Supplementary Information

Figure S1. Characterization of TAK1 expression.

(A) Sections through the cortical bone of Tak1<sup>osx</sup> and control mice showing TAK1 expression in osteoblasts.

(B) Sections through the chondroepiphysis of Tak1<sup>osx</sup> and control mice showing TAK1 expression in hypertrophic chondrocytes.

(C) Kinetics of TAK1 expression in human mesenchymal stem cells under osteoblast differentiating conditions.

Figure S2. Characterization of Tak1<sup>osx</sup> mice

(A) Alizarin red-stained skulls of Tak1<sup>osx</sup> and a littermate control demonstrating reduced calvarial mineralization in p20 mice.

(B) X-rays of Tak1<sup>fl/fl</sup>, Tak1<sup>+/osx</sup>, and Tak1<sup>osx</sup> mice at p20.

(C) Hematoxlin and eosin stained coronal section of the tibia in 3 week old Tak1<sup>osx</sup> and control mice. Tak1<sup>osx</sup> mice display osteopenia and a delay in the formation of the secondary center of ossification.

(D) In situ hybridization for the indicated probes from Tak1<sup>β<sub>β</sub></sup> and Tak1<sup>osx</sup> mice. Images are high magnification versions of Figure 2B. The signal is viewed as black over a hematoxlin and eosin-stained background. Original magnification 100X. Osx was visualized by darkfield microscopy.

Figure S3. Analysis of osteoclast differentiation and activity in Tak1<sup>osx</sup> mice.
(A) Tartrate Resistant Acid Phosphatase (TRAP) staining of trabecular bone below the growth plate of the tibia in 3 week old Tak1<sup>osx</sup> and Tak1<sup>+/osx</sup> mice. TRAP-positive osteoclasts stain a magenta color.

(B,C) Calvaria and tibias were isolated from female Tak1<sup>fl/fl</sup> and Tak1<sup>osx</sup> mice, RNA-extracted, and RNA levels of Rank ligand (Rankl) and Opg genes were analyzed by quantitative PCR. Values are mean + SD.

(D) Fasting serum collagen I C-terminal telopeptide (CTX) levels, a reflection of osteoclast activity in vivo, were determined from 3 week old female Tak1<sup>osx</sup> mice by quantitative ELISA. *, p=.001; **, p=.007, both by an unpaired Student’s t-test.

**Figure S4.** Control infection of wt CalvOb with Lentiviral cre.

(A) Primary WT CalvOb were infected with vector or cre lentivirus, cultured under differentiation conditions, and incubated with Alamar Blue solution. Cell viability was analyzed by colorometric assay. Values are mean + standard deviation (SD).

(B) WT CalvOb infected by vector or cre lentivirus were cultured for 6 days under differentiation conditions and ALP activity was analyzed by colorometric assay. Values are mean + SD.

(C) RNA levels of the indicated genes were analyzed by quantitative PCR on WT CalvOb infected by vector or cre lentivirus. Values are mean + SD.

(D) WT CalvOb infected by vector or cre lentivirus were serum starved for 12 hours before BMP2/7 stimulation for the indicated times, and then immunoblotted with antibodies specific to phospho-SMAD1/5/8 and phospho-p38. Immunoblotting
analysis with antibodies specific to GAPDH and TAK1 was performed as a control.

**Figure S5.** Various signaling pathways in TAK1-deficient osteoblasts.

(A) Quantitative PCR analysis for BMP-responsive gene induction. *Tak1^fl/fl* CalvOb infected by vector or cre lentivirus were treated with or without BMP2/7 for 6 hours and total RNA was extracted for quantitative PCR analysis. Values are mean + SD.

(B) Primary *Tak1^fl/fl* CalvOb (upper) and immortalized *Tak1^fl/fl* CalvOb (lower) were infected by vector or cre lentivirus. 2 day after transduction, cells were transfected with 3TP-lux and *Renilla* luciferase vectors, cultured under differentiation condition, and then serum starved for 12 hours before treatment with TGFβ. Results are expressed as relative luciferase activity normalized by *Renilla* control. Values are mean + SD.

(C) *Tak1^fl/fl* CalvOb infected by vector or cre lentivirus were serum starved for 12 hours before TGFβ stimulation at different timepoints, and then immunoblotted with the indicated antibodies. Immunoblotting with antibodies specific to GAPDH or HSP90 was performed as a control.

