Supplementary Information for

Administration of BMP2/7 *in utero* partially reverses Rubinstein Taybi Syndrome-like skeletal defects induced by *Pdk1* or *Cbp* mutations in mice

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This file contains Supplementary Table S1 and Supplementary Figures S1-S11.

Supplementary Table S1. Genotype of offspring derived from breeding of *Pdk1*$^{fl/+}$ *osx* and *Pdk1*$^{fl/fl}$ parents

	Total	**Flox/Flox**	**Flox/+**	**Flox/+;Osx**	**Flox/Flox;Osx**
P20 and older	134	39	51	42	2
P4-5	29	8	14	7	0
P2-3	76	20	23	14	12 + 7 (dead)
P1	67	20	15	14	18
E18.5	47	9	15	4	19
E16.5	32	6	8	10	8

Legends for Supplementary Figures:

Figure S1. Expression of PDK1 in the calvaria.

Immunohistochemistry for PDK1 showing expression in the sagittal suture (A), nasal bone (B), and zygomatic arch (D) of P2 *Pdk1*$^{fl/fl}$ and *Pdk1*^{osx} mice as diagramed. The osteogenic front (OF) and sutural mesenchyme (SM) are labeled.

Figure S2. Characterization of *Pdk1*^{osx} mice and embryos.

(A) Pictures of empty stomach in P2 *Pdk1*$^{fl/fl}$ and *Pdk1*^{osx} neonates. **(**B, C) Alizarin Red/Alcian Blue-stained skeletal preps demonstrating calvarial hypomineralization and clavicular hypoplasia in E18.5 and E16.5 *Pdk1*$^{fl/fl}$ and *Pdk1*^{osx} embryos. Arrows indicate the zygomatic and nasal bone. (D) Hematoxlin and eosin stained longitudinal section of the femur in E18.5 and E16.5 *Pdk1*$^{fl/fl}$ and *Pdk1*^{osx} embryos. Original magnification 40x. (E) Alizarin Red/Alcian Blue-stained skeletal preps demonstrating abnormal skeletogenesis in E18.5 *Pdk1*$^{fl/fl}$ and *Pdk1*^{dm1} embryos. Scale bars indicate 1mm. (F) Representative FACS profiles on pre-gated live CD45^{-} Ter119^{-} lineage cells harvested from long bones of E17.5 *Pdk1*$^{fl/fl}$ and *Pdk1*^{osx} embryos.

Figure S3. Further characterization of *Pdk1*^{dm1} mice .

(A) Alizarin Red/Alcian Blue-stained skeletal preps demonstrating abnormal skeletogenesis in P0 *Pdk1*$^{fl/fl}$ and *Pdk1*^{dm1} embryos. Scale bars indicate 2mm. (B) Safarin O-stained sections of tibia from E18.5 *Pdk1*^{dm1} embryos. Additionally shown are areas of delayed cartilage ossifcation with persistent cartilage (top right) and an area of densely-

packed osteoblast-lineage cells embedded in an osteoid matrix (bottom right). Original magnification of all images 400X.

Figure S4. Characterization of *Osx-cre* mice.

(A) Alizarin Red/Alcian Blue-stained skeletal preps of calvaria, clavicles, and hind limbs in P2 *Osx-cre* neonates. Arrows indicate the zygomatic and nasal bones. (B, C) Ossification of calvaria and femurs of 4 week old *Osx-cre* mice was analyzed by μCT. Displayed are three-dimensional reconstructions of calvaria (C), trabecular bone and midshaft cortical bone (B, left). Values displayed are bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and cortical thickness (C. Th). Values are mean + SD (B, right). (D) Measurement of nonfasting serum glucose (left) and serum insulin (right) in P2 $Pdk1^{fl/fl}$ and $Pdk1^{osx}$ mice. "*" indicates a significant difference by the Student's t-test, $p < 0.05$.

Figure S5. Impaired osteoblast differentiation *in vivo* and *in vitro* cultured CalvObs.

(A) Sections from the nasal bone (upper left), zygoma (right), and calvarium (bottom left) of P2 $Pdk1^{fl/fl}$ and $Pdk1^{osx}$ neonates were by *in situ* hybridization. (B) $Pdk1^{fl/fl}$ CalvOb were transduced with control (con) or Cre-expressing (ΔPDK1) lentivirus. RNA transcript (upper) and protein (day 7, lower) levels of *Runx2* were analyzed by quantitative PCR and immunoblotting with anti-RUNX2 antibody. (C, D) $Pdk1^{fl/fl}$ CalvOb were transduced with control or Cre-expressing lentivirus and cultured for 7 days. RNA transcript and protein levels of *Pdk1* were analyzed by quantitative PCR and immunoblotting (C). Cell viability was measured with Alamar Blue (upper).

Alternatively, *Pdk1*$^{fl/fl}$ CalvOb infected by control or Cre-expressing lentivirus were cultured for 6 days and ALP activity was analyzed (lower) (D). (E, F) *Pdk1*$^{fl/fl}$ CalvOb were infected with either control or Cre-expressing lentivirus together with WT or kinase-dead PDK1 (KD) expressing lentivirus and for ALP activity (E). Alternatively, RNA transcript levels of the indicated genes were analyzed by quantitative PCR (F).

Figure S6. PI3K/PDK1 inhibitor treatment of CalvOb and hMSCs.

(A) Primary WT CalvObs were cultured in the presence of DMSO, PI3K inhibitor (LY294002, Calbiochem), PDK1 inhibitor (B-3012, Echelon Biosciences) or AKT inhibitor (IV, Calbiochem). (B, C) hMSCs were treated with PDK1 or PI3K inhibitors for 6 days while placed under osteoblast differentiation conditions. They were then assayed for ALP activity (B) or for expression of osteoblast marker genes (C).

Figure S7. Various signaling pathways in PDK1-deficient osteoblasts.

(A) *Pdk1*$^{fl/fl}$ CalvOb were infected with control (con) or Cre-expressing (ΔPDK1) lentivirus, cultured for 6 days under differentiation conditions in the absence or presence of IGF-1 (25ng/ml), and analyzed by Von Kossa staining for mineralization. (B) FGF signaling transduction in PDK1-deficient CalvObs. *Pdk1*$^{fl/fl}$ CalvOb were infected with control or Cre-expressing lentivirus and cultured under differentiation conditions. RNA transcript levels of *Sprouty1* (*Spry1*) and *Sprouty2* (*Spry2)* were analyzed by quantitative PCR. (C) BMP signaling transduction in PDK1-deficient CalvObs. The cells were treated with or without BMP2/7 (100ng/ml) for 6 hours and RNA transcript levels of the indicated genes were analyzed by quantitative PCR. (D) TGFβ signaling transduction in

PDK1-deficient CalvObs. The cells were transfected with 3TP-lux and *Renilla* luciferase gene, and luciferase activity was analyzed 6 days after culture in differentiation medium. Results were normalized to a *Renilla* control. (E) PDK1-deficient CalvObs were serum starved for 12 hours prior to stimulation with FGF2 (10ng/ml), BMP2/7 (100ng/ml), or TGFβ (2ng/ml) at different timepoints and immunoblotted with the indicated antibodies. Protein amount of HSP90 was used as a loading control. (F) PDK1-deficient CalvObs were stimulated with IGF-1 (25ng/ml) and insulin (10nM) at different timepoints and immunoblotted with the indicated antibodies. Protein amount of HSP90 was used as a loading control. These experiments were performed together with Figures 2E and F. (G) C3H10T1/2 osteoblasts were transduced with the Top flash lucfierase reporter concurrently with the construct encoding xWNT/Fz. These cells were concurrently treated with the indicated inhibitors and activity reported 36 hours later. (H) CalvOb were prepared as in (A) and then blotted for β-Catenin (bottom panel). Alternatively, the activity of β-Catenin was examined with the Top flash luciferase reporter (top panel).

