Xia et al., http://www.jcb.org/cgi/content/full/jcb.201207154/DC1

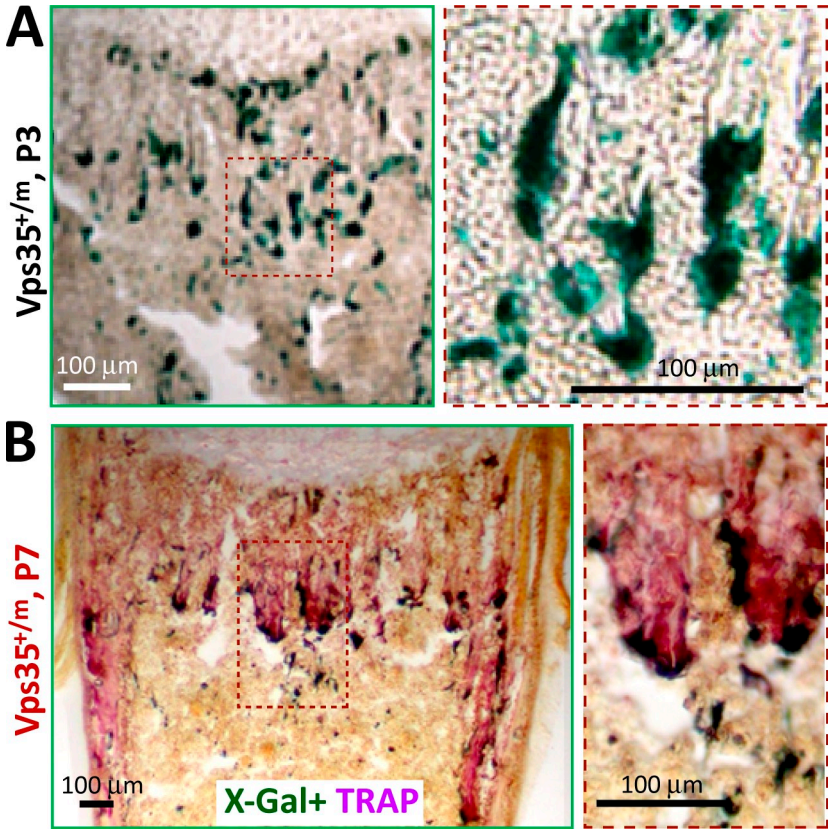

Figure S1. **Enzymatic LacZ activity in P3 Vps35$^{+/m}$ femur and co-distribution of LacZ with TRAP-positive cells in P7 Vps35$^{+/m}$ femur.** (A and B) Detection of enzymatic LacZ activity in P3 Vps35$^{+/m}$ femur (A) and co-distribution of LacZ with TRAP-positive cells in P7 Vps35$^{+/m}$ femur (B). Images marked with red squares in A and B were amplified and shown on the right.

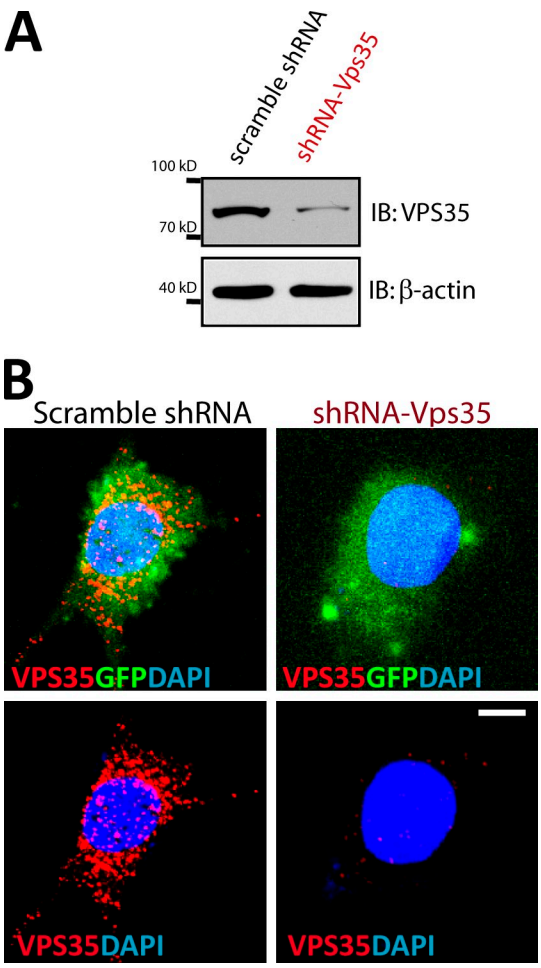

Figure S2. **Suppression of Vps35 expression in Raw264.7 macrophages by lentivirus encoding shRNA-Vps35.** (A and B) Western blot (A) and immunostaining (B) analyses using anti-VPS35 antibodies are shown. IB, immunoblot. Bar, 10 µm.

