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Supplemental Information

p38α MAPK Regulates Lineage Commitment and OPG Synthesis of Bone Marrow Stromal Cells to Prevent Bone Loss under Physiological and Pathological Conditions

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Figure S1

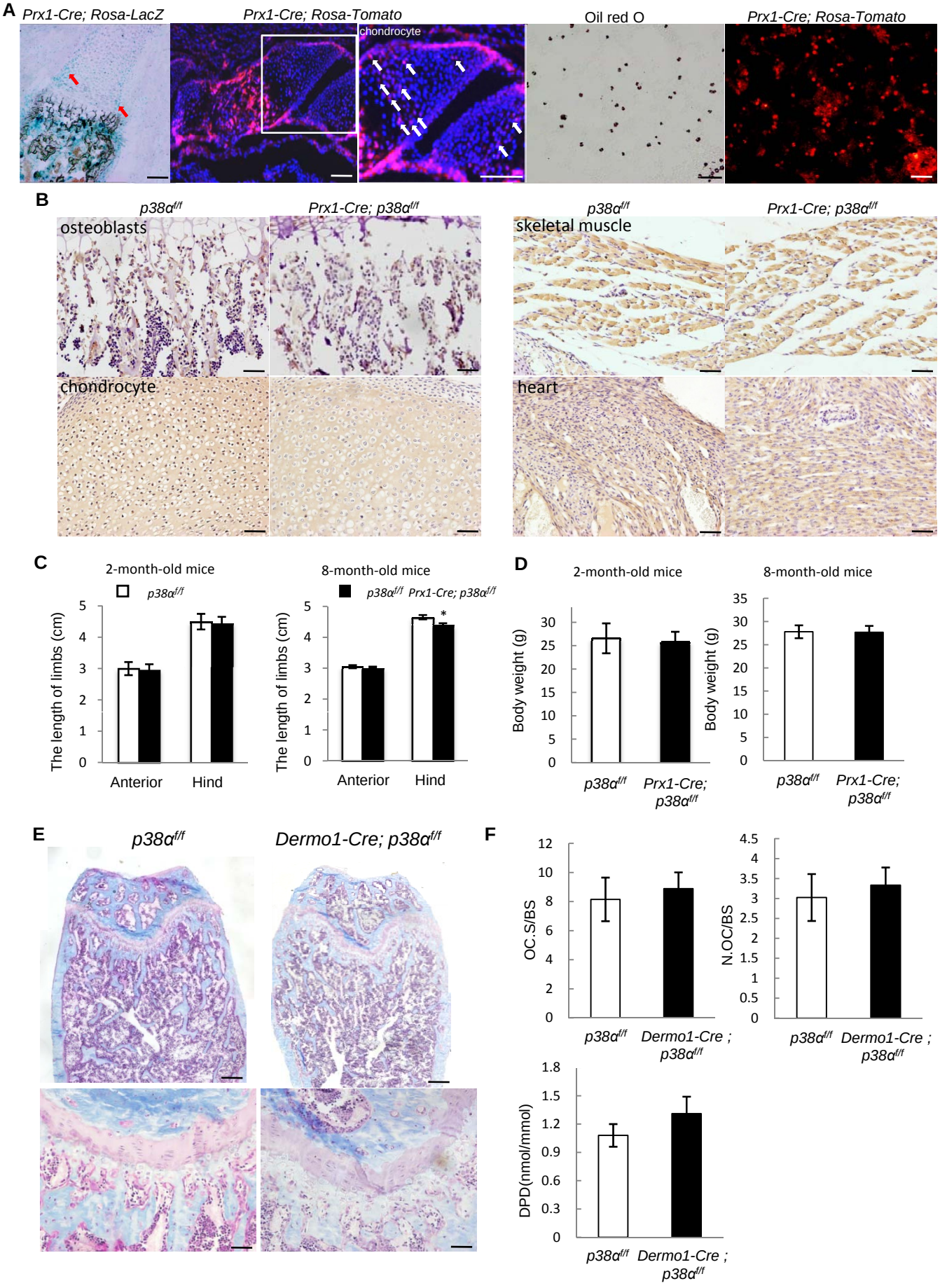

A
Prx1-Cre; Rosa-LacZ
Prx1-Cre; Rosa-Tomato
chondrocyte
Oil red O
Prx1-Cre; Rosa-Tomato
B
p38α f/f
Prx1-Cre; p38α f/f
osteoblasts
chondrocyte
skeletal muscle
heart
C
2-month-old mice
8-month-old mice
p38α f/f
Prx1-Cre; p38α f/f
The length of limbs (cm)
Anterior
Hind
D
2-month-old mice
8-month-old mice
Body weight (g)
p38α f/f
Prx1-Cre; p38α f/f
E
p38α f/f
Dermo1-Cre; p38α f/f
F
OC.S/BS
N.OC/BS
DPD(nmol/mmol)
p38α f/f
Dermo1-Cre ; p38α f/f

