Supplemental Figure 1. RANKL stabilizes 3BP2 and AXIN1 protein levels through suppression of the E3 ubiquitin ligase RNF146. (A and B) qPCR analysis of Axin1 and Sh3bp2 mRNA expression in primary murine macrophages cultured in the presence or absence of RANKL (50 ng/ml), n = 3. (C) Primary murine macrophages were cultured in the presence or absence of RANKL (50 ng/ml). 3BP2 immune complexes were probed with an anti-PAR (poly(ADP-ribosylation)) or anti-3BP2 antibody. P values were determined by unpaired t-test. Data are presented as mean ± SEM. *P < 0.05.
Supplemental Figure 2. RANKL suppresses RNF146 promoter activity through activation of NF-κB. (A) Luciferase activity from the indicated RNF146 promoter constructs transfected in 293T cells. n = 3. (B) Position weight matrix of the NF-κB binding sequence. (C) Predicted NF-κB binding element in the RNF146 promoter is color-coded to match panel B (top), and mutations introduced in the P4 promoter to disrupt NF-κB binding (P4 mutant) are underlined (bottom). (D) Ratio of RANKL-treated to RANKL-untreated luciferase activity obtained from the indicated RNF146 promoter constructs transfected in RAW264.7 cells. Cells were cultured in the presence or absence of RANKL (100 ng/ml). n = 3. P values were determined by ANOVA with Tukey–Kramer's post hoc test. Data are presented as mean ± SEM. *P < 0.05.
Supplemental Figure 3. RNF146 represses osteoclastogenesis and LPS-mediated TNF-α production in bone marrow–derived osteoclast progenitors. (A) Schematic diagrams of the Rnf146loxP/loxP allele. (B) Genotyping PCR of Rnf146+/+, Rnf146fl/+, and Rnf146fl/fl mice using primers shown in the Methods section. The wild-type (WT) product is 322 bp, and the floxed product is 420 bp. (C) qPCR analysis of Rnf146 mRNA expression in primary murine osteoclast progenitors derived from wild-type (WT) and Rnf146fl/fl LysM-Cre (KO) mice. n = 3. (D) Primary murine osteoclast progenitors were derived from wild-type (WT) and Rnf146fl/fl LysM-Cre (KO) mice and lysed. RNF146 immune complexes were probed with an anti-RNF146 antibody. Whole cell lysates (WCLs) were probed with the indicated antibody for western blot analysis. P values were determined by unpaired t-test. Data are presented as mean ± SEM. *P < 0.05.
Supplemental Figure 4. *Rnf146*<sup>fl/fl</sup> *LysM-Cre* mice are osteopenic due to active osteoclastogenesis. (A-D) μCT-derived measurements of cortical major diameter (A), cortical minor diameter (B), cortical periosteal perimeter (C), and cortical endosteal perimeter (D) of 12-week-old wild-type (WT) and *Rnf146*<sup>fl/fl</sup> *LysM-Cre* (KO) mice. n = 6. P values were determined by unpaired t-test. Data are presented as mean ± SEM. *P < 0.05.
Supplemental Figure 5. Stabilization of 3BP2 is required for activated osteoclasts and TNF-α production in Rnf146<sup>fl/fl</sup> LysM-Cre osteoclast progenitors. (A) Schematic diagrams of the Sh3bp2<sup>loxP/loxP</sup> allele. (B) Genotyping PCR of Sh3bp2<sup>+/+</sup>, Sh3bp2<sup>fl/+</sup>, and Sh3bp2<sup>fl/fl</sup> mice using primers shown in the Methods section. The wild-type (WT) product is 290 bp, and the floxed product is 425 bp. (C) Whole cell lysates from primary murine osteoclast progenitors derived from wild-type (WT) and Sh3bp2<sup>fl/fl</sup> LysM-Cre (KO) mice were probed with the indicated antibodies for Western blot analysis. (D and E) TRAP staining (D) or resorption pit assay (E) of osteoclasts derived from wild-type (WT) and Sh3bp2<sup>fl/fl</sup> LysM-Cre (KO) osteoclast progenitors cultured in the presence or absence of RANKL for 7 days. n = 3. P values were determined by unpaired t-test. Data are presented as mean ± SEM. *P < 0.05.
Supplemental Figure 6. Loss of 3BP2 rescued osteopenia observed in Rnf146<sup>fl/fl</sup> LysM-Cre mice. (A-J) μCT-derived measurements of trabecular bone volume: BV/TV (A), trabecular bone mineral density (B), trabecular number (C), trabecular thickness (D), trabecular separation (E), cortical thickness (F), cortical major diameter (G), cortical minor diameter (H), cortical periosteal perimeter (I) and cortical endosteal perimeter (J) of 12-week-old wild-type (WT) and Sh3bp2<sup>fl/fl</sup> LysM-Cre (KO) mice. n = 5-6. P values were determined by unpaired t-test. Data are presented as mean ± SEM. *P < 0.05.
Supplemental Figure 7. The Wnt/β-catenin pathway is impaired in *Rnf146fl/fl LysM-Cre* osteoclast progenitors. (A) Resorption pit assay of cells in Figure 7E. n = 3. (B) Whole cell lysates from cells in Figure 7F were probed with the indicated antibodies for western blot analysis. P values were determined by ANOVA with Tukey–Kramer’s post hoc test. Data are presented as mean ± SEM. *P < 0.05.