Supplemental Figures and Tables

MLK3 regulates bone development downstream of the Faciogenital Dysplasia protein FGD1 in mice

Weiguo Zou[1,6], Matthew B. Greenblatt[1,6], Jae-Hyuck Shim[1], Shashi Kant[2], Bo Zhai[3], Sutada Lotinun[4], Nicholas Brady[1], Dorothy Zhang Hu[1], Steven P. Gygi[3], Roland Baron[4], Roger J. Davis[2], Dallas Jones[1], Laurie H. Glimcher[1,5]

[1] Department of Immunology and Infectious Diseases, Harvard School of Public Health, Department of Medicine, Harvard Medical School, and the Ragon Institute of MGH, Harvard and MIT, Boston, MA 02115, USA
[2] Howard Hughes Medical Institute and Program in Molecular Medicine, Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School, Worcester, MA 01605, USA
[3]Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA.
[4]Department of Oral Medicine Infection and Immunity, Harvard Dental School, Boston, Massachusetts, USA.

[5] Correspondence: lglimche@hsph.harvard.edu; Telephone: 617-432-0622; Fax: 617-432-1223; Address: FXB Rm 205/651 Huntington Ave/Boston, MA 02115

[6] These authors contributed equally to this work

This PDF file includes:

Supplemental Table 1 and 2

Supplemental Figures S1-S7

Supplemental Table 1. Phosphorylation sites identification by MS analysis

Phosphorylation Sites	Vector	MLK3
S28	R.RFS#PPSSSLQPGK.M	R.RFS#PPSSSLQPGK.M
S244	R.QKLDDSKPSLFSDR.L	R.QKLDDS#KPSLFSDR.L
S254	K.LDDSKPSLFSDRLSDLGR.I	K.LDDSKPSLFSDRLS#DLGR.I
S282	R.PSLNSAPSPFNPQGQSQITDPR.Q	R.PSLNSAPS#PFNPQGQSQITDPR.Q
S301	R.QAQSS#PPWSYDQSYPSYL SQM*TSPSIHSTTPLSSTR.G	R.QAQSS#PPWSYDQSYPSYL SQM*TSPSIHSTTPLSSTR.G
S301 and S319	R.QAQSS#PPWSYDQSYPSYL SQM*TSPSIHSTTPLSSTR.G	R.QAQSS#PPWSYDQSYPSYL SQM*TS#PSIHSTTPLSSTR.G
S395	R.QFPSISSLTESRFSNPR.M	R.QFPSISSLTESRFS#NPR.M
S472	Y.QFPM*VPGGDRSPSR.M	Y.QFPM*VPGGDRS#PSR.M

Supplemental Table 1. HEK293 cells were transfected with Myc-Runx2 together with a vector control or a construct encoding MLK3. Myc-Runx2 was immunoprecipitated, run on an SDS-PAGE gel, and the corresponding band was excised (Fig S3). Trypsin digestion was performed and the resulting fragments were analyzed by mass spectroscopy. Phosphorylated residues are indicated by "#".

Supplemental Table 2. Histomorphometry analysis of MLK3 KO mice.

Parameters	WT (n≥ 6)	MLK3 KO (n≥5)	*p* value
BV/TV (%)	3.60±0.67	1.78±0.28*	0.037
Tb.Th (μm)	23.92±1.15	23.74±1.58	0.982
Tb.N (/mm)	1.48±0.25	0.75±0.11*	0.030
Tb.Sp (μm)	787±143	1456±208*	0.020
MS/BS (%)	40.91±2.99	37.84±4.02	0.547
MAR (μm/day)	3.97±0.09	3.12±0.27**	0.0095
BFR/BS ($\mu m^3/\mu m^2$/year)	589±35	424±49*	0.021
BFR/BV (%/year)	4415±305	3148±212**	0.0098
BFR/TV (%/year)	187±33	72±14*	0.016
Ob.S/BS (%)	18.72±3.44	11.58±4.45	0.224
N.Ob/T.Ar (/mm^2)	47.80±8.75	14.25±3.96**	0.007
N.Ob/B.Pm (/mm)	16.74±2.89	11.11±4.52	0.303
OV/TV (%)	0.039±0.010	0.018±0.007	0.112
OS/BS (%)	5.79±1.38	4.45±1.73	0.555
O.Th (μm)	2.00±0.35	2.58±0.69	0.446
Oc.S/BS (%)	2.78±0.84	5.48±1.15	0.081
N.Oc/T.Ar (/mm^2)	2.59±0.86	2.41±0.45	0.864
N.Oc/B.Pm (/mm)	0.89±0.29	1.67±0.30	0.088
ES/BS (%)	1.68±0.54	3.09±0.87	0.182