Figure S2

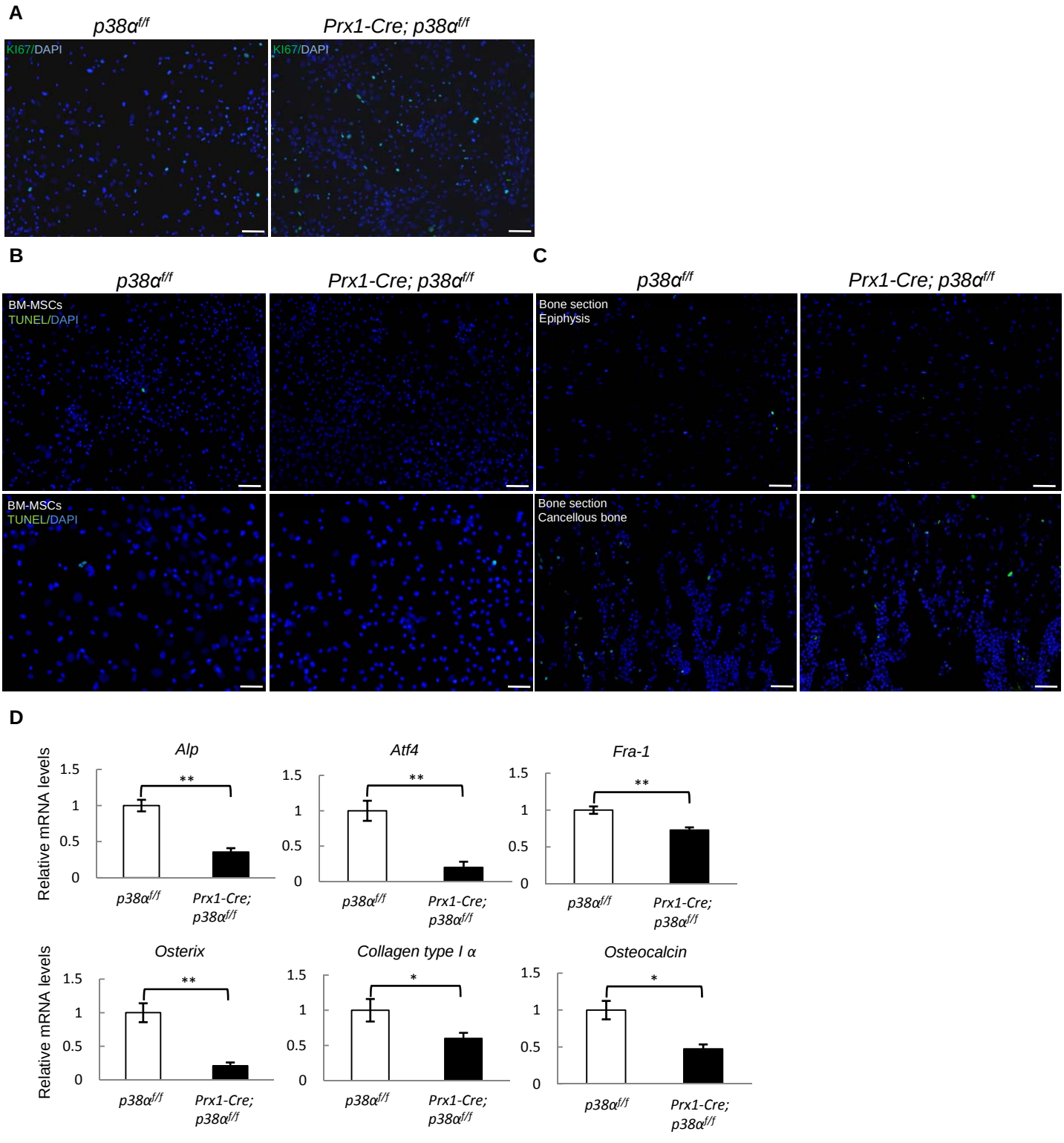

A
p38α f/f
Prx1-Cre; p38α f/f
KI67/DAPI
KI67/DAPI
B
p38α f/f
Prx1-Cre; p38α f/f
BM-MSCs
TUNEL/DAPI
BM-MSCs
TUNEL/DAPI
C
p38α f/f
Prx1-Cre; p38α f/f
Bone section
Epiphysis
Bone section
Cancellous bone
D
Alp
Atf4
Fra-1
Osterix
Collagen type I α
Osteocalcin
Relative mRNA levels
1.5
1
0.5
0
**
*
p38α f/f
Prx1-Cre; p38α f/f

