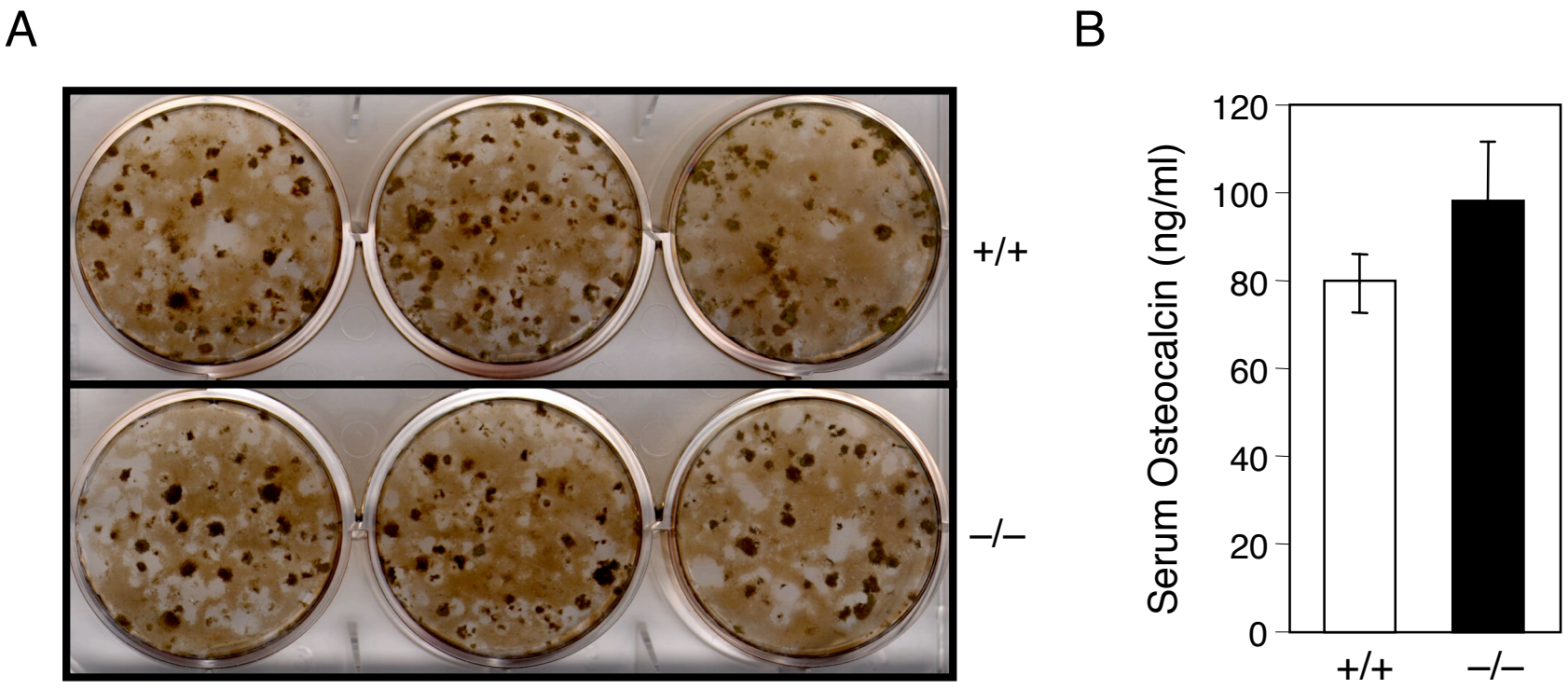

Figure S1. Effect of CYLD deficiency on osteoblastic differentiation and serum osteocalcin. (A) Bone marrow cells (5 X 10^7/well in 6-well plates) were cultured in MEMa medium supplemented with 10% FBS, 1% penicillin/streptomycin, b-glycerophosphate (10mM) and L-ascorbic acid phosphate (50m g/ml) for 21 days, then fixed with 4% paraformaldehyde, and stained by the von Kossa method to detect mineralized bone nodule formation. Cells in the upper three wells were from $CYLD^{+/+}$ mice, and cells in the bottom three wells were from $CYLD^{-/-}$ mice. (B) Sera were collected from $CYLD^{-/-}$ and $CYLD^{+/+}$ mice (4 of each genotype) and subjected to ELISA to measure osteocalcin concentration.