Supplemental Table 2. 3 week old female MLK3 KO mice and wild type control mice were injected with calcein. 2 days later mice were injected with demeclocycline. 1 day later mice were sacrificed and tibias were processed for quantitative histomorphometry.

*$p<0.05$ compared to WT, unpaired *t* test

**$p<0.01$ compared to WT

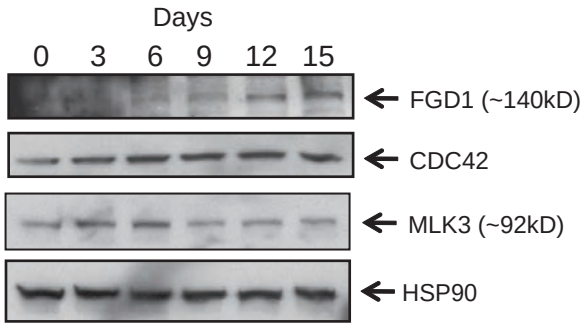

Figure S1. Kinetics of FGD1, CDC42, and MLK3 expression. Human mesenchymal stem cells were placed under osteoblast differentiation conditions for the indicated number of days and blotted for the indicated proteins.

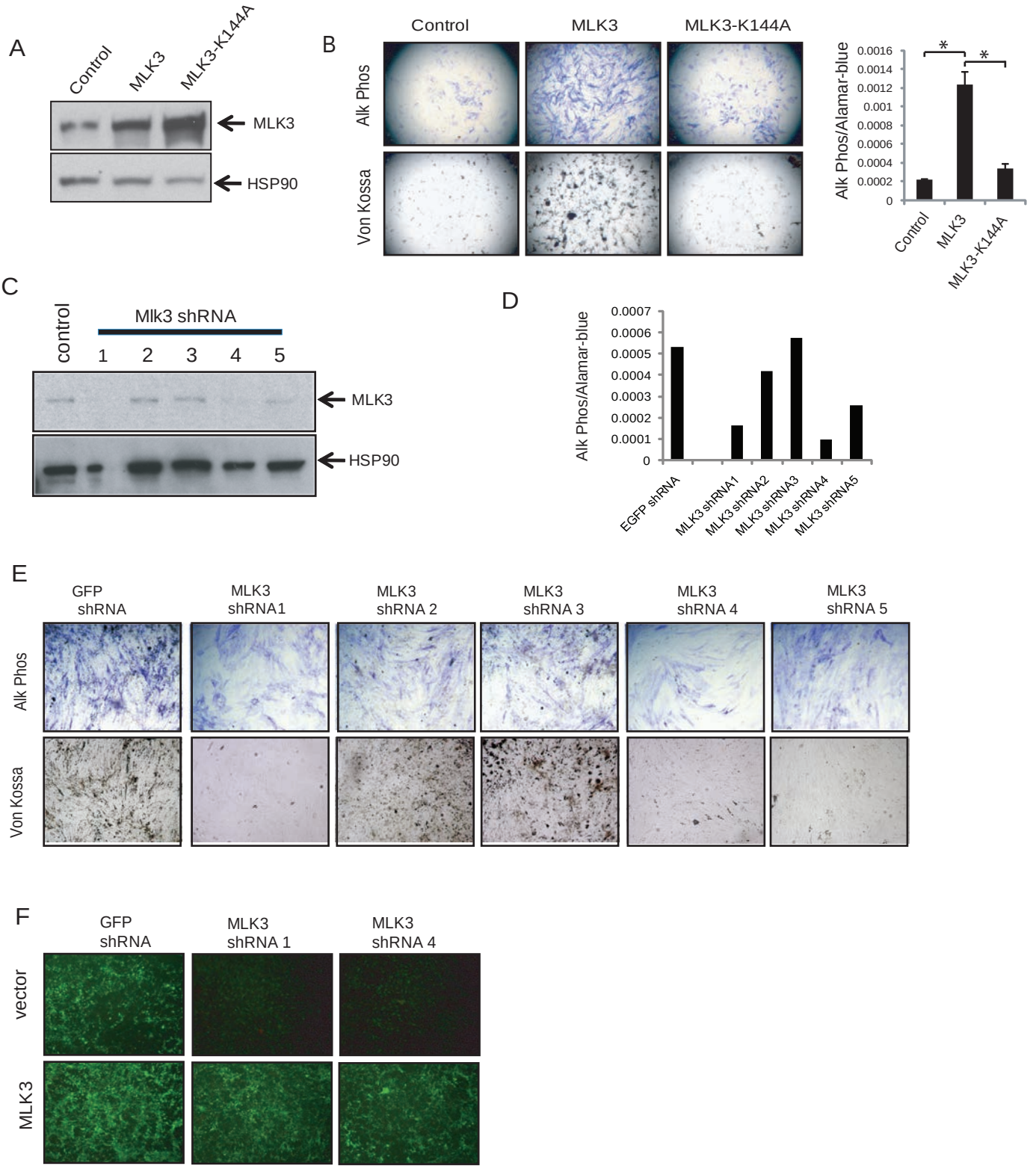

Figure S2. MLK3 drives osteoblast differentiaion in vitro

A) Expression of wild-type MLK3 and kinase-inactive MLK3 K144A from lentivirus was analyzed by Western blot. B) Osteoblast differentiation was analyzed by alkaline phosphatase activity determined by fast blue staining and phosphatase substrate assay following infection of human mesenchymal stem cells (hMSC) with wild-type MLK3 and kinase-inactive MLK3 K144A expressing lentiviruses and subsequent culture for 7 days. * represents $p<0.01$. C) Human mesenchymal stem cells were transduced with 5 different RNAi constructs targeting human MLK3. To validate these, MLK3 protein levels were determined by Western blot. HSP90 was used as a loading control. D and E) Osteoblast differentiation was analyzed by fast blue and phosphatase substrate assay for ALP activity after culture in osteoblast differentiation media for 7 days and Von Kossa staining for mineralization capacity after culture in osteoblast differentiation media for 18 days. F) Osteoblast differentiation was analyzed by calcein staining for mineralization capacity after culture in osteoblast differentiation media for 18 days.