Figure S3

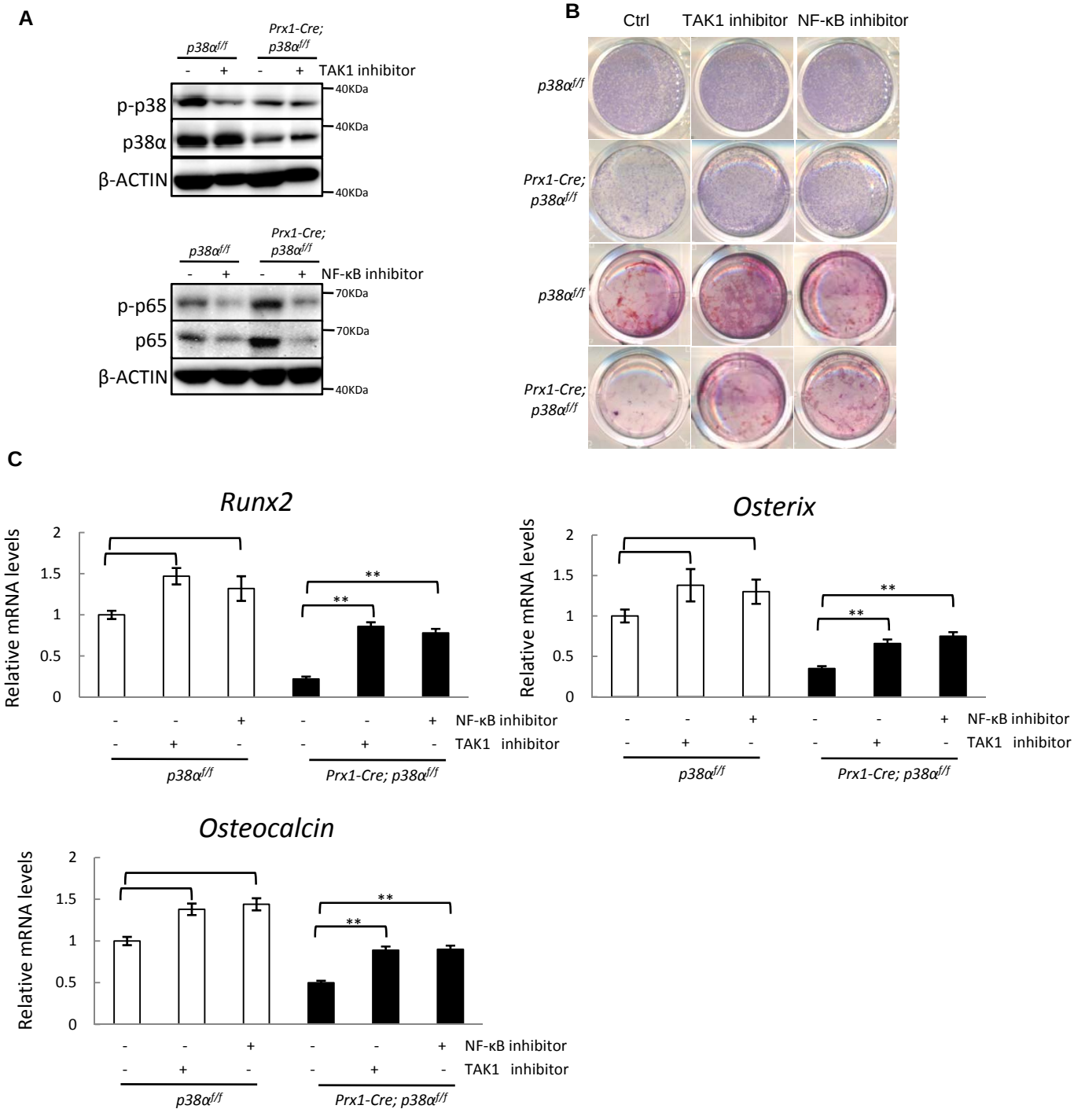

A
p38α f/f
Prx1-Cre; p38α f/f
- + - +
TAK1 inhibitor
p-p38
p38α
β-ACTIN
40KDa
40KDa
40KDa
NF-κB inhibitor
p-p65
p65
β-ACTIN