Figure S8. Impaired AKT activation in *Pdk1*^{osx} mice.

(A) WT CalvObs were treated with the indicated inhibitors for 1hour prior to stimulation with IGF-1 (25ng/ml) for 30 minutes and immunoblotted with antibodies specific to phospho-AKT (T308) and AKT substrates. Protein amount of HSP90 was used as a loading control. (B, C, D) IHC showing phosphorylation levels of AKT (T308), GSK3β (S9), and S6 (S235/236) in a coronal section of the sagittal suture (B), nasal bone (C), and zygomatic arch (D) of P2 *Pdk1*$^{fl/fl}$ and *Pdk1*^{osx} neonates. Original magnification 100X. (E) IHC showing phosphorylation levels of CREB (S133) in a coronal section of the

nasal bone (left) and zygomatic arch (right) of P2 *Pdk1*$^{fl/fl}$ and *Pdk1*^{osx} neonates. Original magnification 100X.

Figure S9. Characterization of CREB knockout mice.

(A) Transcript levels of *Creb* and *Cbp* in PDK1-deficient osteoblasts. *Pdk1*$^{fl/fl}$ CalvOb were infected with control (con) or Cre-expressing (ΔPDK1) lentivirus and cultured under differentiation conditions. RNA transcript levels of the indicated genes were analyzed by quantitative PCR. (B) Alizarin Red/Alcian Blue-stained skeletal preps of E18.5 *Creb*$^{+/+}$ and *Creb*$^{-/-}$ embryos. (C) μCT analysis of calvaria (upper left; right) and Alizarin Red/Alcian Blue-stained skeletal preps of the clavicles (lower left) from 2 week old *Pdk1*$^{fl/+}$, *Pdk1*$^{fl/+}$*osx*, *Creb*$^{+/-}$, and *Pdk1*$^{fl/+}$*osx; Creb*$^{+/-}$ mice. (D) CREB phosphorylation by various stimuli in PDK1-deficient CalvObs. The cells were stimulated with FGF-2 (top), isoproterenol (middle), or forskolin (bottom) at different timepoints and immunoblotted with anti-phospho-CREB (S133) antibody. Protein amount of HSP90 was used as a loading control. (E) CalvOb were prepared as in Figure S9A and their size determined by the forward-scatter on a flow cytometer.

Figure S10. PDK1 and CREB regulate BMP2 expression.

(A) hMSCs (top panel) or CalvOb (bottom panel) were cultured under osteoblast differentiating conditions and treated with the indicated inhibitors for 24 hours and the transcript levels of BMP2 measured by quantitative PCR. (B) Transcript levels of *Bmp2* in PDK1-deficient osteoblasts. *Pdk1*$^{fl/fl}$ CalvOb were infected with control (con) or Cre-expressing (ΔPDK1) lentivirus and cultured under differentiation conditions. RNA

transcript levels of the indicated genes were analyzed by quantitative PCR. (C) C3H10T1/2 cells were transfected with the BMP2-Luc (-150/+165) and *Renilla* luciferase genes along with a different concentration of VP16/CREB (top panel) or with the combination of Flag-CREB (wt) and CBP (bottom panel). Results are expressed as relative luciferase activity normalized to *Renilla* control. (D) C3H10T1/2 cells were transfected with the BMP2-Luc (-2712/+165 or -150/+165) and *Renilla* luciferase genes along with vector, Flag-CREB (wt), or VP16/CREB. Results are expressed as relative luciferase activity normalized to *Renilla* control.

Figure S11. Quantification of Western blotting results.

The relative band intensity from the indicated Western blots was measured. The second set of values reflects the results of an independent replicate experiment.

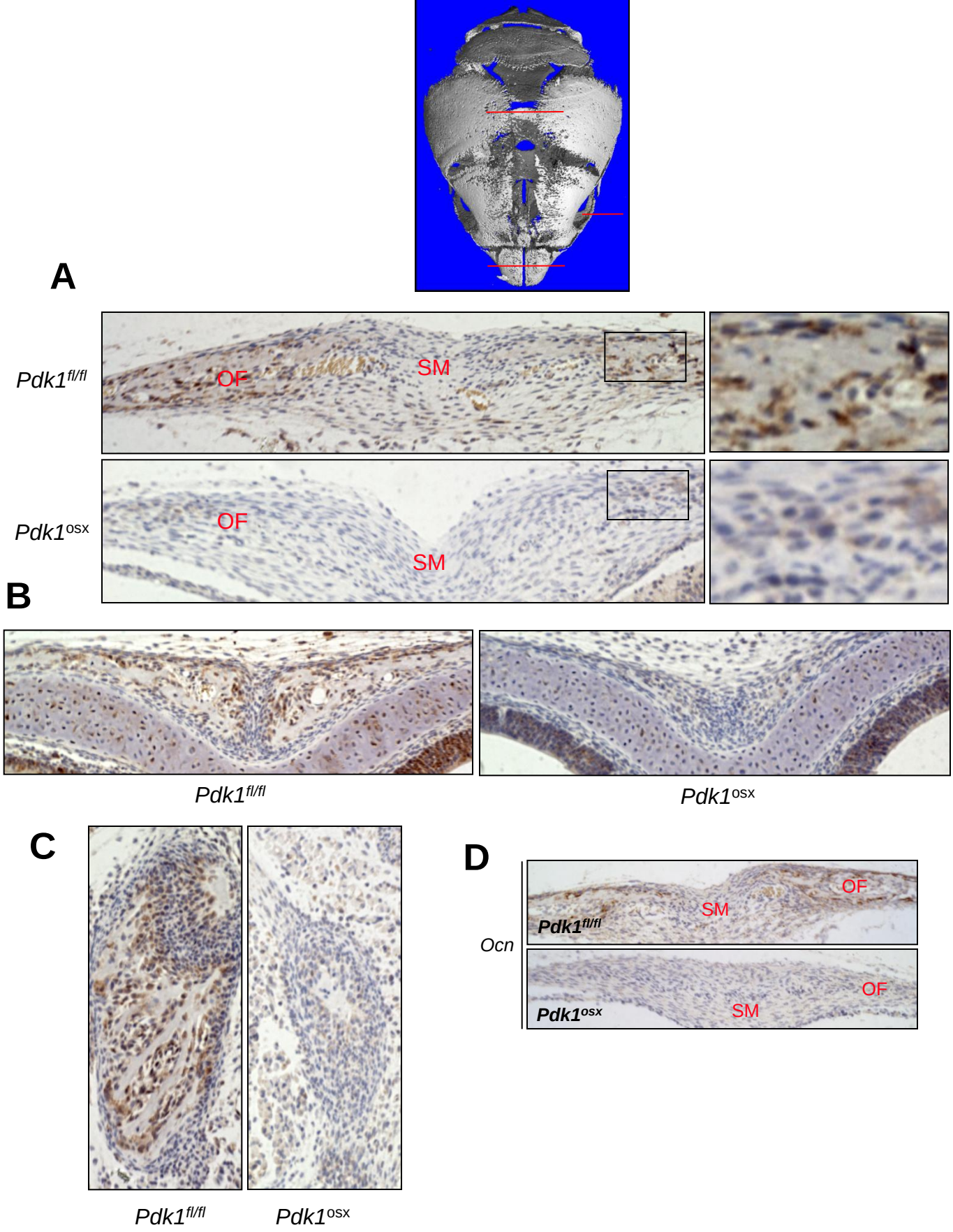


Figure S1. Expression of PDK1 in the calvaria.
Immunohistochemistry for PDK1 showing expression in the sagittal suture (A), nasal bone (B), and zygomatic arch (D) of P2 *Pdk1*$^{fl/fl}$ and *Pdk1*^{osx} mice as diagramed. The osteogenic front (OF) and sutural mesenchyme (SM) are labeled.