(D) *Tak1^fl/fl* CalvOb infected by vector or cre lentivirus were transfected with Top flash-luc and *Renilla* luciferase vectors together with vector or xWNT8/Fz5 fusion protein, and cultured under differentiation condition. Results are expressed as relative luciferase activity normalized to *Renilla* activity. Values are mean + SD.
(E) Immunohistochemistry for β-catenin showing equivalent expression and localization in a calvarial osteogenic front along the sagittal suture in Tak1^osx and Tak1^fl/fl control mice.

(F) RNA levels of Sprouty2 and Dusp6 were analyzed by quantitative PCR on Tak1^fl/fl CalvOb infected by vector or cre lentivirus. Values are mean + SD (left). Alternatively, cells were serum starved for 12 hours before treatment with FGF2, and immunoblotted with anti-phospho-ERK1/2 antibody (right). Immunoblotting with antibodies specific to HSP90 and TAK1 was performed as a control.

**Figure S6.** Expression of MKK3, MKK6, and p38 isoforms.

(A) Quantitative PCR analysis for the indicated gene expression in various tissues.

he; heart, kid; kidney, cor; cortex, mus; muscle, cer; cerebrum.

(B) Quantitative PCR analysis for the indicated gene expression in calvaruium and tibia from Tak1^fl/fl and Tak1^osx mice.

(C) Primary wt CalvOb were cultured under differentiation conditions and total RNAs were extracted at day 0, 10, and 20 for quantitative PCR analysis.

**Figure S7.** Characterization of Mkk3^−/−Mkk6^+/− and p38β^−/− mice.

(A, B) Hematoxlin and eosin stained coronal section of the tibias. 4 week old wild type, Mkk3^−/−Mkk6^+/−, Mkk3^−/− (A) and p38β^−/− (B) mice display osteopenia and a delay in the mineralization of the secondary center of ossification.
(C) TRAP stain of the trabecular bone below the growth plate of the tibia in 4 week old wild type, Mkk3\(^{-/-}\), Mkk6\(^{+/+}\) and Mkk3\(^{-/-}\)Mkk6\(^{+/+}\) mice. TRAP-positive osteoclasts stain a magenta color.

(D) Fasting CTX levels were determined from 5 week old female WT, Mkk3\(^{-/-}\), and Mkk3\(^{-/-}\)Mkk6\(^{+/+}\) mice by quantitative ELISA. *, p=.02 by an unpaired Student’s t-test.

(E) TRAP stain of the trabecular bone below the growth plate of the tibia in 4 week old wild type and p38\(\beta^{-/-}\) mice.

**Figure S8.** Skeletal phenotype of Mkk6\(^{-/-}\) mice.

(A) Femurs from 4-week old female Mkk6\(^{-/-}\) mice and background, age, and sex matched controls were analyzed by \(\mu\)CT.

(B) 3-dimensional reconstructions of \(\mu\)CT scans of cortical bone (top) and trabecular bone (middle) from femurs from 4 week old Mkk6\(^{-/-}\) mice. Also, skulls of p4 mice were scanned and analyzed for the degree of calvarial mineralization (bottom).

**Figure S9.** Runx2 activation by p38 MAP kinases

(A) Tak1\(^{\beta^{osx}}\) and Tak1\(^{osx}\) CalvOb were infected with Flag-tagged MKK6s (glu or K82A) expressing lentiviruses, and the expression was analyzed by immunoblotting with anti-Flag antibody.

(B) Tak1\(^{\beta^{fl}}\) CalvOb infected by vector or cre lentivirus were serum starved for 12 hours before BMP2/7 stimulation, and then immunoblotted with anti-phospho-
p38 antibody. Myc-Runx2 expression was performed by immunoblotting with anti-Myc antibody.

(C) C2H10T1/2 cells were transfected with OSE2-luc and Renilla luciferase vectors together with different concentration of p38α in the absence or the presence of Runx2.

(D) HEK293 cells were transfected with Myc-Runx2, Flag-p38α, and Flag-MKK6-glu or Flag-MKK6-K82A as indicated and immunoprecipitated with anti-flag antibody. Myc-Runx2 mobility was analyzed by immunoblotting with an anti-Myc antibody.

(E) Primary Tak1fl/fl CalvOb were infected with either vector or cre lentivirus together with Myc-Runx2 expressing lentivirus and cultured under differentiation conditions. Nuclear extracts were prepared and Runx2 DNA binding activity to OSE2 DNA was analyzed by EMSA (top). As a control, free probe (FP) was run without the addition of nuclear extracts. Expression of Myc-Runx2 protein was analyzed by immunoblotting with anti-Myc antibody (bottom).

