Supporting Information

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SI Materials and Methods

Whole-Mount Skeletal Analysis. Skeletons of 18.5-d fetuses and adults were fixed in 95% ethanol for 24 h. Detection of mineralization by alizarin red S and Alcian blue (Sigma) were performed as previously described (1).

Histology and Immunohistochemistry. For histological analysis, E18.5 and neonatal mice were killed and whole animals were fixed in 10% formalin/PBS solution and paraffin-embedded, and coronal sections were made of the calvaria to assess ALP and von Kossa staining. ALP staining was carried out by deparaffinizing the coronal sections, washing in AP buffer (100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, 0,05% Tween, pH 9.5), staining with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma) to detect ALP activity, and counterstaining with fast red. Von Kossa staining of coronal sections to detect mineralization was achieved by deparaffinization, immersion in 5% silver nitrate solution, and exposure to UV light for 15 min, followed by counterstaining with fast red. For detection of Ki67, primary rat antimouse antibody (DakoCytomation) was incubated for 30 min. After incubation with an HRP-conjugated secondary antibody, staining was visualized with DAB peroxidase substrate.

BrdU Indirect Immunofluorescence. Primary cell preparations (≈5,000 cells/chamber) were seeded in eight-chambered tissue culture slides and cultured for 1 h in the presence of 10 μM BrdU before being collected and processed for anti-BrdU immunofluorescence. Briefly, cells were fixed for 20 min in 4% paraformaldehyde in PBS solution and permeabilized for 30 min in 2% BSA/0.2% Triton X-100 in PBS solution. Chamber slides were treated at room temperature with biotinylated anti-BrdU antibody (BD PharMingen) diluted 1:200 in 2% BSA/0.2% Triton X-100 in PBS solution for 1 h. Slides were washed and incubated for 30 min at room temperature with Alexa Fluor 546-conjugated goat anti-mouse (Invitrogen) diluted 1:100 in PBS solution. DNA was stained with Hoechest 33258 nuclear stain for 5 min. Cells were rinsed three times with high-salt PBS solution and 1 time with PBS solution and mounted with Fluoromount-G mounting medium (Southern Biotech).

1. Komori T, *et al.* (1997) Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* 89:755–764.

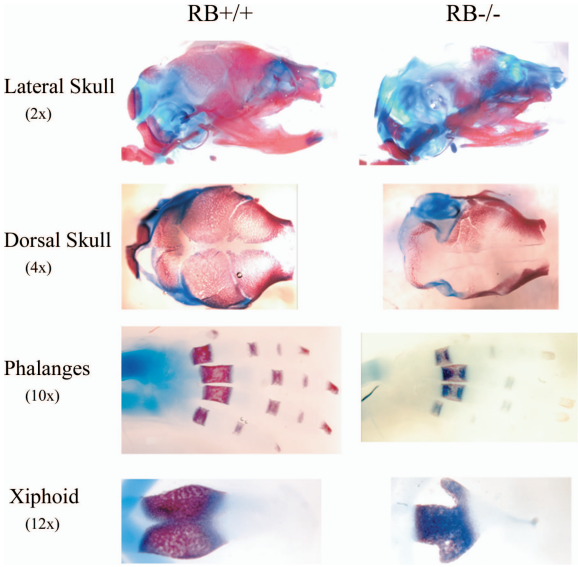

Fig. S1. *In vivo* mineralization defect in *Col3.6-Cre; Rb*$^{f19/f19}$ mice. Whole skeletons were stained with alizarin red (mineralized tissue) and Alcian blue (cartilage). Conditional RB1-KO mice (RB$^{-/-}$) at E18.5 show significant defects in the mineralization of the cranial vault and in the appearance of ossification centers of the distal metacarpals, distal and proximal phalanges, and in the xiphoid process of the distal sternum compared with WT (RB$^{+/+}$) litter-mates.