70KDa
70KDa
40KDa
B
Ctrl
TAK1 inhibitor
NF-κB inhibitor
p38α f/f
Prx1-Cre; p38α f/f
p38α f/f
Prx1-Cre; p38α f/f
C
Runx2
Osterix
Osteocalcin
Relative mRNA levels
0
0.5
1
1.5
2
**
**
- - + - - + NF-κB inhibitor
- + - - + - TAK1 inhibitor
p38α f/f
Prx1-Cre; p38α f/f

Figure S4

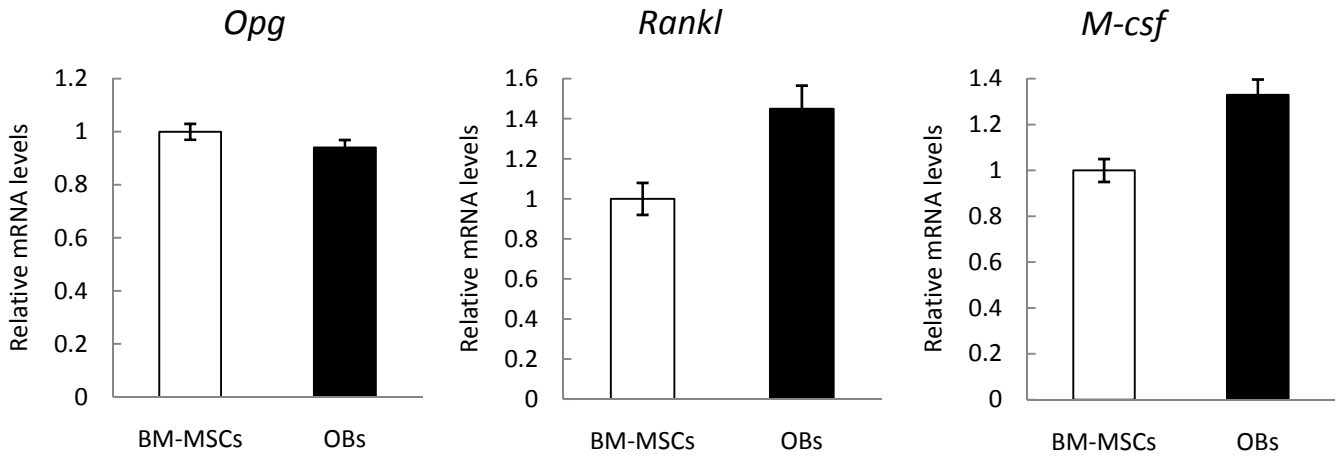
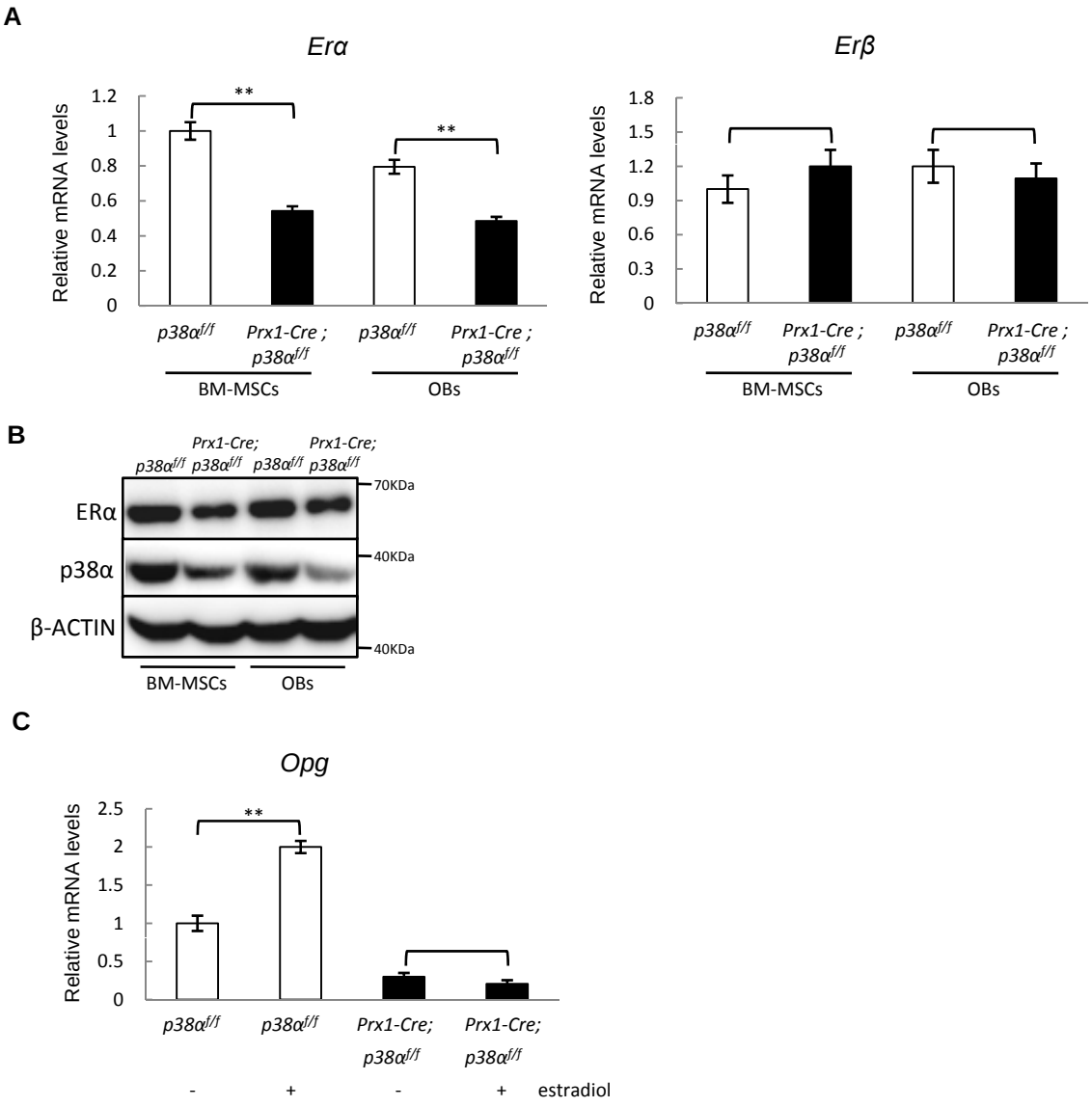