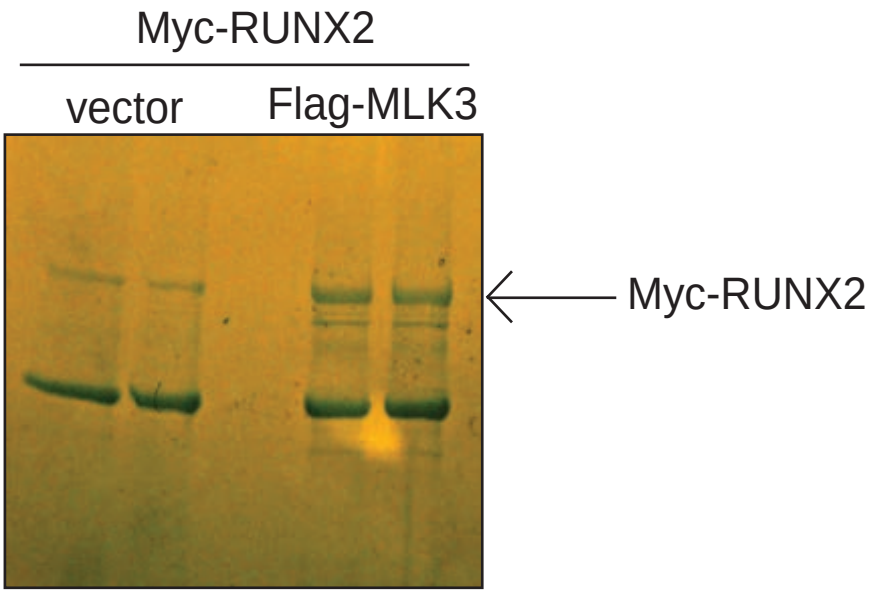

Figure S3. MLK3 is upstream of p38 in vitro.
A) A Coomassie-stained SDS-PAGE gel showing the input used for the determination of MLK3-induced Runx2 phosphorylation sites by mass spectroscopy in Figure 3D. Myc-Runx2 protein was purified from 293T cells using Myc beads from Santa Cruz.