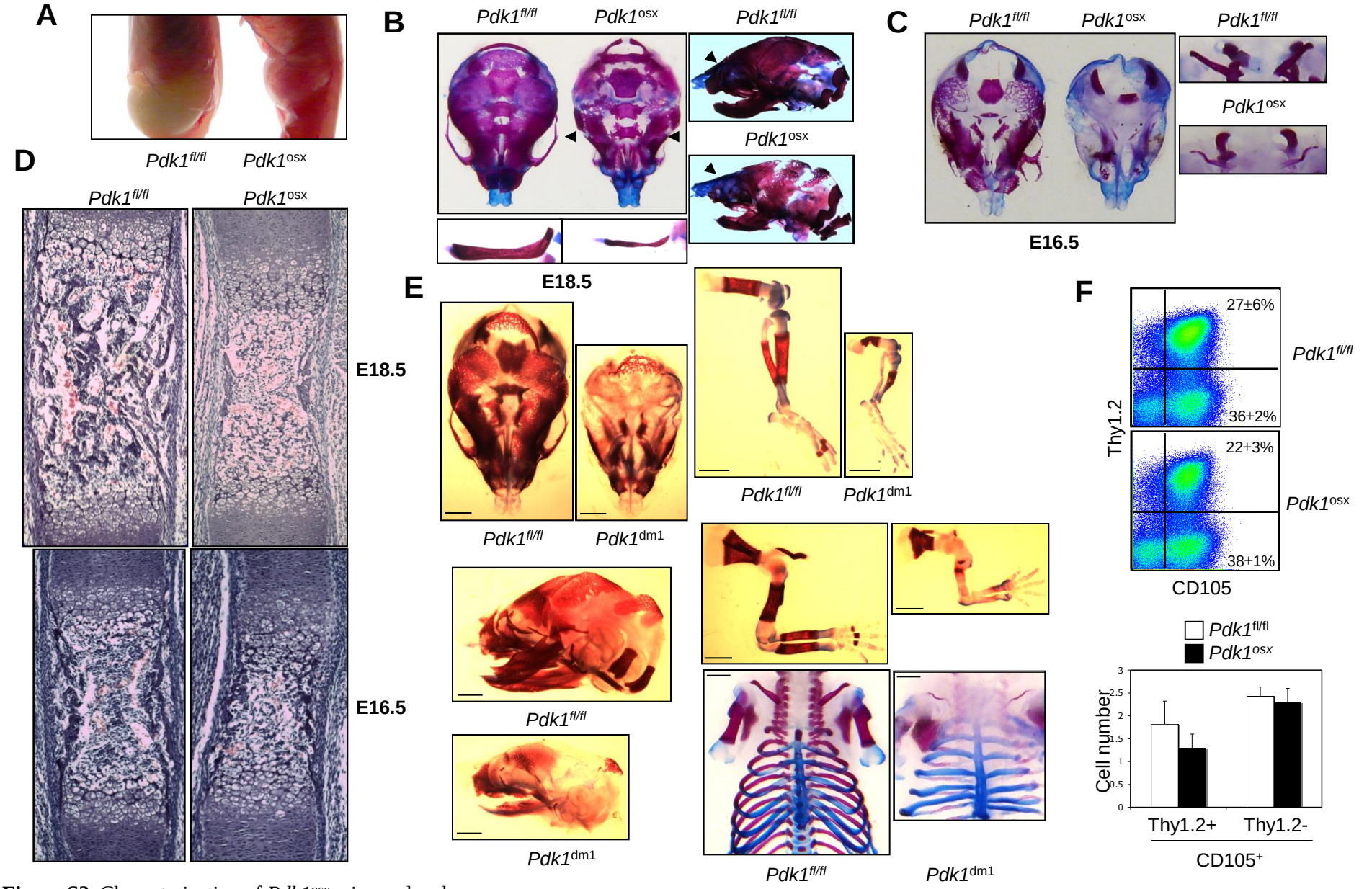


Figure S2. Characterization of *Pdk1*^{osx} mice and embryos.
(A) Pictures of empty stomach in P2 *Pdk1*$^{fl/fl}$ and *Pdk1*^{osx} neonates. **(**B, C) Alizarin Red/Alcian Blue-stained skeletal preps demonstrating calvarial hypomineralization and clavicular hypoplasia in E18.5 and E16.5 *Pdk1*$^{fl/fl}$ and *Pdk1*^{osx} embryos. Arrows indicate the zygomatic and nasal bone. (D) Hematoxlin and eosin stained longitudinal section of the femur in E18.5 and E16.5 *Pdk1*$^{fl/fl}$ and *Pdk1*^{osx} embryos. Original magnification 40x. (E) Alizarin Red/Alcian Blue-stained skeletal preps demonstrating abnormal skeletogenesis in E18.5 *Pdk1*$^{fl/fl}$ and *Pdk1*^{dm1} embryos. Scale bars indicate 1mm. (F) Representative FACS profiles on pre-gated live CD45^{-} Ter119^{-} lineage cells harvested from long bones of E17.5 *Pdk1*$^{fl/fl}$ and *Pdk1*^{osx} embryos.

