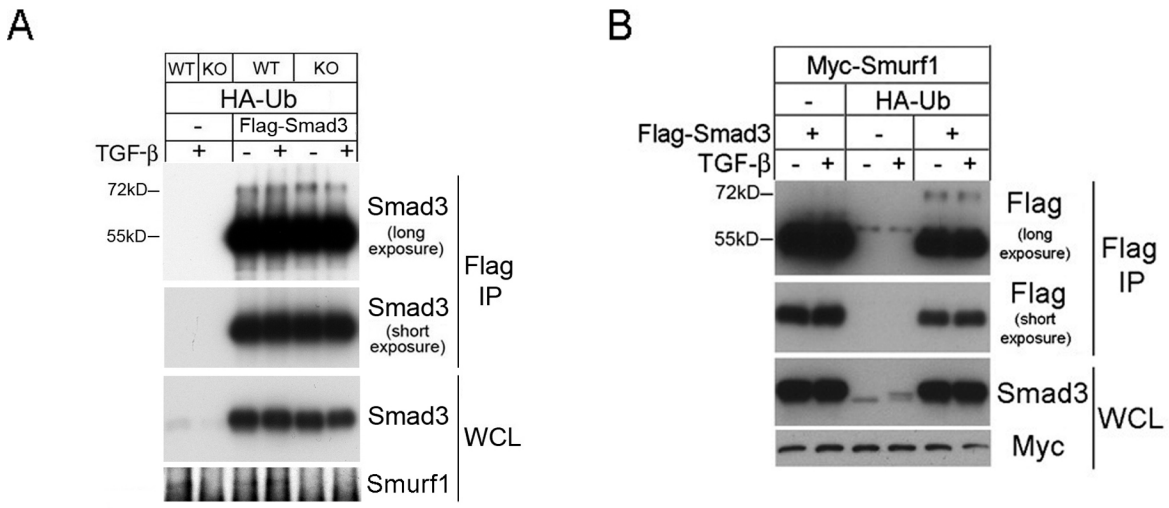

Figure S4

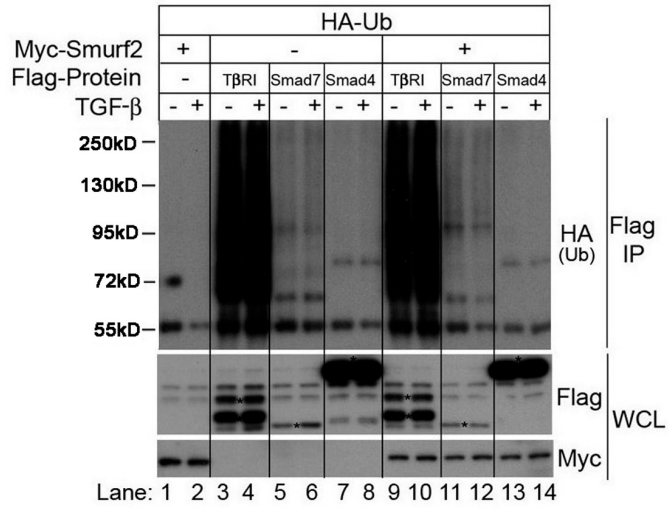

Figure S5

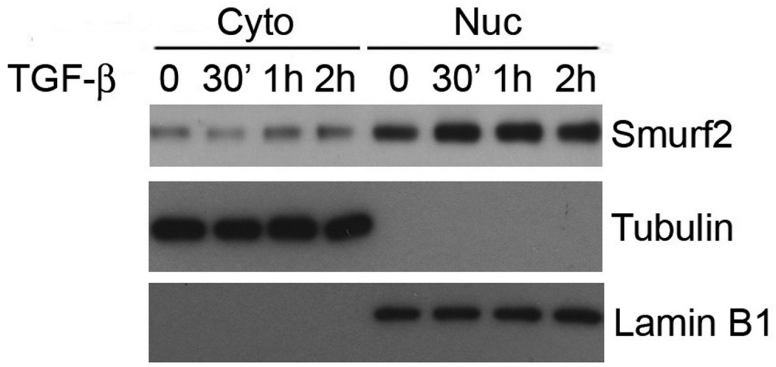

Figure S6