Supplemental Information

Inventory of Supplemental Information

Supplemental information includes three figures, Supplemental Experimental Procedures, and Supplemental References.

Figure S1 provides additional data to support neogenin regulation of chondrogenesis during skeleton development. This is an extension of Figure 1 and is described in page 4.

Figure S2 demonstrates a requirement of neogenin in Smad1/5/8 phosphorylation in vivo, an extension of Figure 4 (see page 9).

Figure S3 is related to Figure 6, providing a control evidence for an un-changed BMP receptor expression in neogenin mutant chondrocytes (see page 10).

Supplemental Figure Legends

Figure S1. Impaired embryonic digit development in neogenin mutant mice. (A) Western blot analysis of neogenin expression in indicated tissue lysates from *neogenin*$^{+/+}$ and $^{m/m}$ mice at age of postnatal day 13 (P13). **(B)** Images of *neogenin*$^{+/+}$, $^{+/m}$, and $^{m/m}$ mice taken at postnatal day 25 (P25). **(C)** Body weight in *neogenin*$^{+/+}$, $^{+/-}$, and $^{m/m}$ mice measured at the indicated ages. **(D)** Defects of digit and sternal calcification in neogenin mutant embryos. *Neogenin*$^{+/+}$ and $^{m/m}$ embryos (~E14.5) were stained with alizarin red and alcian blue. Mineralized bones were stained as red, and non-mineralized bones/chondrocyte matrix were stained as blue. Arrows indicate the phalanges and sternum structures where mutant embryos showed a defect.

Figure S2. Reduced Smad1/5/8 phosphorylation in neogenin mutant growth plate. (A) Immunostaining analysis of p-Smad1/5/8 in the radius of wild type (+/+) and neogenin mutant (m/m) mice at P1. Bar, 120 μm. **(B)** Higher power magnification of p-Smad1/5/8 staining at each zone of growth plates. Bar, 10 μm.

Figure S3. BMP receptor expression in wild type and neognein mutant chondrocytes. (A) Real time PCR analysis of BMP receptor (IA, IB, and II) expression in wild type and neogenin mutant chondrocytes. The results were normalized by GAPDH as internal control, and presented as means plus/minus SD. **(B)** Western blot analysis of BMP receptor expression in chondrocytes

from the wild type and neogenin mutant mice. **(C)** Immunostaining analysis of BMP receptor (IA and II) distribution in wild type and neogenin mutant chondrocytes. Bar, 50 μm.

Supplemental Experimental Procedures.

Materials

The following antibodies, neogenin (rabbit polyclonal), BMPR1A and BMPR2 (goat polyclonal), caveolin-1 (monoclonal) were obtained from Santa Cruz Biotechnology Inc.(Santa Cruz, CA). The phospho-Smad1/5/8, Smad1, phospho-Smad2, Smad2, phosphorylated-ERK1/2 (p-MAPK), ERK1/2, phospho-Stat1 and Stat1 were purchased from Cell Signaling Inc. The anti-BMPR1A and BMPR2 antibodies were also purchased from Orbigen. In addition, we have generated rabbit anti-neogenin antibody using the GST-C-terminal fusion protein as described previously (Xie et al., 2005), and rabbit anti-RGMc antibody using the peptide of CDYEARFSRLHGRAPG as the antigen. Antibodies against actin, Flag (M2), and secondary antibodies (anti-rabbit IgG-HRP, anti-goat IgG-HRP, and anti-mouse IgG-HRP) were purchased from Sigma. The following reagents, FGF, TGF-β, Methyl-β-cyclodextrin (MCD), were from Sigma (St. Louis, MO). rhBMP2 was obtained from R&D Systems (Minneapolis, MN, USA).

Expression plasmids

The plasmid encoding neogenin has been described previously (Ren et al., 2004). The plasmid encoding RGMc was generated by subcloning its cDNA into the mammalian expression vector of pcDNA3-signal-Flag. HA-ALK3, HA-ALK6, and 9xSBE-Luc plasmids were kindly provided by Drs. X. Cao (Johns Hopkins Medical School) and X-M. Shi (Medical College of Georgia).

X-Gal staining analysis

Dissected limbs were fixed in 4% paraformaldehyde (PFA) on ice for 45 minutes. Rinsed with cold PBS three times for 5 minutes each, tissues were subject to 15% sucrose for 1 hour and 30% sucrose for 3 hours to overnight. Tissues were embedded in OCT for cryosecting. Cryosections were re-fixed with 4% PFA. Following rinsing with PBS, sections were stained with X-gal staining solution (1 mg/ml X-gal in dimethylformamide, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM MgCl2, 0.02% Nonidet P-40 (NP-40) and 0.01% Na

deoxycholate) for 10 min to 6 hrs. Sections were then rinsed and counterstained in Nuclear Fast Red and mounted for image analysis.

