Supplemental Data

Ubiquitin Ligase Smurf1 Controls Osteoblast Activity and Bone Homeostasis by Targeting MEKK2 for Degradation

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Supplemental Experimental Procedures

Creation of *Smurf1*-Deficient Mice

Mouse Smurf1 genomic DNA was isolated from a BAC clone identified in the 129 mouse genomic library (Incyte Genomics, Palo Alto, CA). A 2.5 kb BamH1- and a 11 kb XhoI-digested *Smurf1* genomic DNA were cloned into plasmid *pLoxP*^{neo} (Yang et al., 1998) at 3' and 5' end of a neomycin resistant gene, respectively, to generate the targeting vector (Fig. 1A). After linearization with NotI, the targeting construct was electroporated into 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 picked and genomic DNAs from these clones were analyzed by Southern blot using a 5' external probe (shown in Fig. 1A) and BamHI digested genomic DNA. Homologous recombination in ES cells removed Exons 6, 7 and 8, and forced Exon 5 to be spliced directly to Exon 9 (Fig. 1A-D). 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 target inactivated *Smurf1* allele. All mice were maintained and handled according to protocols approved by the Animal Care and Use Committee of National Cancer Institute. Routine genotyping was carried out by PCR using primers E8F (5'-gagcaaaggacaacagtgcagg-3') and E8R (5'-ggggatcctggggtcatgccatg-3') for wild type, and I5F (5'-tttgtagaggggtgtgtcgg-3') and NeoR (5'-ccagactgccttgggaaaagc-3') for mutated Smurf1 alleles.

RT-PCR Analysis

Total RNA was isolated using TRIzol (Invitrogen). For RT-PCR analysis, 2.0 μg total RNA was converted into cDNA using ThermoScript RT-PCR system (Invitrogen) and about 1/10 of cDNA was used in each PCR reaction. Real-time PCR was performed with a 45 cycle, two-step PCR program in an ABI prism 7700 using TaqMan Universal PCR master mix (ABI biosystem). Sequences of each amplifying primer pair for osteoblast specific genes and the corresponding TaqMan probe were listed in supplemental table 2. Amplicon size and reaction specificity were confirmed by agarose gel electrophoresis. RNA transcript in each sample was assayed 3 times and its expression level was normalized to that of Hprt transcript as the internal control. Each experiment was repeated using samples obtained from at least two different mice.

In Situ Hybridazation Analysis

Digoxigenin-UTP-labeled antisense probes were synthesized using T7 RNA polymerase (Roche). In situ hybridazation was performed according to standard procedures on bone sections embedded in paraffin after fixed overnight with neutral buffered 4% PFA followed by decalcification for 10 days in 0.5 M EDTA. The labeled probe was visualized with an anti-digoxygenin-AP antibody and BCIP/NBT substrate (blue/purple). Sections were counterstained with fast nuclear red.

Antibodies

The following antibodies were used in this study: rabbit anti-Smad1/5 (upstate) or mouse anti-Smad2/3 (BD Transduction Lab), rabbit anti-MEKK1 (Zymed), rabbit anti-MEKK2 or MEKK3 (Abgent), mouse anti-JNK1 (Pharmingen), mouse anti-ubiquitin (FK2) (Biomol), mouse anti-HA (HA11, Covance), horse-radish-peroxidase conjugated anti-HA (3F10, Roche), anti-FLAG POD and anti-FLAG antibody conjugated agarose (Sigma). Rabbit

anti-BMPRIA, anti-BMPRIB, anti-TAK1, anti-Smurf1(H60), goat anti-p38 MAPK, anti-Runx2/CBFA1, and mouse anti-Myc (9E10) were from SantaCruz Biotech. Rabbit anti-phospho-JNK, anti-phospho-c-Jun, and anti-phospho-p38 were from Cell Signaling Technology. Rabbit anti-phospho-Smad1/5 and anti-phospho-Smad2 antibodies were provided by Drs. C-H. Heldin and P. ten Dijke (Faure et al., 2000).

Plasmids

Myc-tagged Smurf2 and Smurf2(DN) (containing a point mutation C716G, Zhang et al., 2001), 6xMyc-tagged Smurf1(DN) (containing a point mutation C710A, Ebisawa et al., 2001), FLAG-tagged Runx2/CBFA1 (Alliston et al., 2001), HA-tagged MEKK2, MEKK2(KM) and MEKK2(CT) (Cheng et al., 2000; Su et al., 2001), HA-tagged JNKK2-JNK1 (Zheng et al., 1999) were each described in the reference given. Myc-tagged Smurf1 and Smurf1 (ΔHECT, a truncation containing amino acids 1-351) were constructed via subcloning the corresponding human Smurf1 cDNA into mammalian expression vector pRK5.