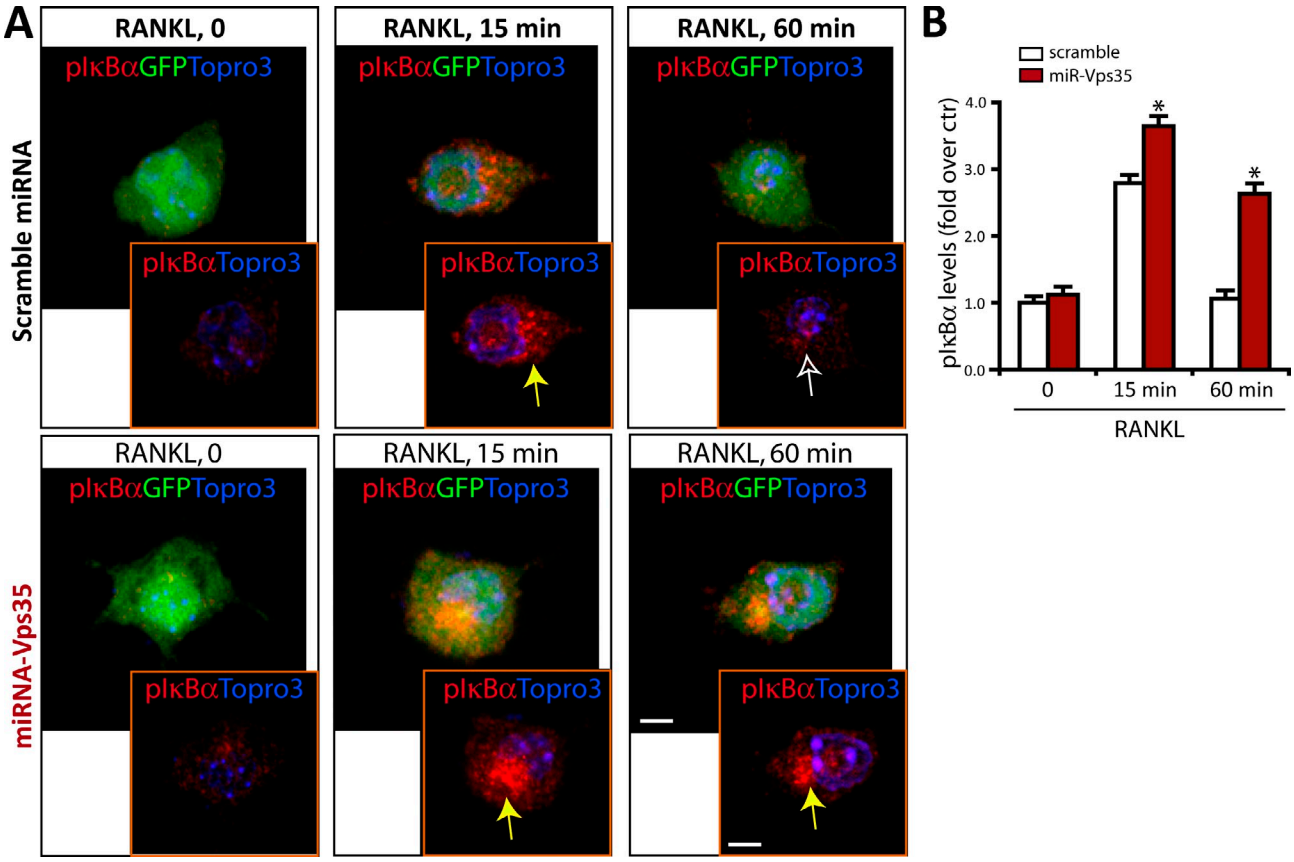

Figure S3. **Sustained RANKL-induced phosphorylation of IκB-α in Vps35-depleted macrophages.** (A and B) Immunostaining analysis of phosphor–IκB-α was performed in the control and Vps35-depleted Raw264.7 cells stimulated with 100 ng/ml RANKL for the indicated time. Representative images are shown in A. The immunofluorescence signals were quantified and presented in B. The values of means ± SD (n = 10) are shown. *, $P < 0.05$, significant difference from control. Bars, 10 µm. Note that upon RANKL stimulation for 60 min, phosphor–IκB-α signals at the perinuclei areas of Raw264.7 cells remained at intensity in Vps35-depleted, but not control, culture. The insets show the same cell without GFP signal. The yellow arrows indicate positive staining signals at the perinuclei areas, and the open arrow indicates reduced staining signal. ctrl, control.