Transfection and luciferase assays

The primary cultured chondrocytes were transiently transfected indicated plasmids using Lipofectamine 2000 (Invitrogen) per manufacturer's instruction. 24 hours after transfection, cells were washed and treated with BMP2 for additional 24 hours. Cells were washed with PBS and lysed in lysis buffer (Promega) for 20 min at room temperature. 50 μl of lysate were used to determine relative luciferase activity (firefly luciferase activity divided by Renilla luciferase activity) using a dual luciferase assay system (Promega). The data of luciferase activity were represented as an average of four independent cell isolations, each performed in triplicate.

Immunofluorescence confocal microscopy

Bone sections and culture cells on glass were fixed in 4% paraformaldehyde (PFA), and permeablized with 0.1% Triton X-100 for 15 minutes at room temperature, and immunostained as described previously (Zhou et al., 2008; Zhou et al., 2006). The sections or cells were incubated with 10% BSA in PBS for 1 h at room temperature to block background staining and then incubated with indicated primary antibodies. After washing three times, the samples were incubated with fluorescence labeled secondary antibodies at room temperature for 1-2 hours. After washing with PBS for three times, sections or cover glasses were mounted. Confocal microscopy was performed with a Zeiss (Thornwood, NY) confocal microscope.

Quantitative real time RT-PCR analysis

Total RNA was prepared from cultured cells derived from wild type and neogenin$^{m/m}$ mice using Trizol (Invitrogen). cDNAs were synthesized from 2 μg of total RNA using SuperScript III First Strand Synthesis System (Invitrogen) in a volume of 20 μl. The reaction mixture was adjusted to 100 μl with distilled water. Quantitative PCR was performed to measure the relative expression levels of the interest, using the Platinum SYBR Green Kit (Bio-Rad). Samples were normalized to GAPDH expression.

Supplemental References

Ren, X.R., Ming, G.L., Xie, Y., Hong, Y., Sun, D.M., Zhao, Z.Q., Feng, Z., Wang, Q., Shim, S., Chen, Z.F., *et al.* (2004). Focal adhesion kinase in netrin-1 signaling. Nature neuroscience *7*, 1204-1212.