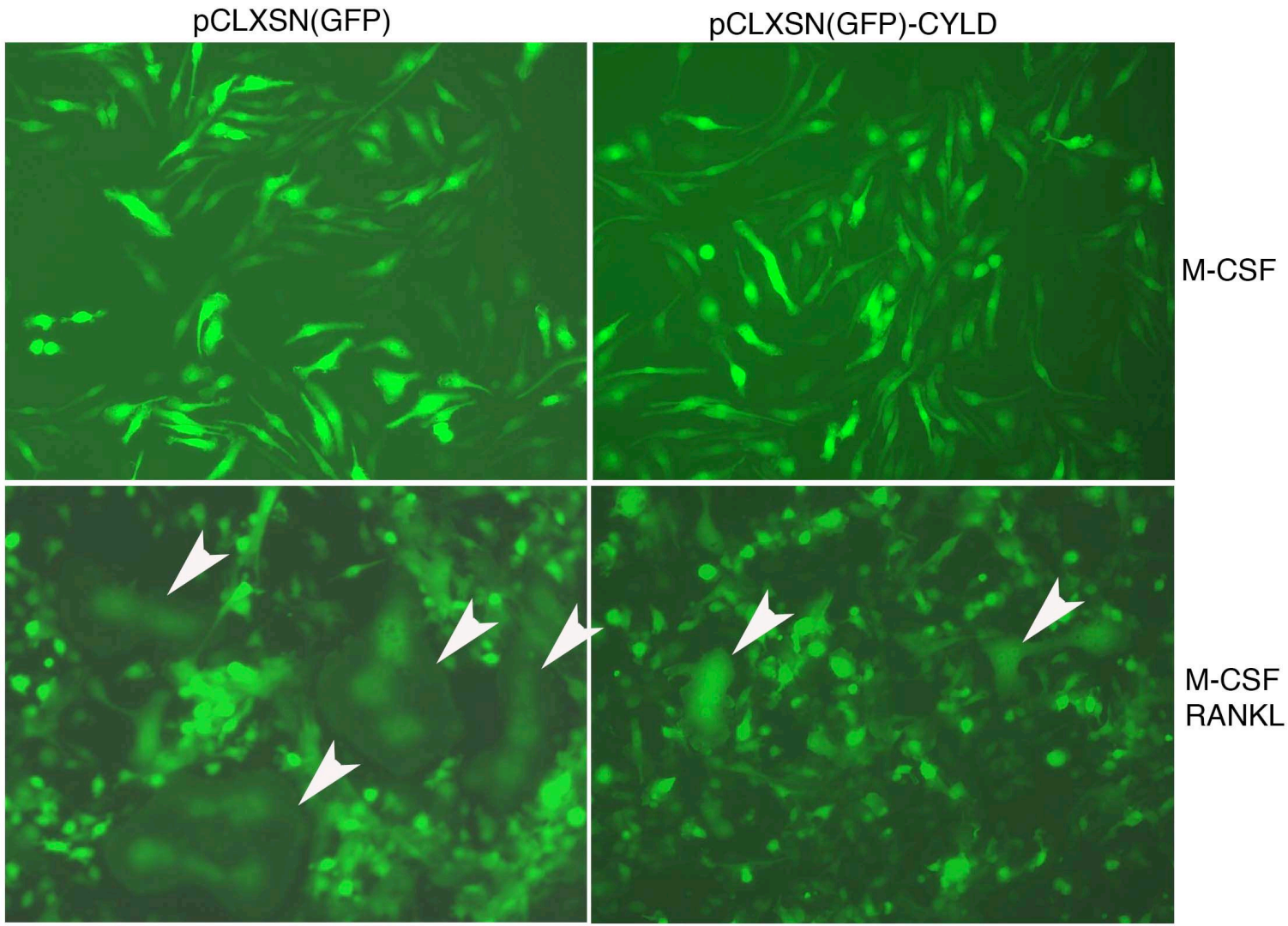

Figure S2. Inhibition of osteoclast differentiation by overexpressed CYLD. Wildtype bone marrow derived macrophages were infected with a retroviral vector carrying a GFP marker gene, pCLXSN(GFP), or the same vector encoding CYLD, pCLXSN(GFP)-CYLD. Infected cells were cultured for 4 days in DMEM media supplemented with either MCSF or MCSF plus RANKL and subjected to fluorescence microscopy. CYLD-infected cells form substantially fewer and smaller osteoclasts (arrowheads).