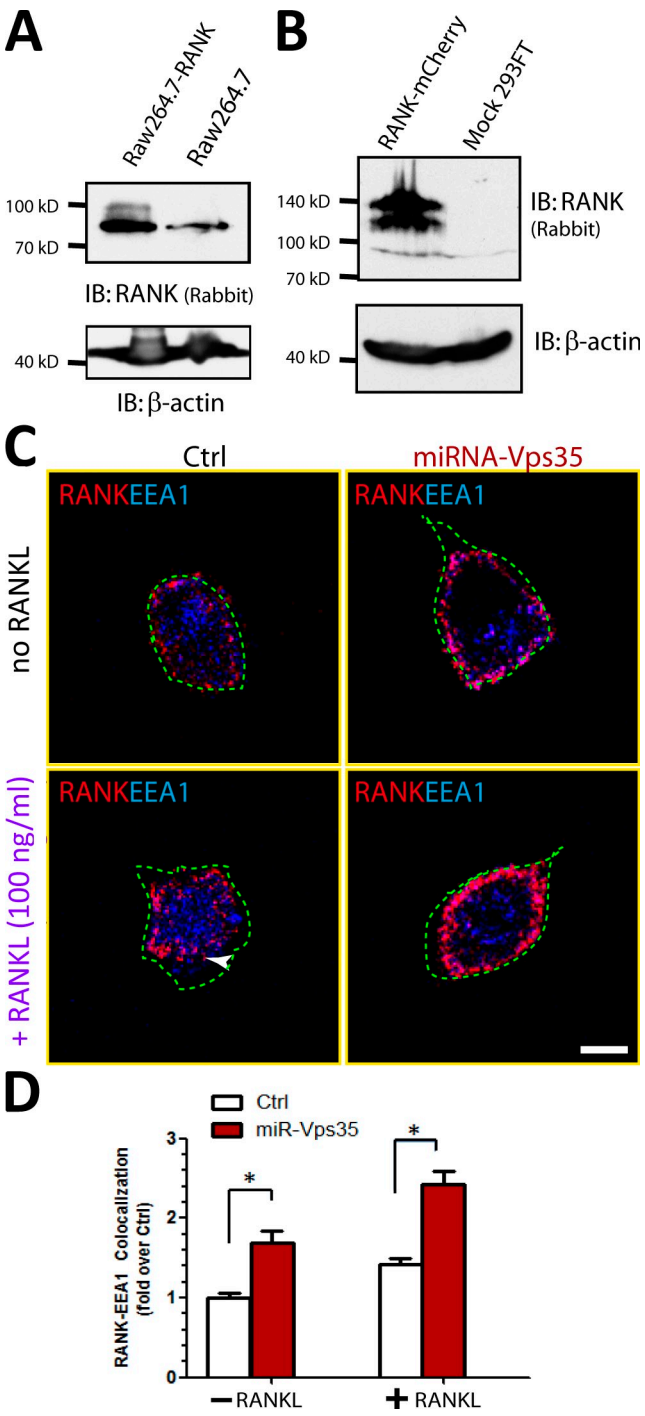

Figure S4. **RANK antibody specificity.** (A and B) The polyclonal anti-RANK (from Cell Signaling Technology) antibody specifically recognizes endogenous RANK protein (~80 kD) in lysates of Raw264.7 cells and Raw264.7 cells stably expressing RANK (A) and exogenous RANK-mCherry fusion protein in lysates of transfected HEK 293T cells (B) by Western blot analyses. IB, immunoblot. (C) Increased RANK in early endosomes in Vps35-depleted Raw264.7 cells. Confocal representative images of coimmunostaining analyses are shown. The dotted lines indicate the borders of the cell. The arrowhead indicates the colocalization signal. Bar, 10 µm. (D) The quantitative analysis of RANK-EEA1 colocalization signal over total RANK in miRNA-expressing cells is shown (means ± SD, *n* = 10). *, P < 0.05, significant difference from control. Ctrl, control.