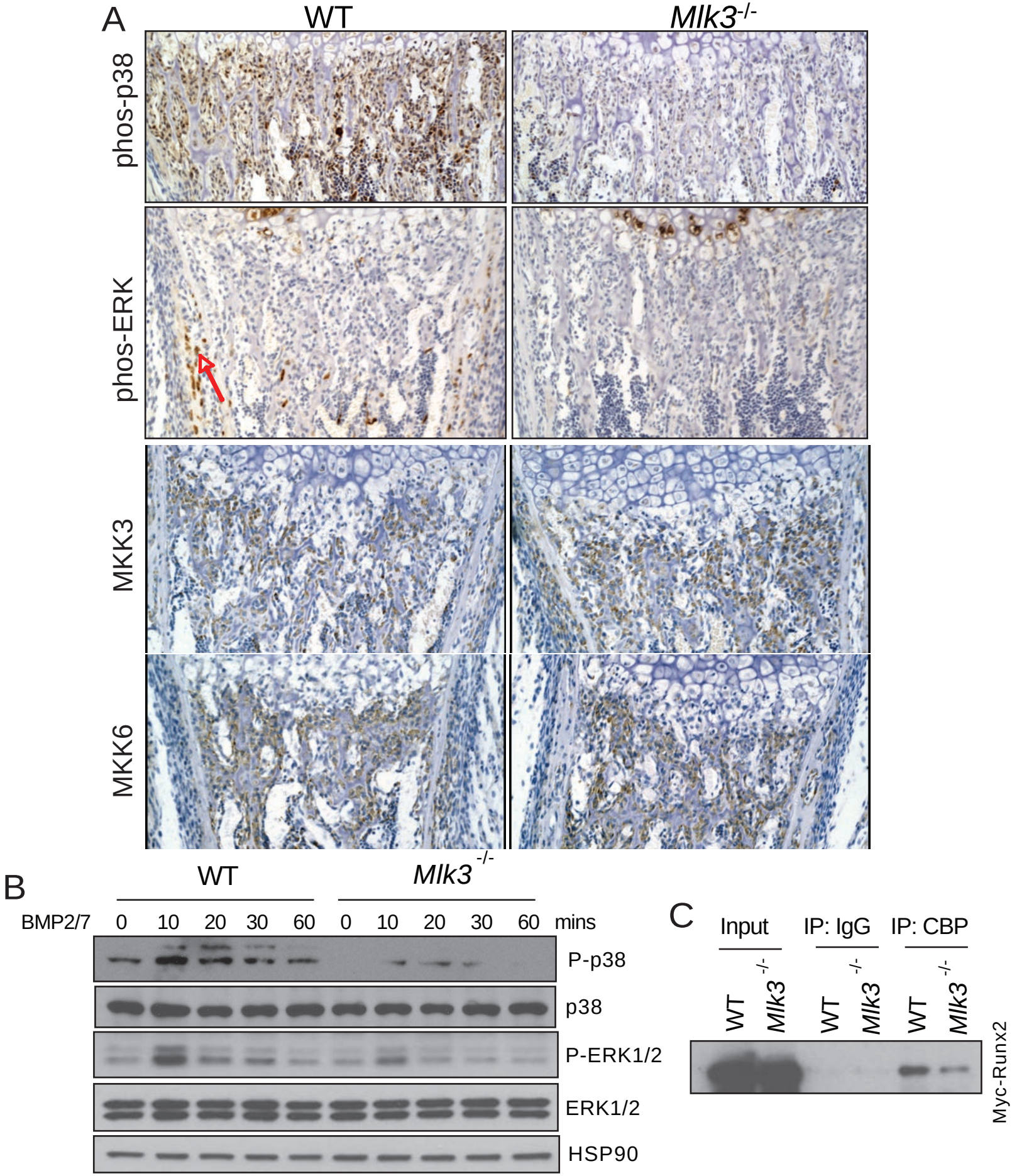

Figure S4. MLK3 is upstream of p38 and ERK MAPK in osteoblasts in vivo.
A)Immunohistochemistry was performed for the indicated proteins on sections from the proximal tibias of 5 day old Mlk3-/- and control mice. B) Primary calvarial osteoblasts from *Mlk3*-/- and WT mice were stimulated with BMP2/7 for the indicated times. Lysates were made and blotted with the indicated antibodies. C) Myc-Runx2 was expressed with a lentiviral vector in WT and Mlk3-/- calvarial osteoblasts. Lysates were made, immunoprecipitated with anti-CBP, and blotted with anti-myc-Runx2.