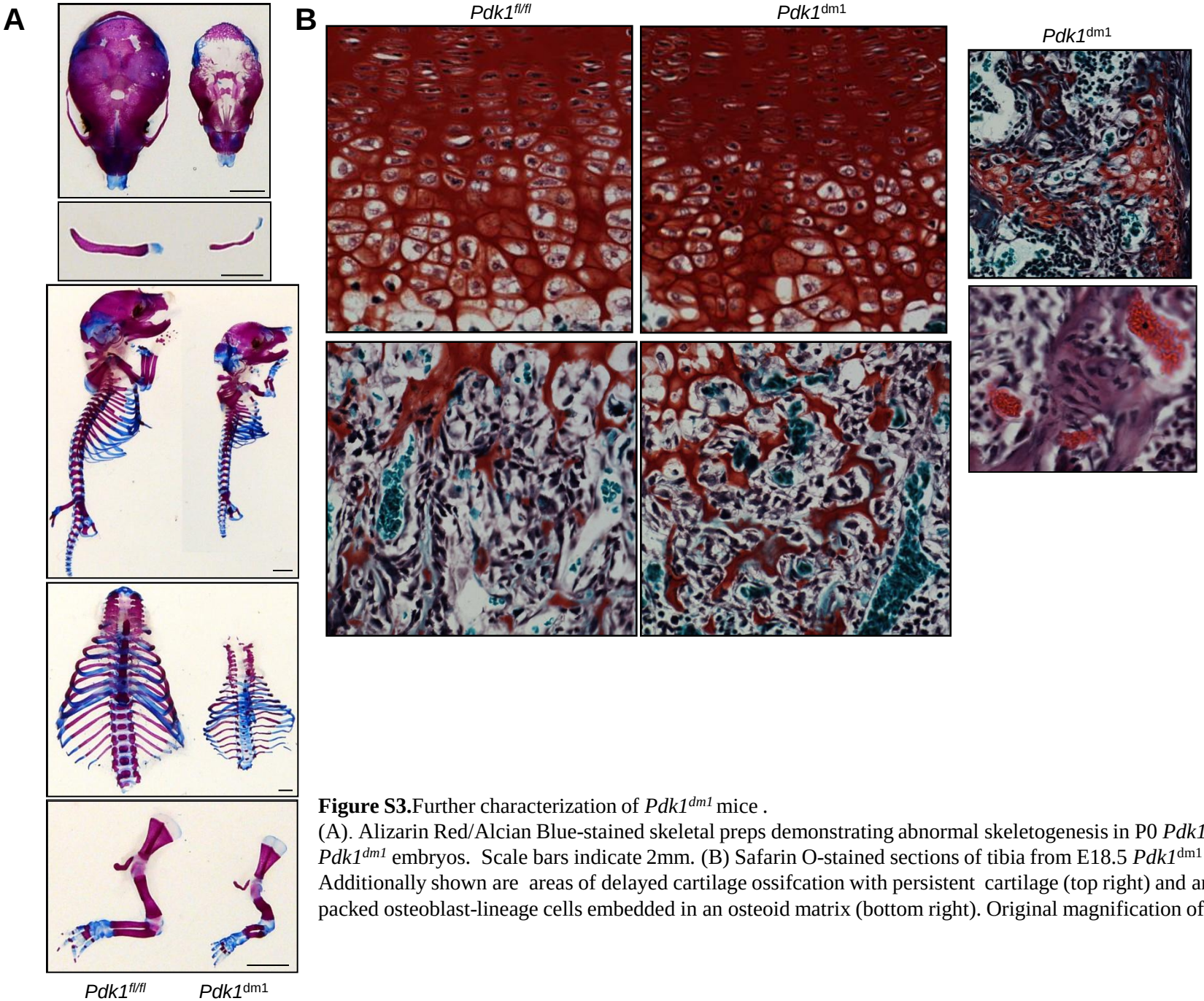


Figure S3. Further characterization of *Pdk1^{dm1}* mice .
(A). Alizarin Red/Alcian Blue-stained skeletal preps demonstrating abnormal skeletogenesis in P0 *Pdk1$^{fl/fl}$* and *Pdk1^{dm1}* embryos. Scale bars indicate 2mm. (B) Safarin O-stained sections of tibia from E18.5 *Pdk1*^{dm1} embryos. Additionally shown are areas of delayed cartilage ossifcation with persistent cartilage (top right) and an area of densely-packed osteoblast-lineage cells embedded in an osteoid matrix (bottom right). Original magnification of all images 400X.

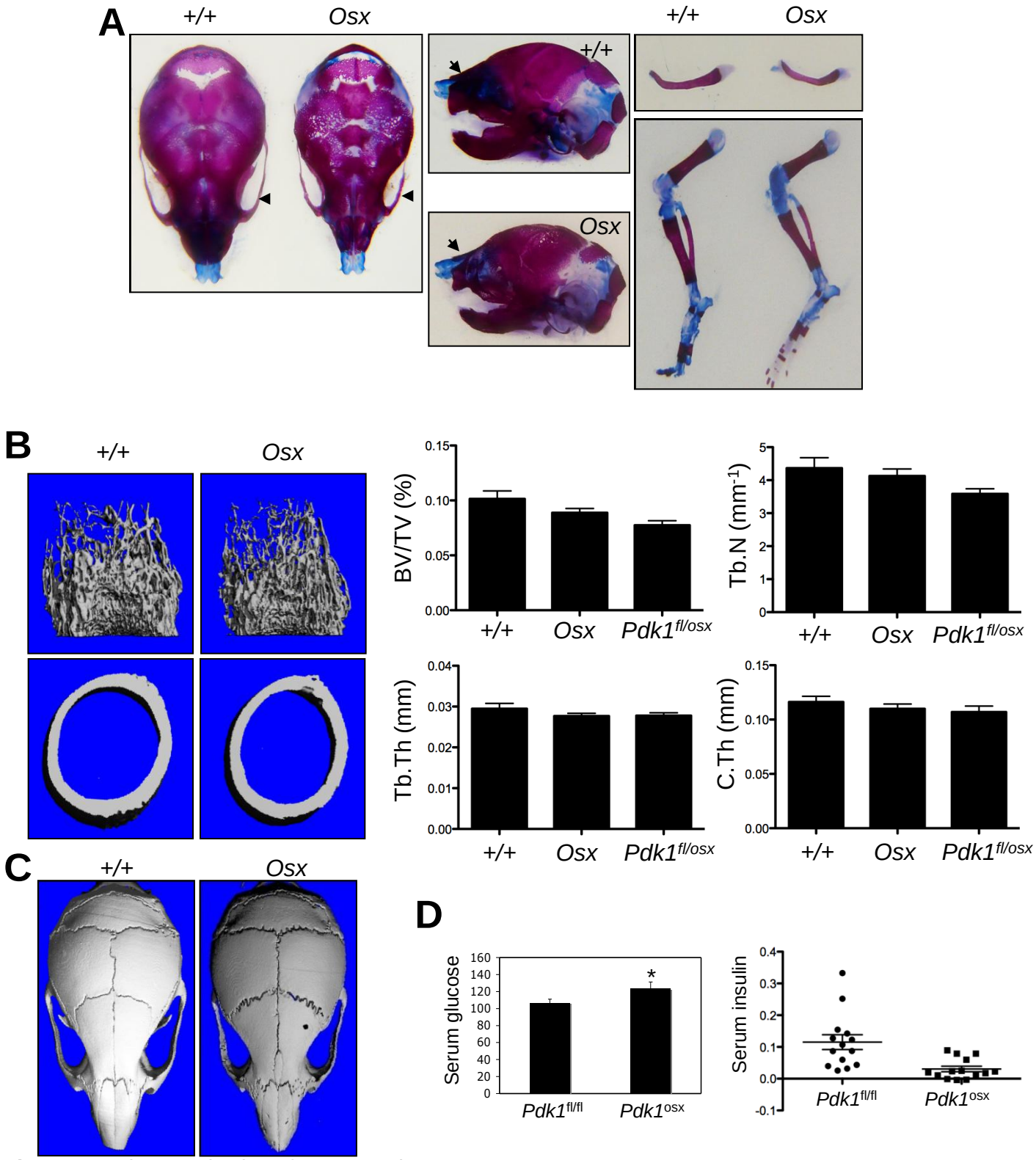
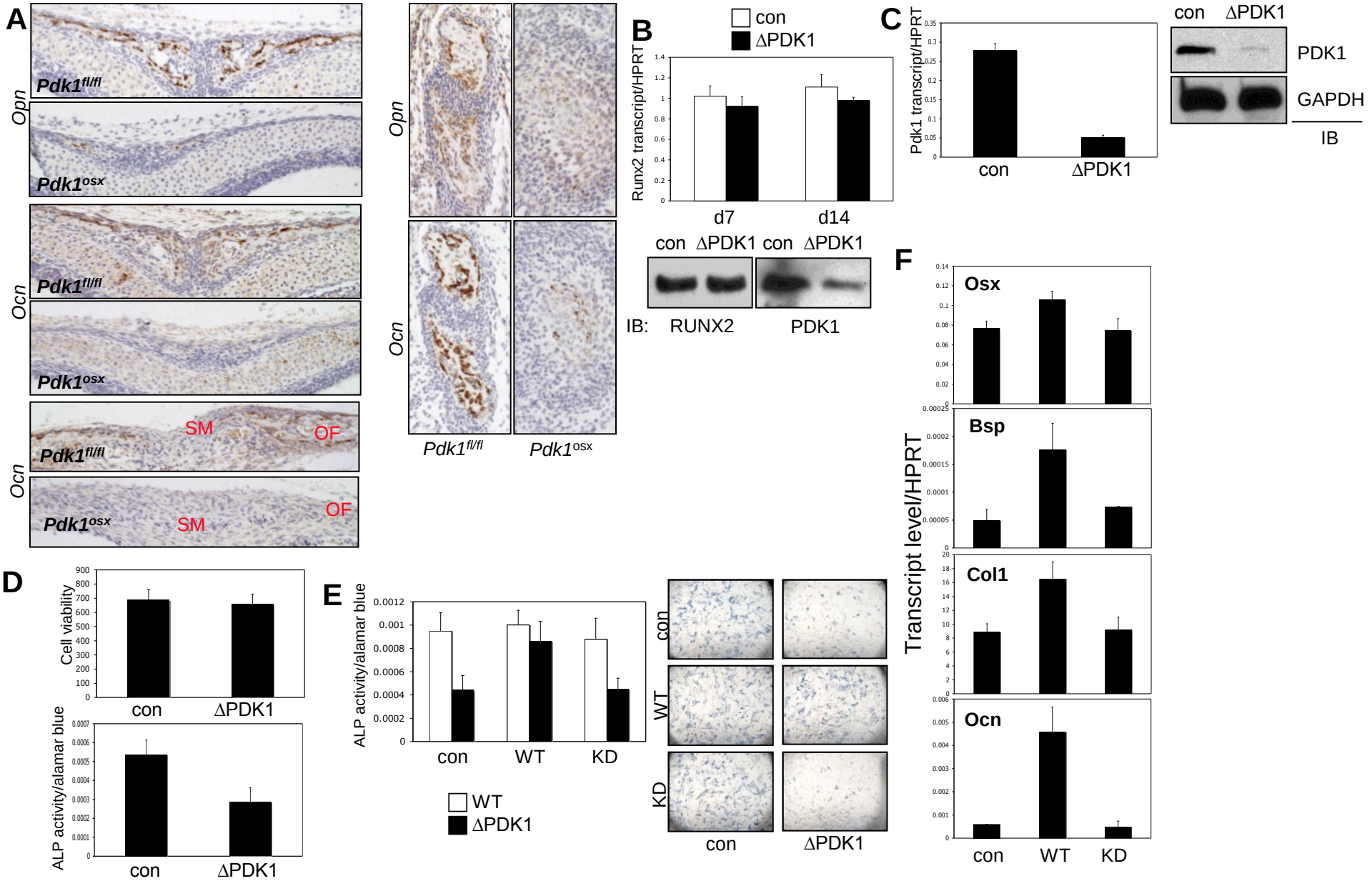