Opg
Relative mRNA levels
1.2
1
0.8
0.6
0.4
0.2
0
BM-MSCs
OBs
Rankl
Relative mRNA levels
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
BM-MSCs
OBs
M-csf
Relative mRNA levels
1.4
1.2
1
0.8
0.6
0.4
0.2
0
BM-MSCs
OBs

Figure S5


A
Erα
Erβ
Relative mRNA levels
**
**
p38α f/f
Prx1-Cre ; p38α f/f
BM-MSCs
OBs
B
Prx1-Cre; p38α f/f
ERα
p38α
β-ACTIN
70KDa
40KDa
40KDa
BM-MSCs
OBs
C
Opg
Relative mRNA levels
**
p38α f/f
Prx1-Cre; p38α f/f
estradiol

Supplemental Figure Legends

Figure S1. Ablation of *p38α* in *Prx1*+ BM-MSCs leads to shortened limb length, related to Figure 1. (A) Lineage tracing of *Prx1*+ cell in the osteoblasts, chondrocytes, and bone marrow fat in mouse. New-born *Prx1-Cre; Rosa-tdTomato* and *Rosa-LacZ* mice were used (Scale bar, 100 μm). The bone marrow smear was used to detect bone marrow adipocytes in adult *Prx1-Cre; Rosa-tdTomato* (Scale bar, 50 μm). Arrows indicate chondrocytes. (B) Immunohistochemical staining shows that p-p38 was greatly reduced in the bone but not in the heart or skeletal muscle of *Prx1-Cre; p38α$^{f/f}$* mice. Scale bar, 50 μm. (C) The hind limb was slightly shortened in 8-month-old *Prx1-Cre; p38α$^{f/f}$* mice. (D) Two or eight-month-old *Prx1-Cre; p38α$^{f/f}$* mice showed normal body weight. Data represent means ± SEM of eight independent experiments, *$p<0.05$ when the value in mutant mice was compared to that of control mice. (E) *Dermo1-Cre; p38α$^{f/f}$* mice did not show a significant defect in growth plate. Upper panel: Scale bar, 200 μm. Bottom panel: Scale bar, 50 μm. (F) *Dermo1-Cre; p38α$^{f/f}$* mice did not show a significant defect in bone resorption rate. Data represent means ± SEM of eight independent experiments.

Figure S2. BM-MSCs isolated from *Prx1-Cre; p38α$^{f/f}$* mice showed enhanced proliferation and defective osteogenic differentiation without affecting apoptosis, related to Figure 2. (A) *p38α-/-* BM-MSC cultures showed an increase in the number of KI67 positive cells. Scale bar, 100 μm. (B) *p38α-/-* BM-MSC cultures showed no alteration in TUNEL positive cells compared to WT BM-MSC cultures. (C) *Prx1-Cre; p38α$^{f/f}$* mouse femur sections showed no alteration in TUNEL positive cells compared to WT mouse. Scale bar, 100 μm. (D) Quantitative PCR results revealed that *p38α-/-* BM-MSC cultures showed a decrease in the mRNA levels of osteogenic differentiation markers. Data represent means ± SEM of three independent experiments, *$p<0.05$, **$p<0.01$.

Figure S3. Inhibition of TAK1 or NF-κB with small molecule compounds rescued the osteogenic differentiation defect of *p38α-/-* BM-MSCs, related to Figure 3. WT and

p38α-/- BM-MSCs were cultured in osteoblast differentiation medium for 4 days in the presence of TAK1 inhibitor (5Z-7-Oxozeaenol, 0.1 μM) or NF-κB inhibitor (BAY11-7082, 10μM). (A) Western blot results show that inhibition of TAK1 led to a decrease in p38 MAPK activation (upper panel) and inhibition of NF-κB led to a decrease in p65 phosphorylation (bottom panel). (B) The ALP staining results showed that inhibition of TAK1 or NF-κB with small molecule compounds rescued the osteogenic differentiation defect of *p38α-/-* BM-MSCs. (C) Quantitative PCR results confirmed that inhibition of TAK1 or NF-κB with small molecule compounds rescued the osteogenic differentiation defect of *p38α-/-* BM-MSCs. Data represent means ± SEM of three independent experiments, **p<0.01.

Figure S4. BM-MSCs and osteoblasts did not show a significant difference in expression of *Opg*, *Rankl*, or *M-csf*, related to Figure 4. BM-MSCs and differentiated osteoblasts (induced by BMP2) cultures were collected, from which total RAN was isolated. Quantitative PCR was used to determine the mRNA levels of *Opg*, *Rankl*, and *M-csf*, with *Actin* as an internal control. Data represent means ± SEM of three independent experiments.

Figure S5. *p38α-/-* BM-MSCs and osteoblasts showed decreased expression of ERα but not ERβ, related to Figure 6. (A) BM-MSCs and osteoblasts cultures were collected, from which total RNA was isolated. Quantitative PCR was used to determine the mRNA levels of *Erα* and *Erβ*. (B) Western blot showed that *p38α-/-* BM-MSCs and osteoblasts expressed decreased levels of ERα at the protein levels compared to control counterparts. (C) *p38α-/-* osteoblasts also showed a decrease in *Opg* expression in response to estradiol. Data represent means ± SEM of three independent experiments, **p<0.01.