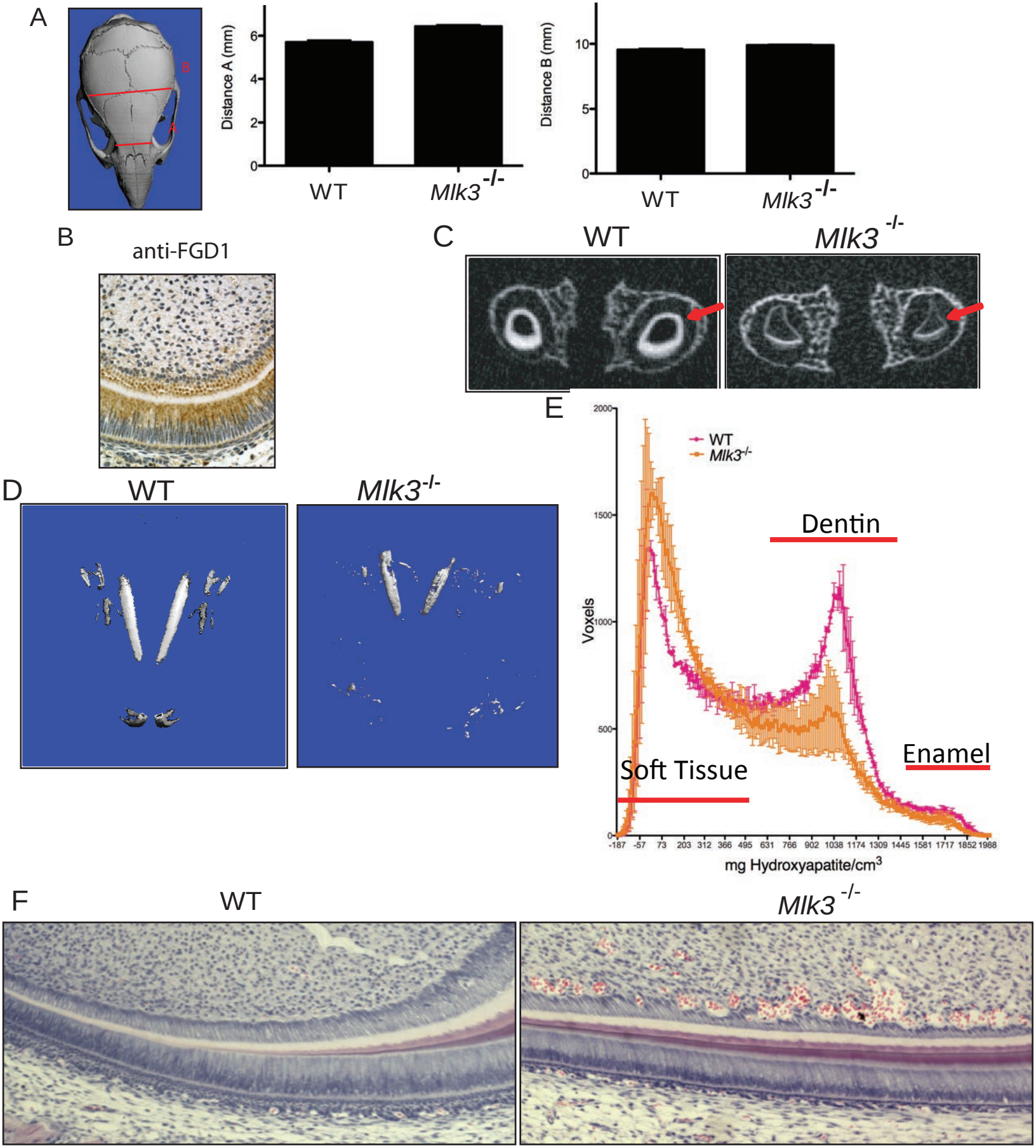

Figure S5. Analysis of the teeth of Mlk3-/- mice.

A) The distances between the anterior (distance A) and the posterior (distance B) sites of insertion of the zygomatic arch were measured. For both distance A and B, $p<0.001$. B) Immunohistochemistry for FGD1 on coronal sections of mandibular incisors from WT P3 mice. Original magnification 400X. C) 2D coronal images of the mandibular incisors of 5 day old Mlk3-/- and control mice, showing a triangular morphology and a decrease in dentin thickness in the Mlk3-/- incisors. D) 3D reconstructions of the teeth of 5 day old Mlk3-/- and control mice, thresholded to only display enamel densities. E) A histogram of denisites from contoured mandibular incisors, confirming a decrease in the volume of tooth dentin. F) Longitidunal sections of mandibular incisors of P3 mice. Note vascular invasion of the basolateral odonotlbast layer. Original magnification 100X.