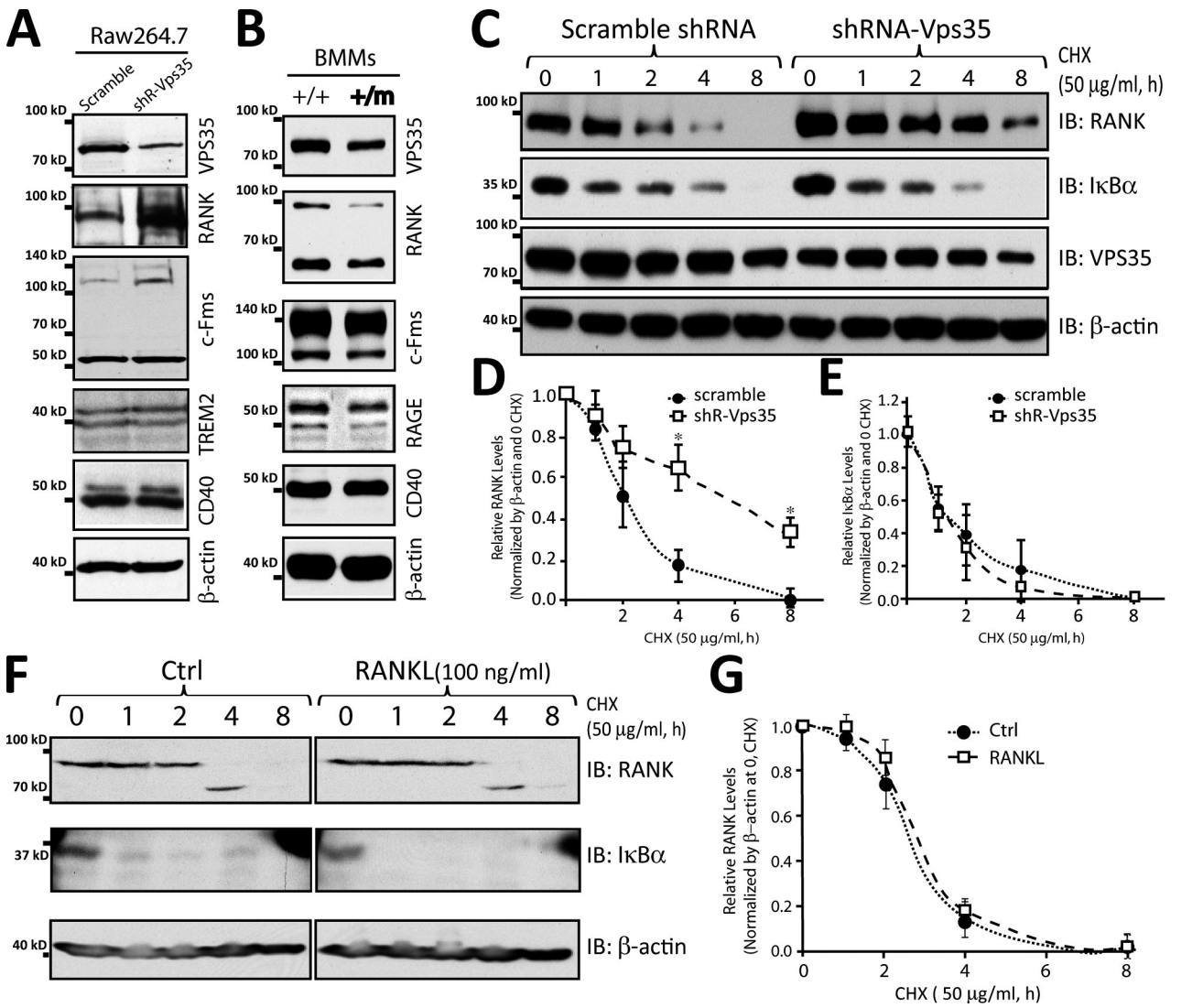

Figure S5. **Increased RANK protein levels and impaired RANK degradation in Vps35-depleted Raw264.7 cells, but not Vps35$^{+/m}$, BMMs.** (A) Increased RANK levels, but not TREM2 or CD40, in Vps35-deficient Raw264.7 cells were revealed by Western blot analyses. (B) RANK levels were slightly reduced in Vps35$^{+/m}$ BMMs. (C) Impaired RANK degradation in Vps35-deficient Raw264.7 cells was determined by Western blot analysis of cell lysates treated with 50 μg/ml cycloheximide (CHX) for the indicated time. (D and E) Quantification analysis of data from C revealed the time-dependent degradation of RANK (D) and IκB-α (E) in control and Vps35-deficient cells. (F and G) RANK degradation in Raw264.7 cells was unaffected by RANKL. In D, E, and G, data were quantified from three different experiments, and the values of means ± SD (n = 3) are presented. *, $P < 0.05$, significant difference from control. IB, immunoblot; Ctrl, control.