Supplemental Table S1. Histomorphometry parameters of 3-month-old *Dermo1-Cre; p38α$^{f/f}$* and control mice, related to Table 1. Data represent means ± SEM of eight independent experiments, * p<0.05 when the value of mutant mice was compared to that of control mice.

	p38α$^{f/f}$	***Dermo1-Cre; p38α$^{f/f}$***
BV/TV(%)	15.377±1.97	11.11±3.44*
Tb.Ar(%)	13.034±1.45	9.887±2.61*
Tb.Th(mcm)	25.449±2.02	20.333±3.13*
Tb.Sp(mcm)	238.154±20.86	377.625±116.59*
Tb.N(#/mm)	4.655±1.11	3.289±0.99*
MAR(mcm/d)	1.521±0.142	1.339±0.076*
BFR(mcm/d)	70.445±4.125	60.356±3.865*
OB.S/BS(%)	15.489±0.706	13.011±1.42*

Supplemental experimental procedures

Mouse genotyping

Genomic DNA was extracted from mouse tails and used for genotyping by PCR using the following sets of primers. *p38α*$^{f/f}$ -F: 5'-TCCTACGAGCGTCGGCAAGGTG-3'; *p38α*$^{f/f}$ -R: 5'-AGTCCCCGAGAGTTCCTGCCTC-3'; *Cre*-F: 5'-TTTCCCGCAGAACCTGAAGA-3'; *Cre*-R: 5'-GGTGCTAACCAGCGTTTTCGT-3'. *Rosa-LacZ*: *Rosa26WT*-F: 5'-GGAGCGGGAGAAATGGATATG-3'; *Rosa26WT*-R: 5'-AAAGTCGCTCTGTGTTAT-3'; *Rosa26*-F: 5'-AAGCACGTTTCCGACTTGAGTTG-3'; *Rosa26*-R: 5'-CATCAAGGAAACCCTGGACTACTG-3'; *Rosa-tdTamato*: oIMR9020: 5'-AAGGGAGCTGCAGTGGAGTA-3'; oIMR9021: 5'-CCGAAAATCTGTGGGAAGTC-3'; oIMR9103: 5'-GGCATTAAAGCAGCGTATCC-3'; oIMR9105: 5'-CTGTTCCTGTACGGCATGG-3'.

Cell Transfection

Cells were plated and transfected with *Creb* siRNA (sc-35111), *Tak1* siRNA (sc-36607), *Nf-κb p65* siRNA (sc-29411), or control siRNA (sc-37007) using Lipofectamine 2000 (Invitrogen). These cells were harvested after 72-96 hours and total RNA and protein were isolated.

Quantitative PCR

Total RNA was isolated from the cells or femurs with Trizol reagent (Invitrogen). Reverse transcription was performed using Transcriptor First strand cDNA synthesis kit (Roche) with random anchored-oligo (dT) 18 primers. Real-time PCRs were performed using FS Universal SYBR Green Master Premix (Roche). Quantification was normalized to the amounts of endogenous *Gapdh*. The primers used for real-time PCR were:

Osteocalcin F: 5'-AGCAGGAGGGCAATAAGGTAGT-3'

R: 5'-ACCGTAGATGCGTTTGTAGGC-3'.

Runx2 F: 5'-TTTAGGGCGCATTCCTCATC-3'

R: 5'-TGTCCTTGTGGATTAAAAGGACTTG-3'

Osterix F: 5'-ACTCATCCCTATGGCTCGTG-3'

R: 5’-GGTAGGGAGCTGGGTTAAGG-3’

C/ebpα F: 5’-TGGACAAGAACAGCAACGAG-3’

R: 5’-AATCTCCTAGTCCTGGCTTG-3’

Pparγ F: 5’-ACTGCCTATGAGCTCTTCAC-3’

R: 5’-CAATCGGATGGTTCTTCGGA-3’

Collagen type Iα F: 5’-CAAGGTCCTTCTGGATCAAGTG-3’

R: 5’-CCTTTATGCCTCTGTCACCTTG-3’

Atf4 F: 5’-TTCCACTCCAGAGCATTCCT-3’

R: 5’-CAGGTGGGTCATAAGGTTTG-3’

Alp F: 5’-TGAGCGACACGGACAAGA-3’

R: 5’-GGCCTGGTAGTTGTTGTGAG-3’

Sox9 F: 5’-AGTCCCAGCGAACGCACATCA-3’

R: 5’-GTCGTATTGCGAGCGGGTGAT-3’

Opg F: 5’-CACCCTGTGTGAAGAGGCCT-3’

R: 5’-GCAGGCTCTCCATCAAGGCA-3’

M-csf F: 5’-CTGACACAGGCCATGTGGAG-3’

R: 5’-GAGAGGGTAGTGGTGGATGT-3’

Rankl F: 5’-GCA CAC CTC ACC ATC AAT GCT-3’

R: 5’-GGT ACC AAG AGG ACA GAG TGA CTT TA-3’

Fra-1 F: 5’-GCAGAAACCGAAGAAAGGAG-3’

R: 5’-CCGATTTCTCATCCTCCAAT-3’.