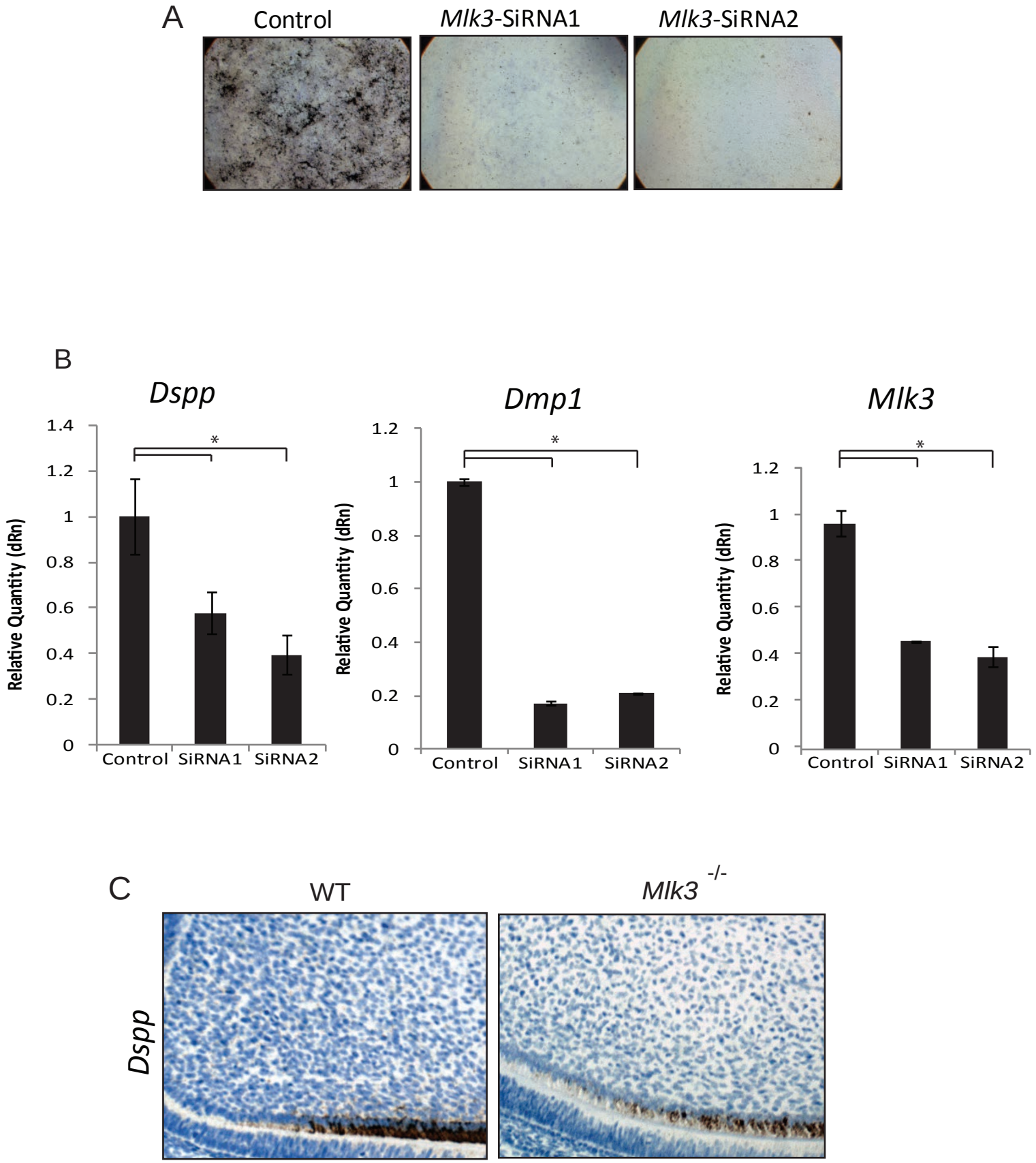

Figure S6. Analysis of gene expression and function in Mlk3-deficinet odontoblasts in vivo and in vitro.
A)The T4-4 odontoblast cell line was cultured for 10 days. Subsequent Von kossa staining demonstrates decreased mineralization after knockdown of Mlk3 with 2 independent SiRNAs (Oinigal magnficiation, 100X. B) T4-4 odontoblasts were prepared as in (A) and then analyzed for expression of the indicated genes by realtime PCR. Values shown are normalized to HPRT and then divided by the control value to display relative expression. * represents $p<0.01$.
C) In situ hybridization for *Dspp* on the mandibular incisors of P3 mice. Original magnification 100X.