Yeast Two-Hybrid Interaction Assay

The LexA based yeast two-hybrid system was utilized as described (Gyuris et al., 1993). Bait plasmid containing MEKK2, MEKK2 (ΔPY) (deleting amino acid residue 165-169) and Smad7 in pEG202 and prey plasmids containing different fragment of Smurf1 in pJG4-5 were generated by PCR and/or subcloning. PEG202-Smad1 plasmid was described before (Zhang et al. 2001). EGY48/pSH18-4 yeast transformants were selected on $Ura^-His^-Trp^-$ plates and protein-protein interactions were determined by scoring for β-galactosidase activity (Wu et al., 1997).

Supplemental References

Deng, C.X., Wynshaw-Boris, A., Zhou, F., Kuo, A., and Leder, P. (1996). Fibroblast growth factor receptor 3 is a negative regulator of bone growth. Cell *84*, 911-921.

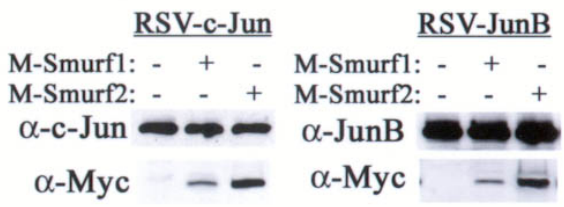
Faure, S., Lee, M.A., Keller, T., ten Dijke, P., and Whitman, M. (2000). Endogenous patterns of TGFβ superfamily signaling during early Xenopus development. Development *127*, 2917-2931.

Gyuris, J., Golemis, E., Chertkov, H., and Brent, R. (1993). Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. Cell, *75*, 791-803.

Wu, R-Y., Zhang, Y., Feng, X.-H., Derynck R. (1997). Heteromeric and homomeric interactions correlate with signaling activity and functional cooperativity of Smad3 and Smad4/DPC4. Mol. Cell. Biol. *17*, 2521-2528.

Yang, X., Li, C., Xu, X., and Deng, C. (1998). The tumor suppressor SMAD4/DPC4 is essential for epiblast proliferation and mesoderm induction in mice. Proc. Natl. Acad. Sci. USA *95*, 3667-3672.