Figure S4. Characterization of *Osx-cre* mice.
(A) Alizarin Red/Alcian Blue-stained skeletal preps of calvaria, clavicles, and hind limbs in P2 *Osx-cre* neonates. Arrows indicate the zygomatic and nasal bones. (B, C) Ossification of calvaria and femurs of 4 week old *Osx-cre* mice was analyzed by µCT. Displayed are three-dimensional reconstructions of calvaria (C), trabecular bone and midshaft cortical bone (B, left). Values displayed are bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and cortical thickness (C. Th). Values are mean + SD (B, right). (D) Measurement of nonfasting serum glucose (left) and serum insulin (right) in P2 *Pdk1*$^{fl/fl}$ and *Pdk1*^{osx} mice. "*" indicates a significant difference by the Student's t-test, $p < 0.05$.



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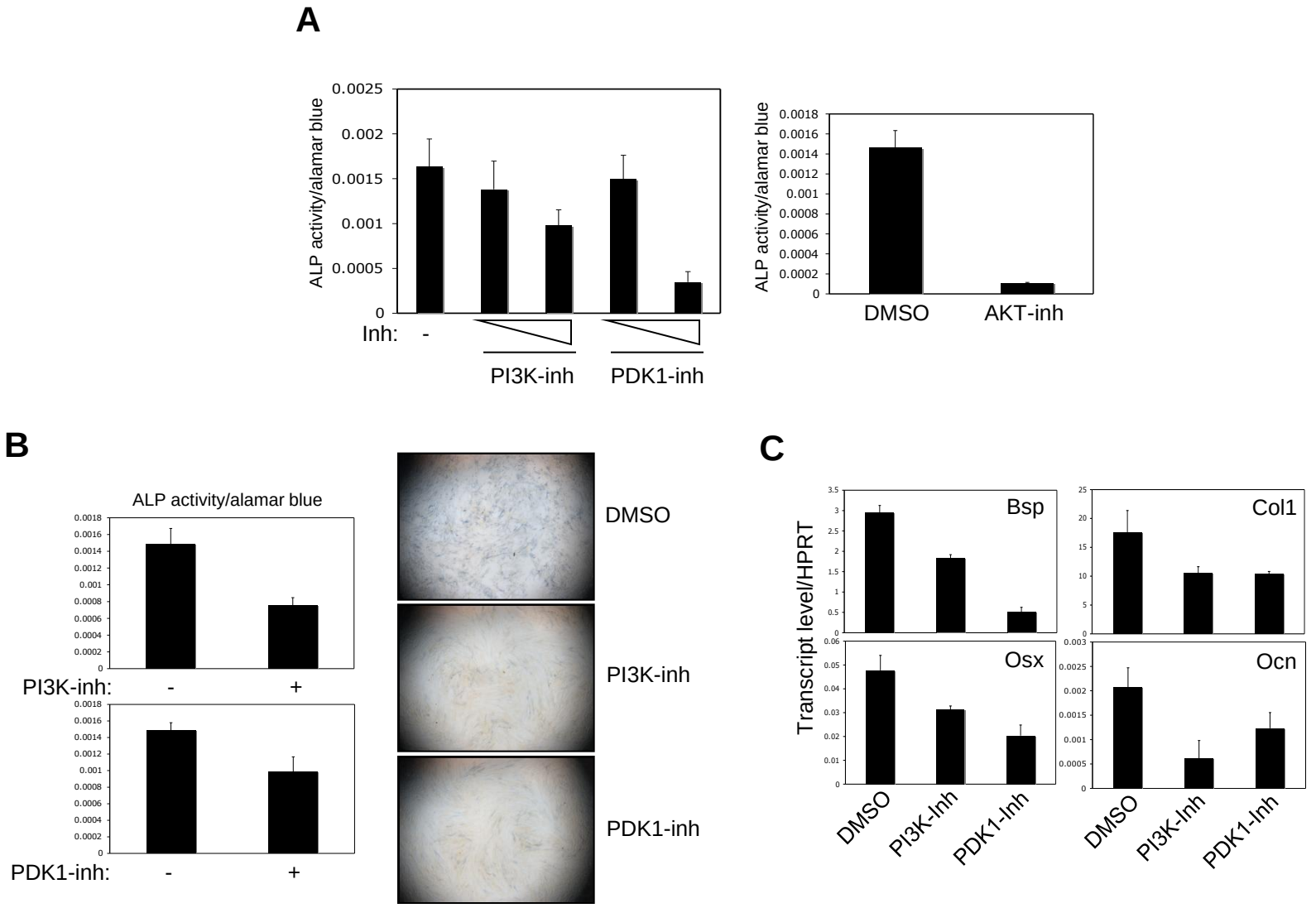

Figure S6. PI3K/PDK1 inhibitor treatment of CalvOb and hMSCs.
(A) Primary WT CalvObs were cultured in the presence of DMSO, PI3K inhibitor (LY294002, Calbiochem), PDK1 inhibitor (B-3012, Echelon Biosciences) or AKT inhibitor (IV, Calbiochem). (B, C) hMSCs were treated with PDK1 or PI3K inhibitors for 6 days while placed under osteoblast differentiation conditions. They were then assayed for ALP activity (B) or for expression of osteoblast marker genes (C).