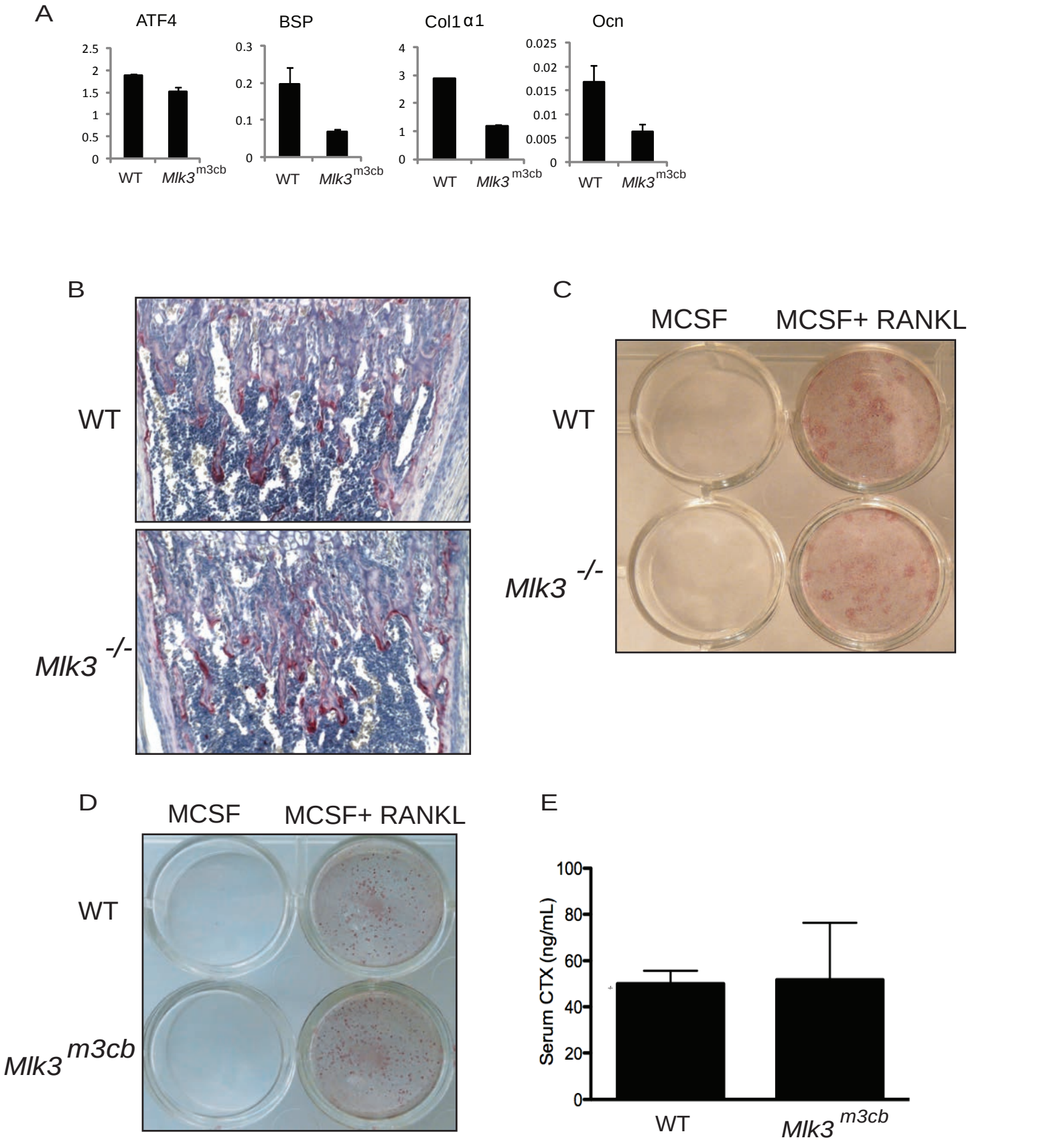

Figure S7. Gene expression in Mlk3m3cb osteoblasts, osteoclast formation and activity are MLK3-independent. A) Mlk3m3cb and control calvarial osteoblasts were isolated and culutred under differentiation conditions for 6 days. Expression of the indicated genes was analyzed by real-time PCR. For ATF4, BSP, Col1 and Ocn, $p<.005$ by Student's t-test. B)Osteoclast formation was assessed by tartrate-resistant acid phosphatase (TRAP) staining on the sections from the proximal tibias of 5 day old Mlk3-/- and control mice. C) Bone marrow cells were isolated from Mlk3-/- and control mice, and then cultured in the presence of M-CSF +/- RANKL for 5 days. Osteoclast differentiation was analyzed by TRAP staining. D) -Splenocytes were isolated from MLK3m3cb and control mice, and then cultured in the presence of M-CSF +/- RANKL for 5 days. Osteoclast differentiation was analyzed by TRAP staining. E) Serum CTX was measured by ELISA. Serum was collected from 4 week old Mlk3m3cb and control mice. p= not significant.