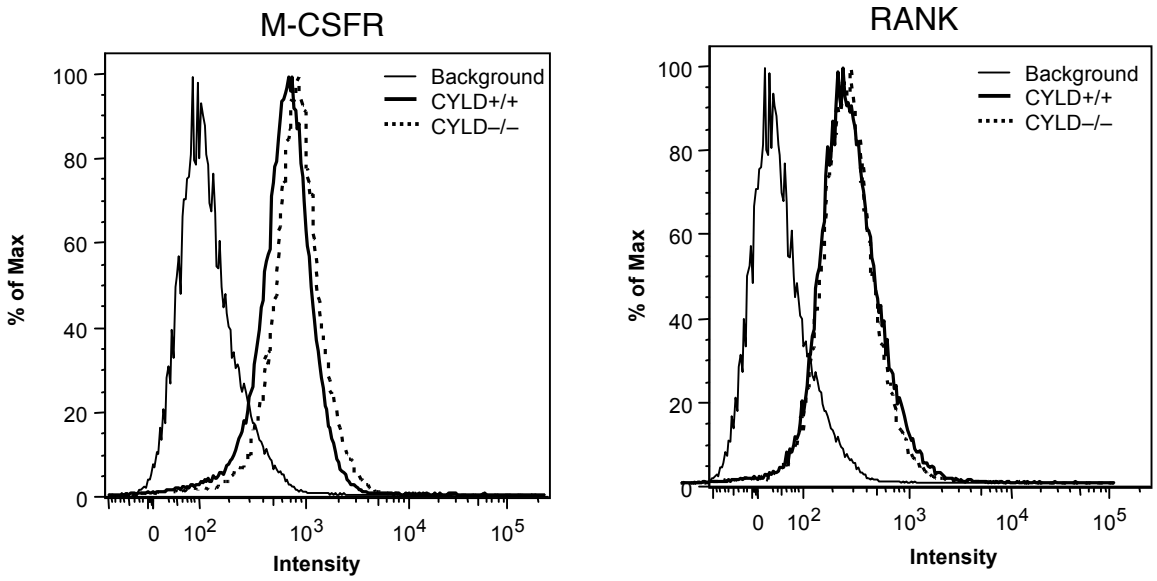

Figure S3. Surface expression of RANK and M-CSF receptor (M-CSFR) on osteoclast precursors. Bone marrow derived macrophages prepared from CYLD$^{+/+}$ and CYLD$^{-/-}$ mice were either unstained (background) or stained with PE-conjugated anti-mouse RANK or PE-conjugated anti-mouse M-CSFR and subjected to flow cytometry analysis.

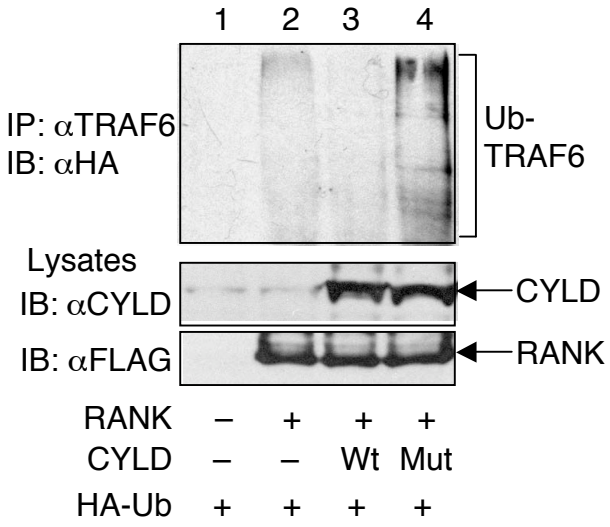

Figure S4. Inhibition of RANK-induced TRAF6 ubiquitination by transfected CYLD. 293 cells were transfected with HA-tagged ubiquitin along with the indicated expression vectors. Endogenous TRAF6 was isolated by IP using anti-TRAF6, and its ubiquitination was detected by IB using HRP-conjugated anti-HA. Expression of CYLD, a catalytically inactive CYLD mutant (1-932), and RANK were monitored by IB.