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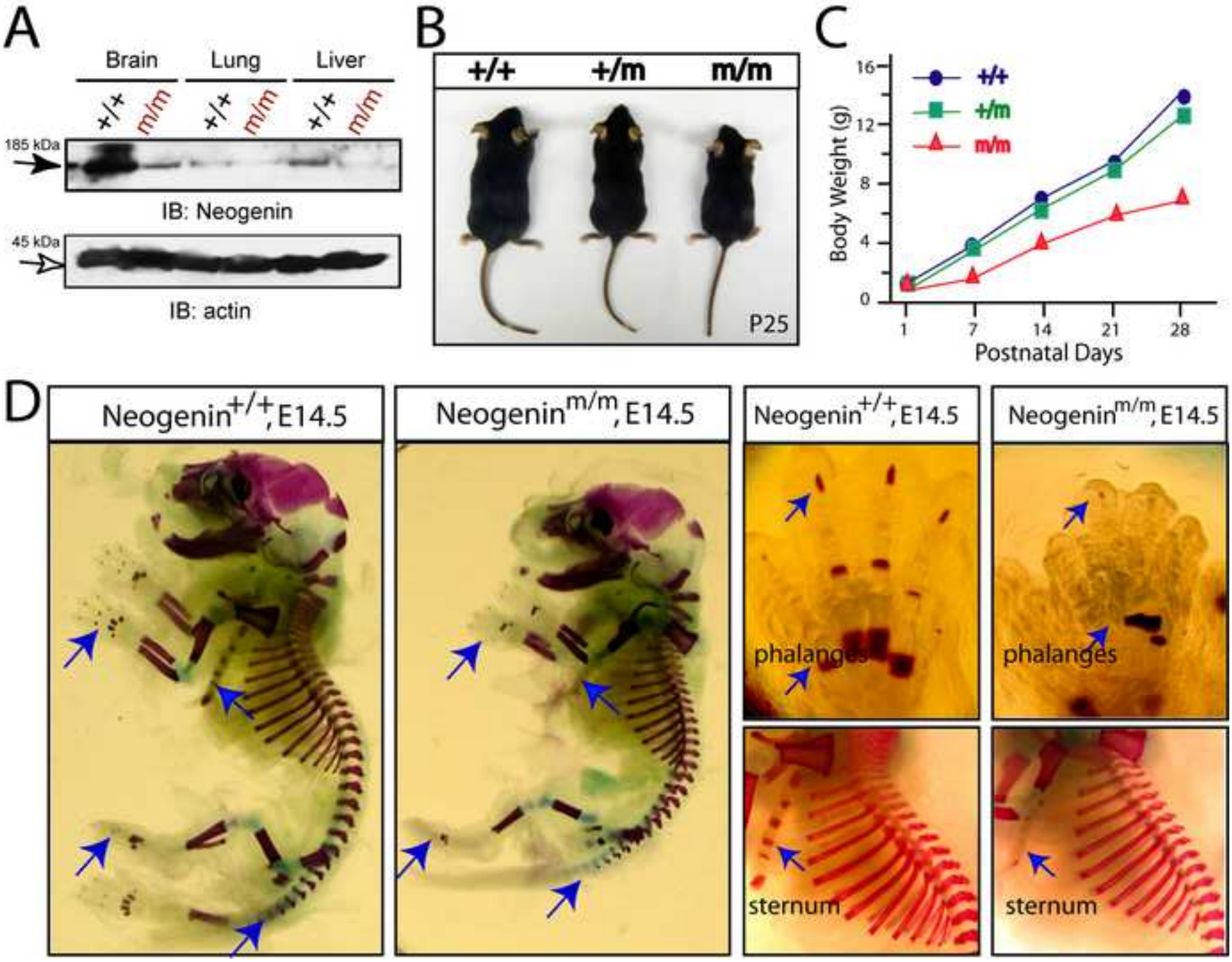
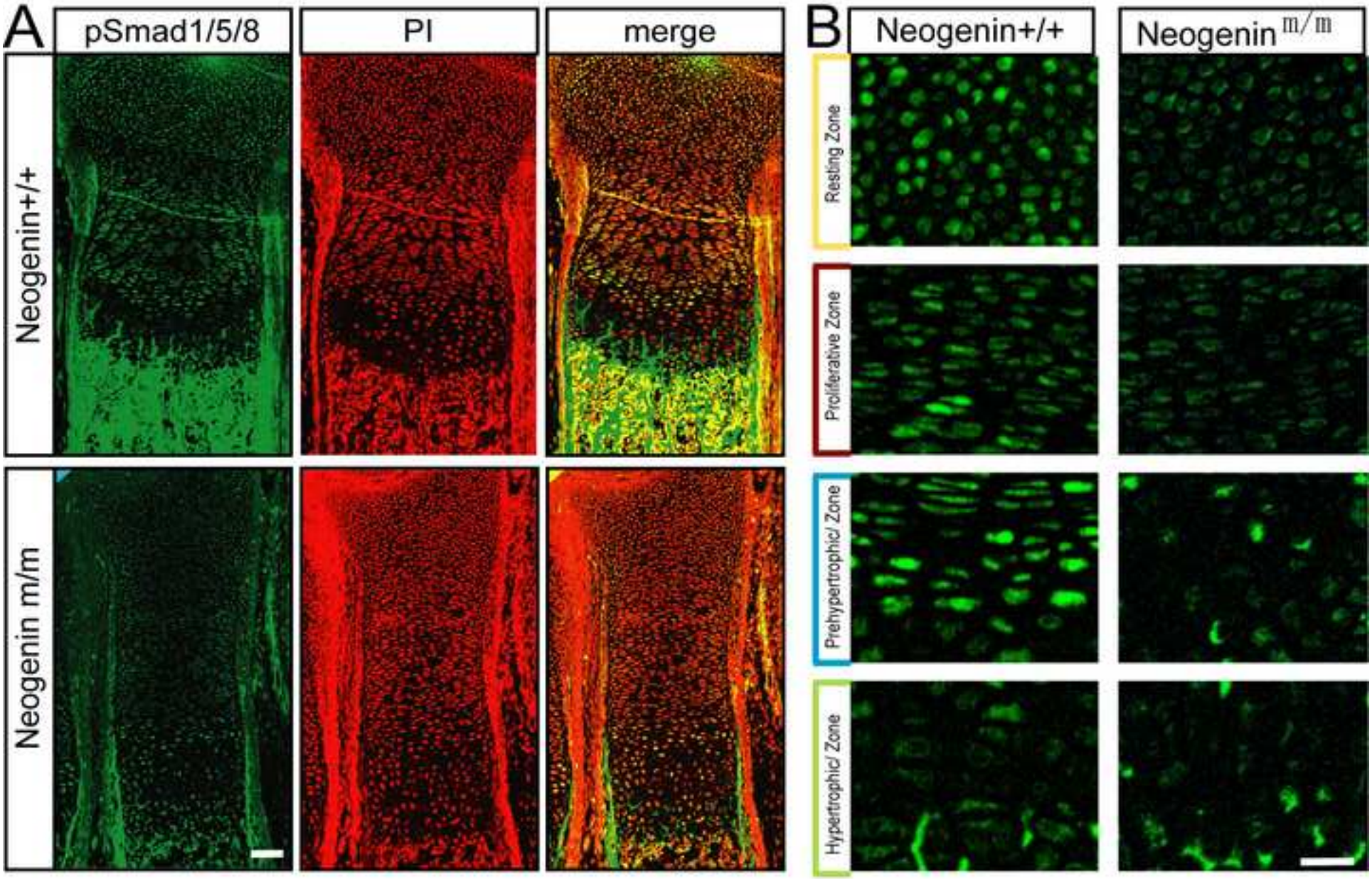
Zhou, Z., Immel, D., Xi, C.X., Bierhaus, A., Feng, X., Mei, L., Nawroth, P., Stern, D.M., and Xiong, W.C. (2006). Regulation of osteoclast function and bone mass by RAGE. The Journal of experimental medicine *203*, 1067-1080.

Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3