Figure S1. Smurfs Do Not Affect Stability of c-Jun and JunB

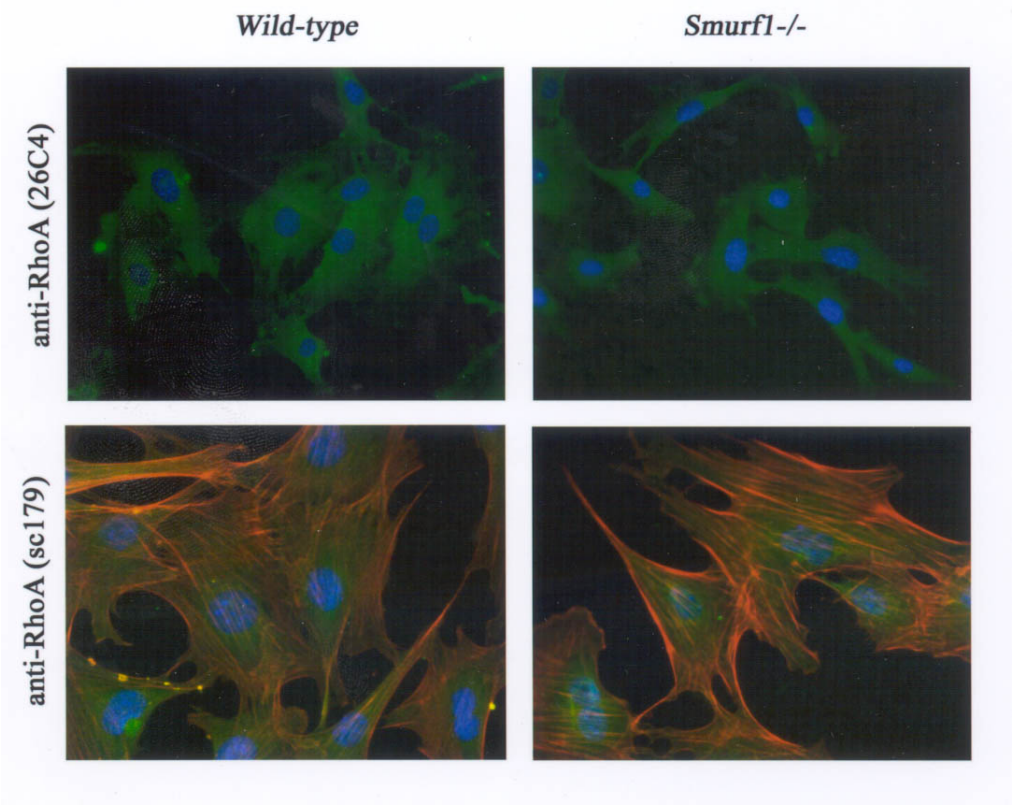


Steady-state levels of exogenous c-Jun or JunB protein in lysates from cells expressing Myc-tagged Smurf1 or Smurf2. Western blot analysis was carried out as in Fig. 6E.

Figure S2. MTT Viability Assay

Calvaria-derived osteoblasts were seeded in 24-well plates. After reaching confluency, these cells were then treated with differentiation medium in the presence or absence of JNK inhibitor SP600125 (10 μM), p38 MAPK inhibitor SB203580 (5 μM) or RhoA-associated kinase inhibitor Y27632 (10 μM) for 15 days to assess the cellular toxicity. Cell viability was determined by incubation for 4 hours with MTT labeling and electron coupling reagent (Roche). Live cells produced a formazan dye. After solubilization, the formazan dye was quantified using spectrophotometer. The absorbance directly correlates to the cell number of metabolically active cells in culture.

Figure S3. Immunofluorescence of RhoA in MEFs



Wild-type or *Smurf1*$^{-/-}$ MEFs were fixed and stained using two different anti-RhoA (SantaCruz Biotech) followed by a FITC-conjugated secondary antibody. As previously observed in fibroblasts (Yonemura et al., Experimental Cell Research 295, 300-314, 2004), RhoA distributed evenly in the cytoplasm of MEFs. Texas Red conjugated phalloidin was used to visualize F-actin. After staining, the cells were mounted using mounting medium containing DAPI to visualize nucleus.

Supplemental table 1. Genotype analysis of offspring from Smurf1 heterozygous intercross

Number with Genotype			
+/+	+/-	-/-	Total
48	101	49	198

Supplemental table 2. Primer and TaqMan probe sequences used for RT-PCR

Gene name (GenBank Accession #)	Primer Sequence (5' to 3')	Probe sequence
Smurf1 (NM_ 029438)	E5F: CTACCAGCGTTTGGATCTAT E6R: TGTCTCGGGTCTGTAAACT E9R: TTCATGATGTGGTGAAGCCG	5'- FAM- TCCCTCAGATACTGATG CAGTTCGTGGCCA – TAMRA-3'
Smurf2 (AY685230)	F: ATGAGCAGGACACACTTACA R: CACCAGTCTGAGTATGTAAGA	5'- FAM- CTCCAGACCTACCGGAA GGCTATGAACAAAG – TAMRA-3'
Runx2 (NM_009820)	F: AGTAGCCAGGTTCAACGAT R: GGAGGATTTGTGAAGACTGTT	5'- FAM- AGCGGACGAGGCAAGA GTTTCACCTTGAC – TAMRA-3'
Osteocalcin (NM_007541, NM_031368)	F: TGAGTCTGACAAAGCCTTCA R: AAGCAGGGTTAAGCTCACA	5'- FAM-CAATAAGGTAGTGAACA GAC TCCGGCGCTAC - TAMRA
Type I Collagen (α1) (NM_007742)	F: GGTATGCTTGATCTGTATCTG R: TCTTCTGAGTTTGGTGATACG	5'- FAM- CGTGCAATGCAATGAAG AACTGGACTGTCCC - TAMRA
Type I Collagen (α2) (NM_007743)	F: AGACTTGACTGTTGCCAAGA R: CTCTTTGTGAGCTTCACCAA	5'- FAM – CCCTTAAAACTAGACT GTTAGCCCGGATGCC – TAMRA
Bone Sialo Protein (NM_008318)	F: GGCCACGCTACTTTCTTTAT R: GAACTATCGCAGTCTCCATT	5'- FAM – TTTCCAGTCCAGGGAG GCAGTGACTCTTCA – TAMRA
Hprt (NM_013556)	F: TATGGACAGGACTGAAAGAC R: TAATCCAGCAGGTCAGCAAA	5'- VIC- CCATCACATTGTGGCCC TCTGTGTGCTCAA – TAMRA

Note: For Smurf1 gene expression, primer pair E5F and E6R was used for real-time RT-PCR while primer pair E5F and E9R was used for static RT-PCR. F: forward; R: reverse.