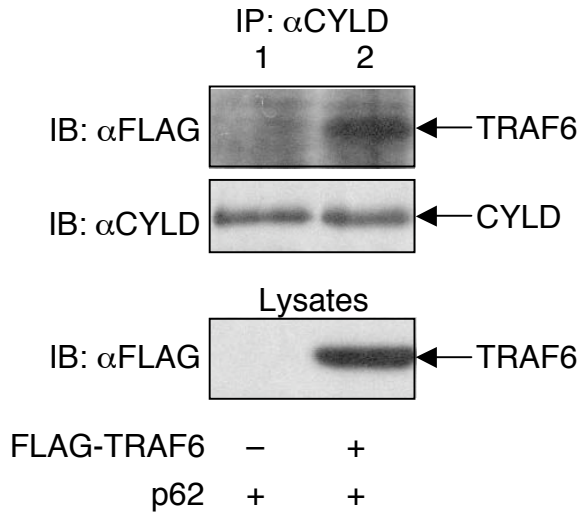

Figure S5. CYLD interacts with TRAF6. 293 cells were transfected with (+) or without (–) the indicated expression vectors. Endogenous CYLD complex was isolated by IP, and the precipitated CYLD and CYLD-associated TRAF6 was detected by IB using anti-CYLD and anti-FLAG, respectively (top two panels). Expression of TRAF6 in the lysates was analyzed by direct IB (bottom panel).

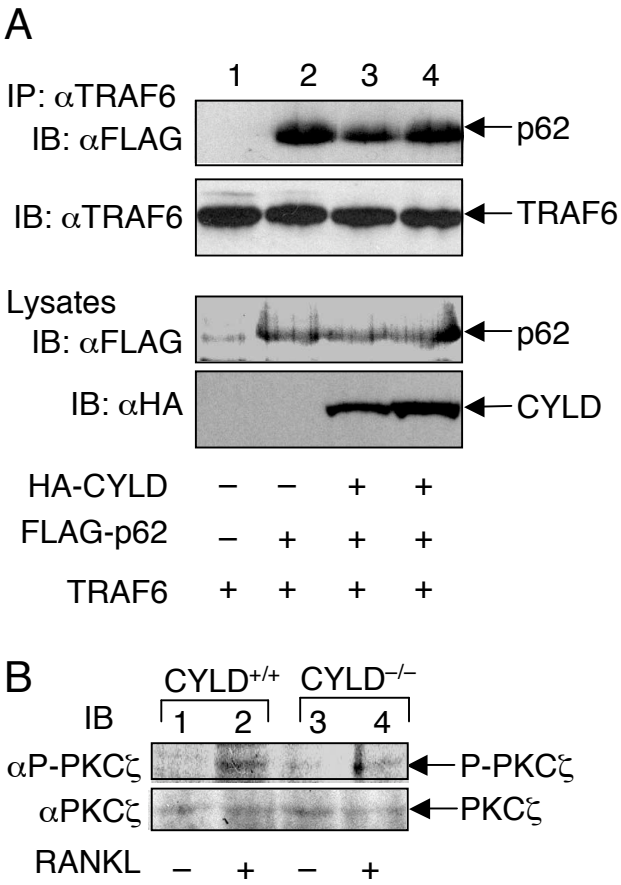

Figure S6. CYLD does not regulate the p62/TRAF6 association or activation of PKCz. (A) 293 cells were transfected with the indicated expression vectors. TRAF6 complexes were isolated by IP using anti-TRAF6, and the associated p62 was detected by IB using anti-FLAG (panel 1). The levels of precipitated TRAF6 and p62 and CYLD in the lysates were monitored by IB. (B) BMDM derived from CYLD+/+ or CYLD–/– mice were cultured for 2 days either in the presence (+) or absence (–) of GST-RANKL. Phosphorylated and total PKCζ were detected by IB using the indicated antibodies.