Erα F: 5’- TCCTTCTAGACCCTTCAGTGA-3’

R: 5’- ACATGTCAAAGATCTCCACCATGCC-3’.

Erβ F: 5’- AAAGCCAAGAGAAACGGTGGGCAT-3’

R: 5’- GCCAATCATGTGCACCAGTTCCTT-3’.

Gapdh F: 5’-CCACAGTCCATGCCATCAC-3’

R: 5’-CATACCAGGAAATGAGCTTGAC-3’.

Western blot analysis

The following antibodies were used: p38α MAPK (Cell Signaling, 9212), p-p38MAPK (T180/182) (Cell Signaling, 9211), p53 (c12) (Cell Signaling, 2524), TAK1 (Cell Signaling, 4505), p-TAK1(T184/187) (Cell Signaling, 4531), CREB (Upstate, 05767), p-CREB (Upstate, 6519), p-NF-κB p65 (Ser536) (Cell Signaling, 3031), NF-κB p65 (Cell Signaling, 4767), Estrogen Receptor α (Abcam, ab37438), NF-κB p50/52 (Santa Cruz, sc-8414), p21 (BD, 556430), p16 (Santa Cruz, sc-1207), and β-ACTIN (Santa Cruz, sc-81178).

Chromatin immunoprecipitation (ChIP) primer sequences

Quantitative PCR was carried out to determine the promoter fragments of *Opg* using the following gene-specific primer sets:

0—-101site F:5’-cagaggcaggcagaggcag-3’R :5’-tgtctatgtagctctgcct-3’

-101—-201site F:5’-gtaaatatttcctattagc-3’ R:5’-catttaaaatcatattaaa-3’

-201—-301site F:5’-tttttacttgctgtctcct-3’ R:5’-acattctgagacatagatt-3’

-301—-401site F:5’-catacctttggagggtag -3’ R:5’-gaagtccctaccctaactt-3’

-401—-501site F:5’-aaatttgtcacatcacatc-3’ R:5’-cttgagctagaagtgcaga-3’

-501—-601site F:5’-acaccttgcctagggaatg-3’ R:5’-cagaattggcctgtgggtc-3’

-601—-701site F:5’-tgtagataatcaatctctc-3’ R:5’-aaacatttttctcaaaatg-3’

-701—-801site F:5’-tcagctaatatcccagaca-3’ R:5’-acttaccatccaaataaac-3’

-801—-901site F:5’-gttgttatcacactgttgt-3’ R:5’-gttcactccatcaagacat-3’

-901—-1001site F:5’-tactttgaactcatgatag-3’ R:5’-tagtgagatgtctcctgag-3’

-1001—-1201siteF:5’-acccagctcctgatagaga -3’ R:5’-tctcaagtcagctgtaggt-3’

-1201—-1301siteF:5’-gttgcctatggcatcttgg-3’ R:5’-gatttgcaaaataaggttc-3’

-1301—-1401siteF:5’-tttaaacgtgccaacagca-3’ R:5’-ttgttggctccttagagtc-3’

-1401—-1501siteF:5’-ccctttatgaaagaggatg-3’ R:5’-tcagaagctagggagaacc-3’

-1501—-1601siteF:5’-caacccaggtaaatatgag-3’ R:5’-attgtcctgaaaaacgact-3’

-1601—-1701siteF: 5’-gccatccctacgcgagagg-3’R:5’-ctttctgggagaaggctga-3’

-1701—-1801siteF:5’-ggtacagtgactgagacat-3’ R:5’-gtacacttgggggagccgc-3’

-1801—-1901siteF:5’-tcagcctctcaccacagg-3’ R:5’-aagaacaaggcagcagctg-3’

-1901—-2001siteF:5’-cagctcagcggtggctttc-3’ R:5’-gcgcggaggcgtgggacaa-3’