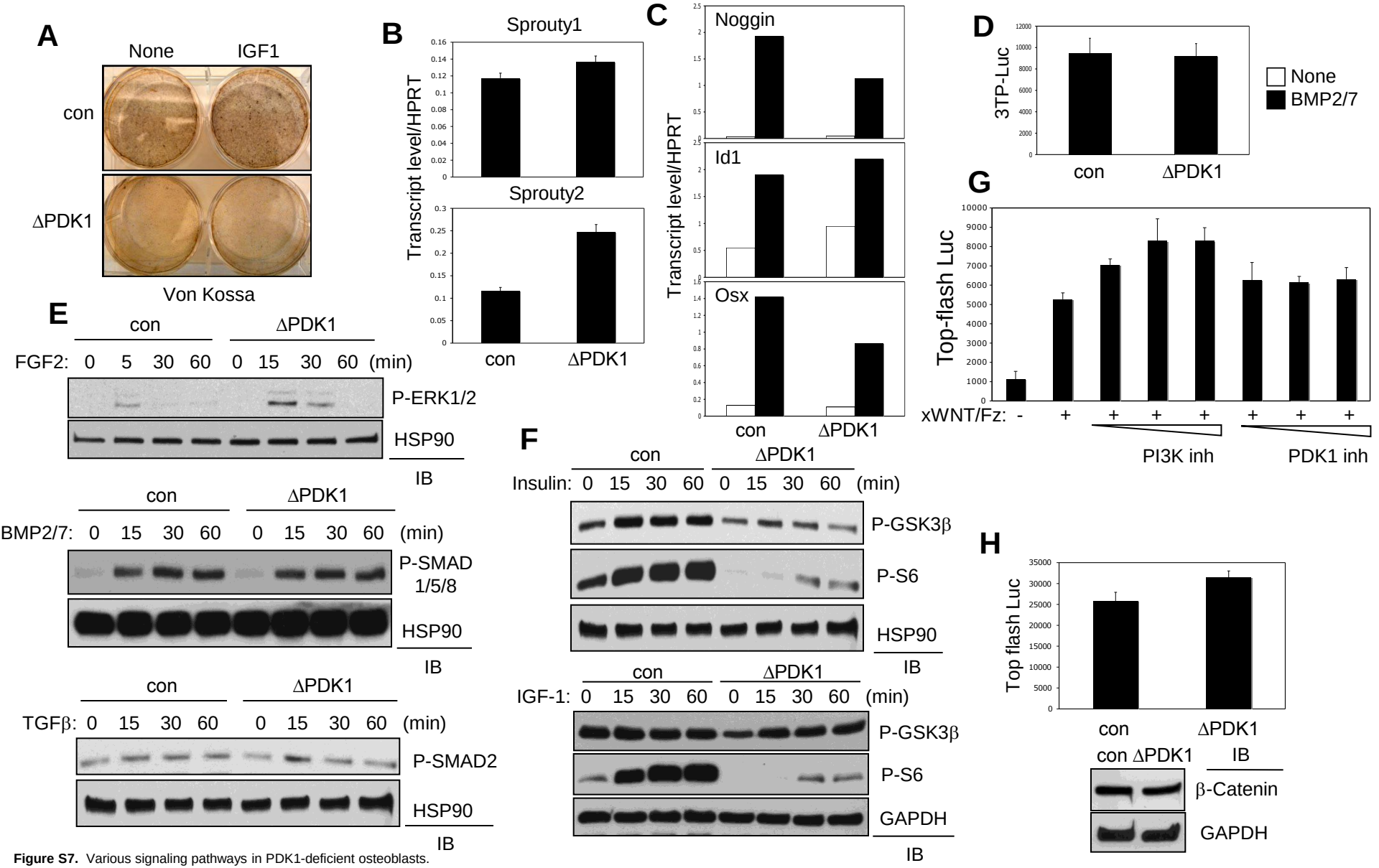

Figure S7. Various signaling pathways in PDK1-deficient osteoblasts.
(A) *Pdk1*$^{fl/fl}$ CalvOb were infected with control (con) or Cre-expressing (ΔPDK1) lentivirus, cultured for 6 days under differentiation conditions in the absence or presence of IGF-1 (25ng/ml), and analyzed by Von Kossa staining for mineralization. (B) FGF signaling transduction in PDK1-deficient CalvObs. *Pdk1*$^{fl/fl}$ CalvOb were infected with control or Cre-expressing lentivirus and cultured under differentiation conditions. RNA transcript levels of *Sprouty1* (*Spry1*) and *Sprouty2* (*Spry2)* were analyzed by quantitative PCR. (C) BMP signaling transduction in PDK1-deficient CalvObs. The cells were treated with or without BMP2/7 (100ng/ml) for 6 hours and RNA transcript levels of the indicated genes were analyzed by quantitative PCR. (D) TGFβ signaling transduction in PDK1-deficient CalvObs. The cells were transfected with 3TP-lux and *Renilla* luciferase gene, and luciferase activity was analyzed 6 days after culture in differentiation medium. Results were normalized to a *Renilla* control. (E) PDK1-deficient CalvObs were serum starved for 12 hours prior to stimulation with FGF2 (10ng/ml), BMP2/7 (100ng/ml), or TGFβ (2ng/ml) at different timepoints and immunoblotted with the indicated antibodies. Protein amount of HSP90 was used as a loading control. (F) PDK1-deficient CalvObs were stimulated with IGF-1 (25ng/ml) and insulin (10nM) at different timepoints and immunoblotted with the indicated antibodies. Protein amount of HSP90 was used as a loading control. These experiments were performed together with Figures 2E and F. (G) C3H10T1/2 osteoblasts were transduced with the Top flash lucfierase reporter concurrently with the construct encoding xWNT/Fz. These cells were concurrently treated with the indicated inhibitors and activity reported 36 hours later. (H) CalvOb were prepared as in (A) and then blotted for β-Catenin (bottom panel). Alternatively, the activity of β-Catenin was examined with the Top flash luciferase reporter (top panel).