(F) HEK293 cells were transfected with HA-CBP, Myc-Runx2, MKK6-glu and p38α as indicated. Cellular lysates were then immunoprecipitated with anti-HA antibody and then immunoblotted with antibodies specific to Myc and HA to demonstrate the interaction between Myc-Runx2 and HA-CBP.

**Figure S10. Functional analysis of Runx2-3SA mutants.**

(A) The ability of recombinant p38α to phosphorylate GST-Runx2 (WT) and GST-Runx2 (3SA) was analyzed by in vitro kinase assay (lower panel). Controls
demonstrating equal Runx2 protein input by coomassie blue staining and lack of signal in the absence of recombinant p38α are provided (upper panels).

(B) HEK293 cells were transfected with HA-CBP, MKK6-glu and p38α together with Runx2-WT or Runx2-3SA as indicated. Cellular lysates were immunoprecipitated with anti-HA antibody and then immunoblotted with antibodies specific to Runx2 and HA to demonstrate the interaction between Runx2 and HA-CBP.
Supplementary Figure. 1
Supplementary Figure. 2
A

Tak1^{+/osx}

Tak1^{osx}

B

**Supplementary Figure. 3**

C

D

Serum CTX (ng/mL)

Tak1^{fl/fl}  Tak1^{osx}  Tak1^{osx}

*  **

Transcript level/HPRT

Tak1^{fl/fl}  Tak1^{osx}  Tak1^{fl/fl}  Tak1^{osx}
Cell viability

![Graph A: Cell viability comparison between vec and cre for ND and OBD conditions.]

![Graph B: ALK phos/Alamar blue comparison between vec and cre for ND and OBD conditions.]

![Graph C: Transcript level/HPRT comparison between vec and cre for Osx, Runx2, and Alp.]

![Graph D: BMP-mediated phosphorylation of SMAD1/5/8, p38, and TAK1 with GAPDH as a loading control.]

Supplementary Figure. 4
A

B

C

D

E

F

Supplementary Figure. 5
Supplementary Figure. 6
Supplementary Figure. 7
Supplementary Figure. 8

A

<table>
<thead>
<tr>
<th>Metric</th>
<th>WT</th>
<th>Mkk6⁻/⁻</th>
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<tbody>
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<td>BV/TV</td>
<td>0.16</td>
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<tr>
<td>C.Th (mm)</td>
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<td>Tb.N (mm⁻¹)</td>
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<td>4</td>
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<tr>
<td>Tb.Th (mm)</td>
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B

WT | Mkk6⁻/⁻
---|---------

Images show bone structure comparisons between WT and Mkk6⁻/⁻.
Supplementary Figure. 9
A

GST-Runx2: - WT 3SA

- p38α

Kinase assay

Coomassie blue

GST-Runx2: - WT 3SA

+ p38α

Kinase assay

Ratio to WT: 1.0 0.56

B

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<tr>
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<th>Runx2-WT</th>
<th>Runx2-3SA</th>
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<tr>
<td>Mkk6-glu:</td>
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<td>- - +</td>
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<tr>
<td>p38α:</td>
<td>- - +</td>
<td>- - +</td>
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<tr>
<td>HA-CBP:</td>
<td>- + +</td>
<td>- + +</td>
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IP: HA

Runx2

HA-CBP

Runx2

IB
**Supplementary Table S1.** Histomorphometry analysis of Tak1^{osx} mice.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Tak1^{ββ} (n=6)</th>
<th>Tak1^{osx} (n=6)</th>
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<tr>
<td>BV/TV (%)</td>
<td>7.91±1.26</td>
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<td>Tb.Th (µm)</td>
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<td>Tb.N (/mm)</td>
<td>2.58±0.22</td>
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<td>Tb.Sp (µm)</td>
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<tr>
<td>MS/BS (%)</td>
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<td>MAR (µm/day)</td>
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<td>5.09±0.69</td>
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<tr>
<td>BFR/BS (µm³/µm²/year)</td>
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<td>672±106*</td>
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<tr>
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<td>5798±905</td>
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<tr>
<td>BFR/TV (%)/year</td>
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<td>Ob.S/BS (%)</td>
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<td>O.Th (µm)</td>
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<td>ES/BS (%)</td>
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3 week old female Tak1^{osx} and Tak1^{ββ} mice were injected with calcein and 2 days later injected with daydemecycline. 1 day later mice were sacrificed and tibias processed for quantitative histomorphometry. *p<0.05 compared to Tak1^{ββ}, unpaired t test.