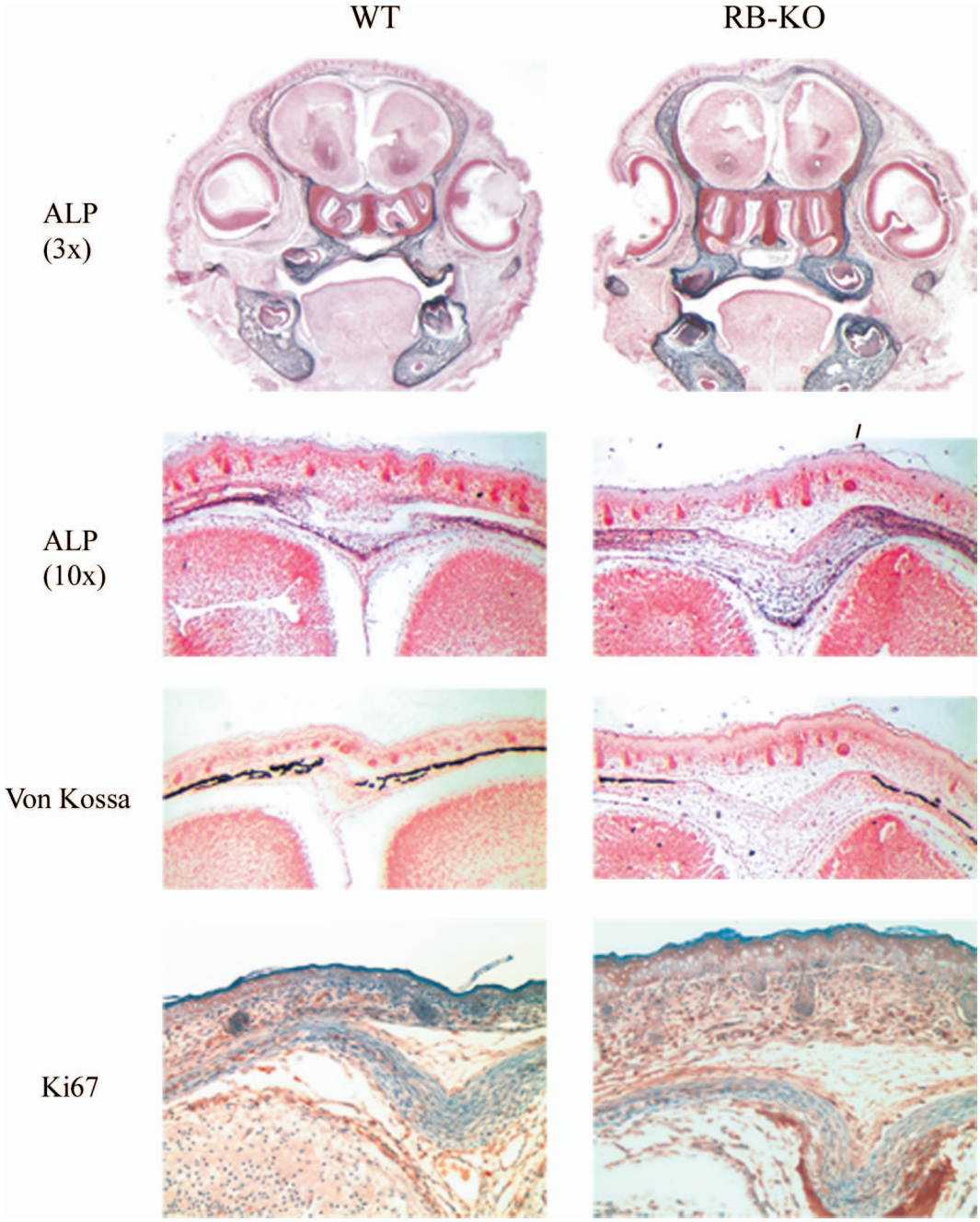

Fig. S2. *In vivo* mineralization defect in *Col3.6-Cre; Rb*$^{f19/f19}$ mice. Coronal sections of *RB1*-WT and null mice. ALP activity (*Top*), mineral deposition (von Kossa, third row), and proliferating cells (Ki67, *Bottom*) were visualized in cranial sections of WT or RB-KO mice. Alkaline phosphatase activity (blue) appears equivalent in both genotypes; however, mineralization as detected by the Von Kossa method (black) is absent at the apical-most area of the calvarium in *RB1*$^{-/-}$ animals. Ki67 staining (brown, *Bottom*) in *RB*-null animals shows a similar level of proliferation compared with that in *RB1*$^{+/+}$ animals. These sections were counterstained with hematoxylin (blue).