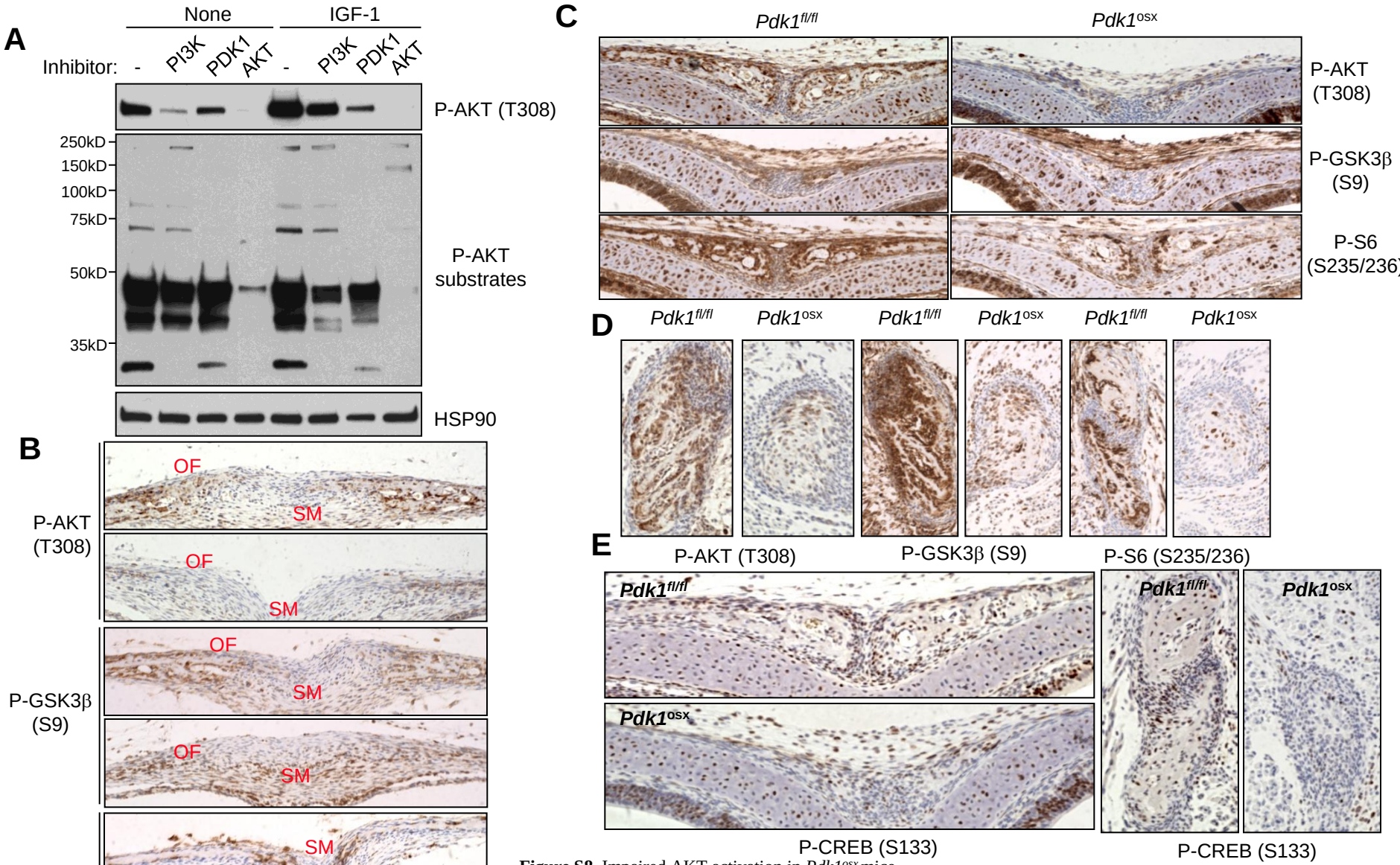

Figure S8. Impaired AKT activation in *Pdk1*^{osx} mice.
(A) WT CalvObs were treated with the indicated inhibitors for 1hour prior to stimulation with IGF-1 (25ng/ml) for 30 minutes and immunoblotted with antibodies specific to phospho-AKT (T308) and AKT substrates. Protein amount of HSP90 was used as a loading control. (B, C, D) IHC showing phosphorylation levels of AKT (T308), GSK3β (S9), and S6 (S235/236) in a coronal section of the sagittal suture (B), nasal bone (C), and zygomatic arch (D) of P2 *Pdk1*$^{fl/fl}$ and *Pdk1*^{osx} neonates. Original magnification 100X. (E) IHC showing phosphorylation levels of CREB (S133) in a coronal section of the nasal bone (left) and zygomatic arch (right) of P2 *Pdk1*$^{fl/fl}$ and *Pdk1*^{osx} neonates. Original magnification 100X.

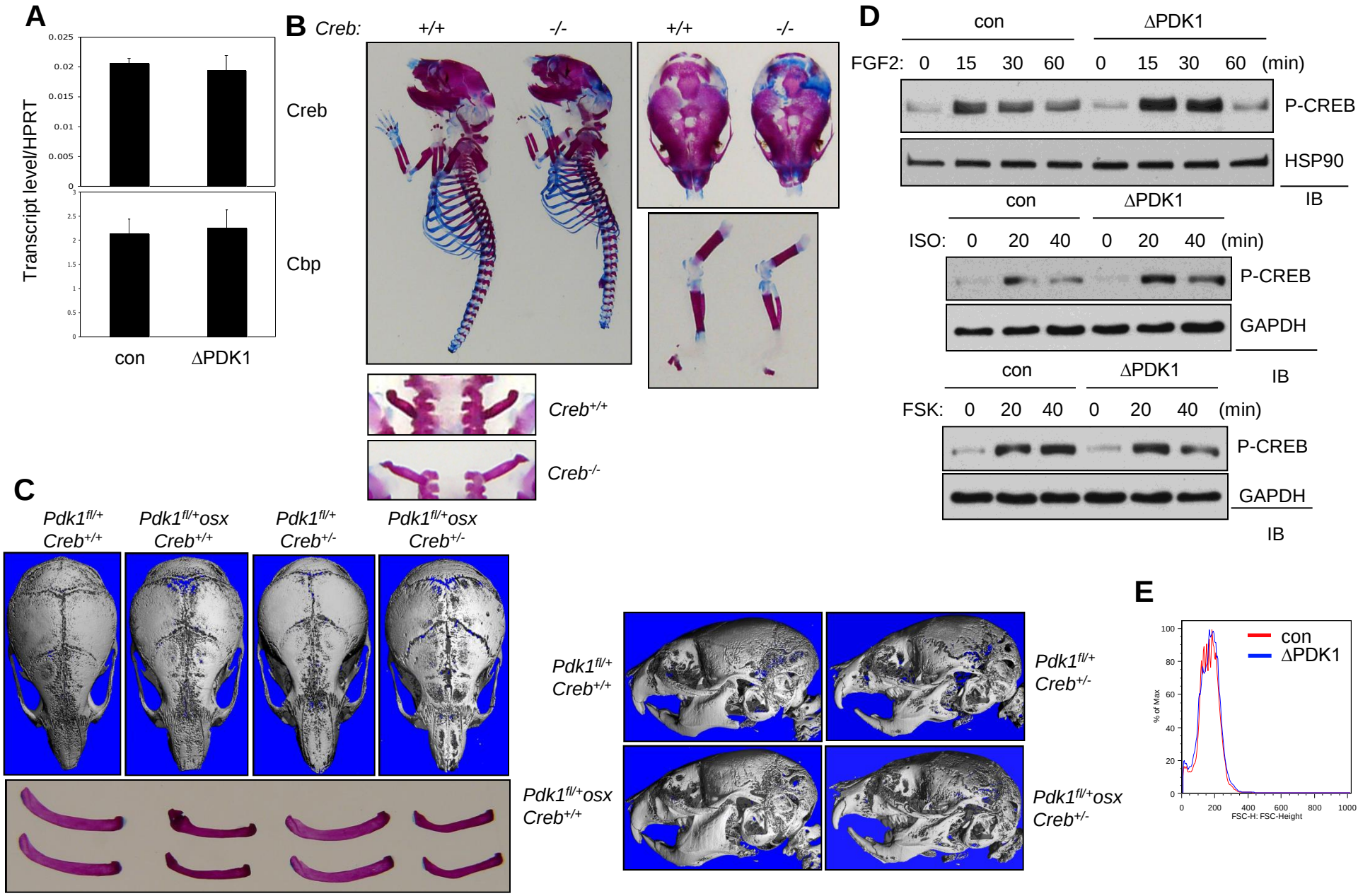

Figure S9. Characterization of CREB knockout mice.
(A) Transcript levels of *Creb* and *Cbp* in PDK1-deficient osteoblasts. *Pdk1*$^{fl/fl}$ CalvOb were infected with control (con) or Cre-expressing (ΔPDK1) lentivirus and cultured under differentiation conditions. RNA transcript levels of the indicated genes were analyzed by quantitative PCR. (B) Alizarin Red/Alcian Blue-stained skeletal preps of E18.5 *Creb*$^{+/+}$ and *Creb*$^{-/-}$ embryos. (C) μCT analysis of calvaria (upper left; right) and Alizarin Red/Alcian Blue-stained skeletal preps of the clavicles (lower left) from 2 week old *Pdk1*$^{fl/+}$, *Pdk1*$^{fl/+}$*osx, Creb*$^{+/-}$, and *Pdk1*$^{fl/+}$*osx; Creb*$^{+/-}$ mice. (D) CREB phosphorylation by various stimuli in PDK1-deficient CalvObs. The cells were stimulated with FGF-2 (top), isoproterenol (middle), or forskolin (bottom) at different timepoints and immunoblotted with anti-phospho-CREB (S133) antibody. Protein amount of HSP90 was used as a loading control. (E) CalvOb were prepared as in Figure S9A and their size determined by the forward-scatter on a flow cytometer.