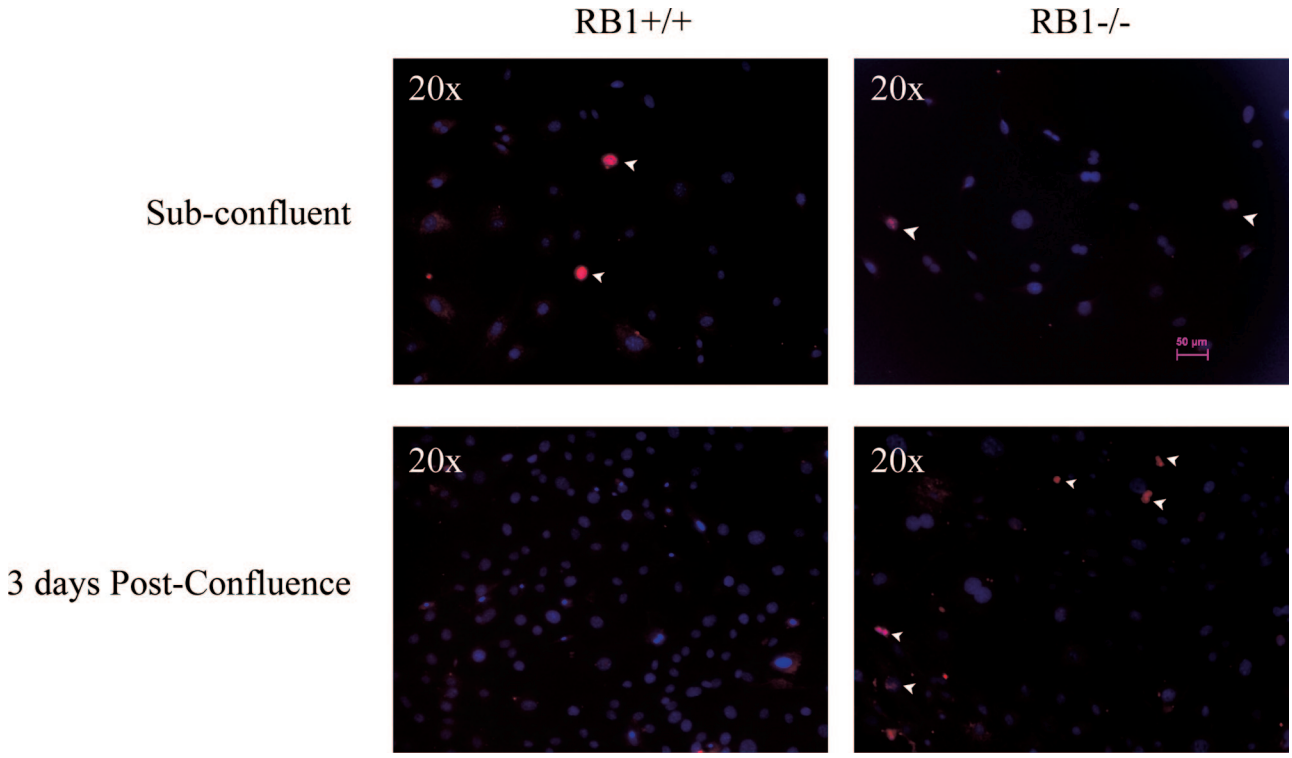

Fig. S3. *In vitro* incorporation of BrdU in post-confluent *RB1*$^{-/-}$ primary cell preparations. (*Top*) Sub-confluent primary calvarial preparations (70% confluence) were treated for 1 h with BrdU and then processed for indirect immunofluorescence as described in *Materials and Methods*. Both *RB1*$^{-/-}$ and *RB1*$^{+/+}$ cell preparations incorporated BrdU at similar rates. The percentage of BrdU-positive cells was 3.7 ± 1.1 for both genotypes, determined in three different visual fields. BrdU-positive cells are indicated by an arrowhead. (*Bottom*) Calvarial cell preparations were treated at 3 days after confluence for 1 h with BrdU and processed for indirect immunofluorescence as described. *RB1*$^{-/-}$ cell preparations continue to incorporate BrdU, whereas *RB1*$^{+/+}$ cells do not. Total nuclei were visualized by DAPI staining. Images shown are representative of three independent cell preparations for each genotype.