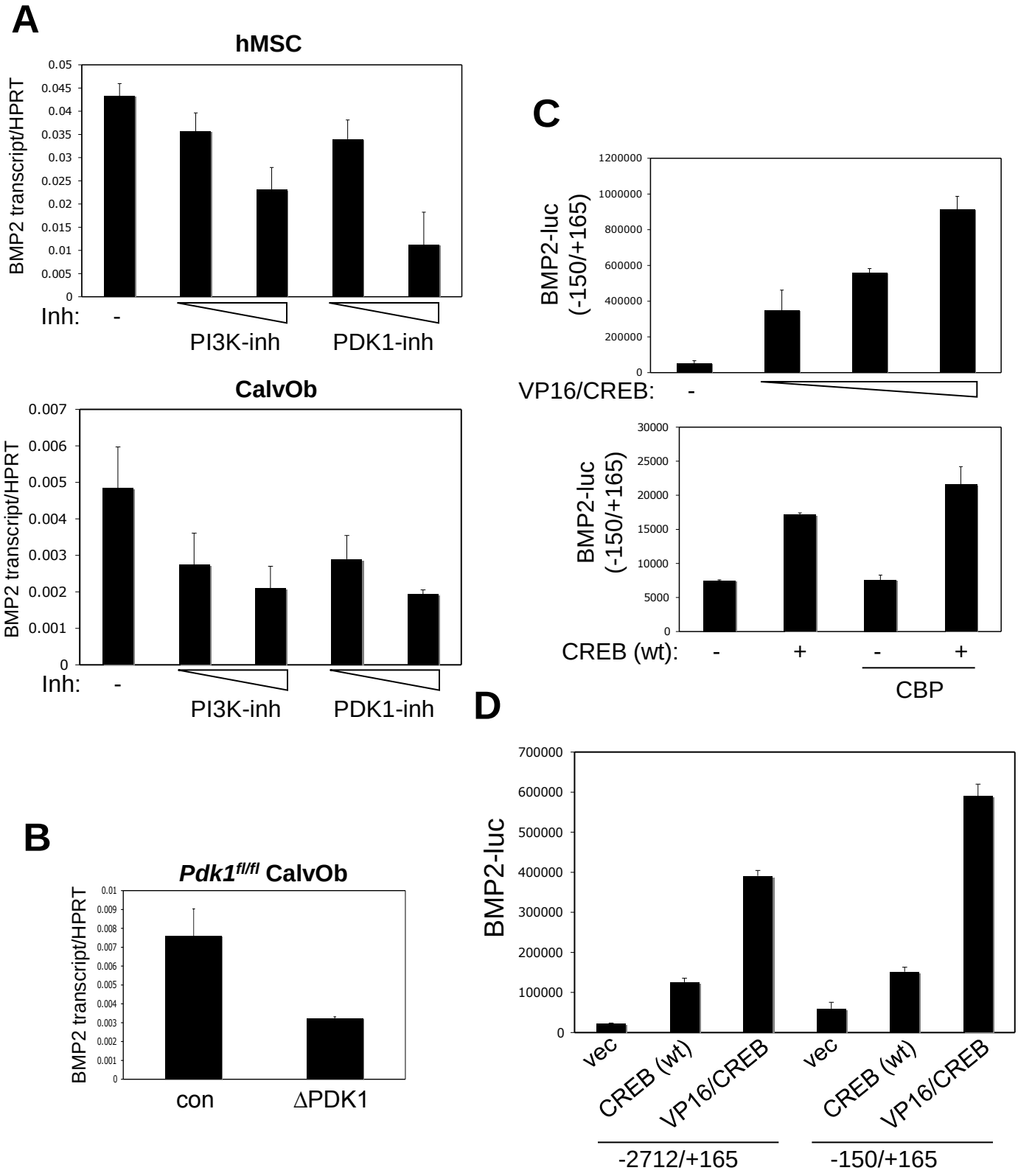

Figure S10. PDK1 and CREB regulate BMP2 expression.
(A) hMSCs (top panel) or CalvOb (bottom panel) were cultured under osteoblast differentiating conditions and treated with the indicated inhibitors for 24 hours and the transcript levels of BMP2 measured by quantitative PCR. (B) Transcript levels of *Bmp2* in PDK1-deficient osteoblasts. *Pdk1*$^{fl/fl}$ CalvOb were infected with control (con) or Cre-expressing (ΔPDK1) lentivirus and cultured under differentiation conditions. RNA transcript levels of the indicated genes were analyzed by quantitative PCR. (C) C3H10T1/2 cells were transfected with the BMP2-Luc (-150/+165) and *Renilla* luciferase genes along with a different concentration of VP16/CREB (top panel) or with the combination of Flag-CREB (wt) and CBP (bottom panel). Results are expressed as relative luciferase activity normalized to *Renilla* control. (D) C3H10T1/2 cells were transfected with the BMP2-Luc (-2712/+165 or -150/+165) and *Renilla* luciferase genes along with vector, Flag-CREB (wt), or VP16/CREB. Results are expressed as relative luciferase activity normalized to *Renilla* control.

Figure. 2E

P-AKT (T308)	0.89 0.89	0.92 1	1 0.33	0.97 0.37	0.27 0	0.15 0	0.21 0	0.3 0
P-AKt (S473)	0.22 0.14	0.95 0.9	0.60 0.88	0.64 0.44	0.33 0.53	0.97 1	0.92 0.97	1 0.95
P-p38	0.4 0.18	0.19 0.7	0.15 0.7	0.23 0.72	0.89 0.75	0.96 0.84	1 0.92	0.83 1
GAPDH	0.94 0.89	0.96 0.99	0.95 0.86	0.94 0.9	1 0.98	0.96 1	0.92 1	1 0.98

Figure. 3A

P-AKT (T308)	1 1	0.11 0.11
P-AKT (S473)	1 1	1.32 1.8
GAPDH	1 1	0.98 1
P-GSK3β	1 1	0.28 0.31
P-S6	1 1	0 0

Figure. 3B

P-CREB (S133)	1 1	0.43 0.46
HSP90	1 0.86	0.98 1

Figure. 3D

P-CREB (S133)	0.19 0	1 1	0.47 0.77	0.31 0.77	0.09 0.11	0.16 0.15	0.14 0.11	0 0
HSP90	1 0.87	0.99 0.92	0.96 0.93	0.95 0.91	0.94 0.94	0.93 0.93	0.94 0.93	0.96 1

Figure. 5B

Oligo pulldown	1 1	0.42 0.34	2.13 2.99	0.64 1.14
Input	1 1	1 1.03	0.99 1.01	1.05 1.01

Figure. 5C

IP:HA IB: Myc	0 0	0.53 0.49	1 1
IB: Myc	0.97 1	1 0.96	1 0.97

Figure. 5D

IP:IgG, CBP IB: Runx2	0.11 0.18	1 1	0.46 0.57
IB: Runx2	0.81 0.84	1 1	0.99 1.03

Figure. 5F

P-SMAD 1/5/8	1 1	0.49 0.32
GAPDH	1 1	0.97 0.96

Figure. 5I

Oligo pulldown	1 1	0.36 0.41
Input	1 0.98	1 1

Figure S11. Quantification of Western blotting results.
The relative band intensity from the indicated Western blots was measured. The second set of values reflects the results of an independent replicate experiment.