SUPPLEMENTAL FIGURES

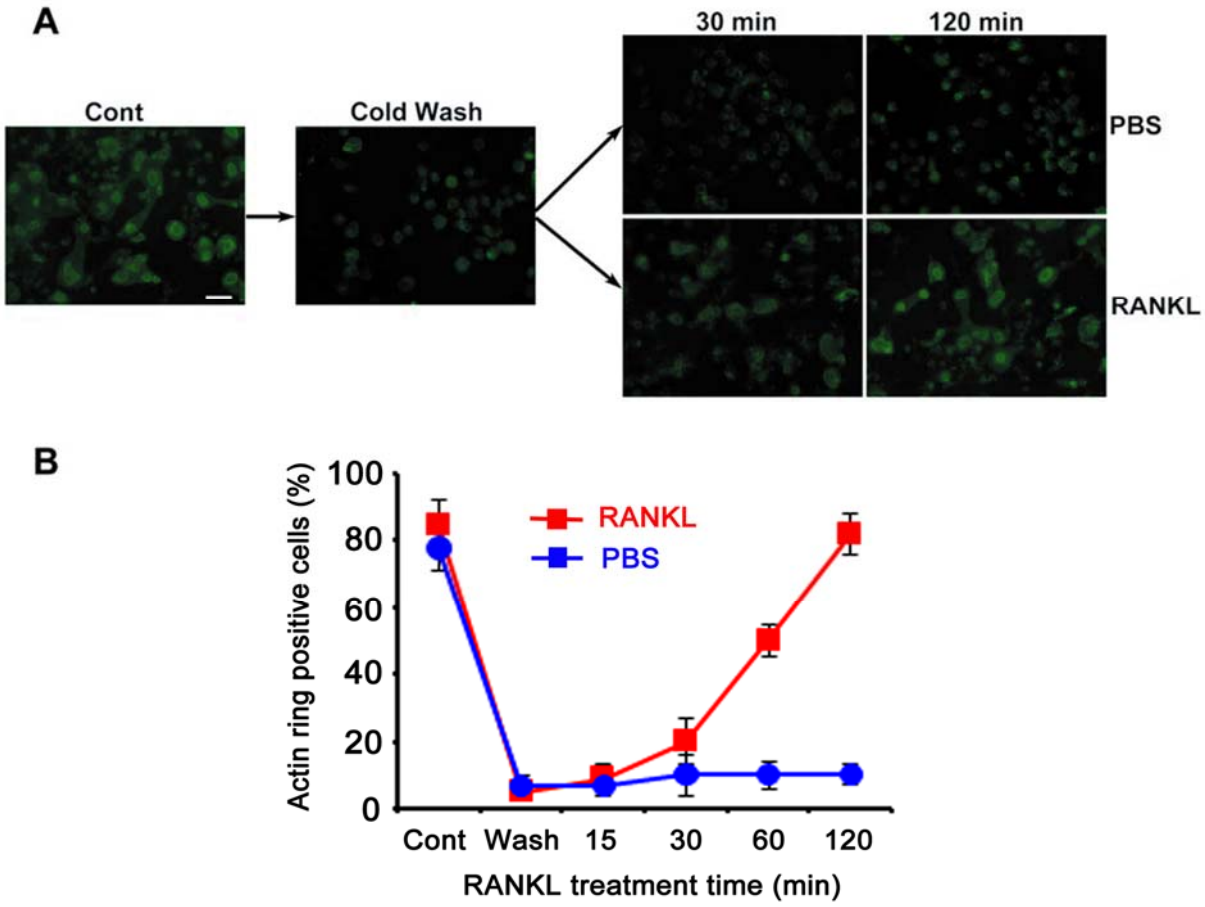

Figure S1. RANKL regulates the osteoclast cytoskeleton. A) Mature osteoclasts, generated from bone-slice residing BMMs cultured in M-CSF and RANKL, for 6 days, were washed in cold, cytokine-free medium, to disrupt actin rings. Following incubation in the same medium, at 37°C for 30 minutes, RANKL (100 ng/ml) or PBS was added with time. Bone slices were fixed, and F-actin was stained with Alexa Fluor 488-phalloidin to visualize actin rings. The experiments were repeated three times. B) % of osteoclasts with actin rings were counted. Scale bar, 200 μm. The data are shown as the mean ± SD of triplicate samples.

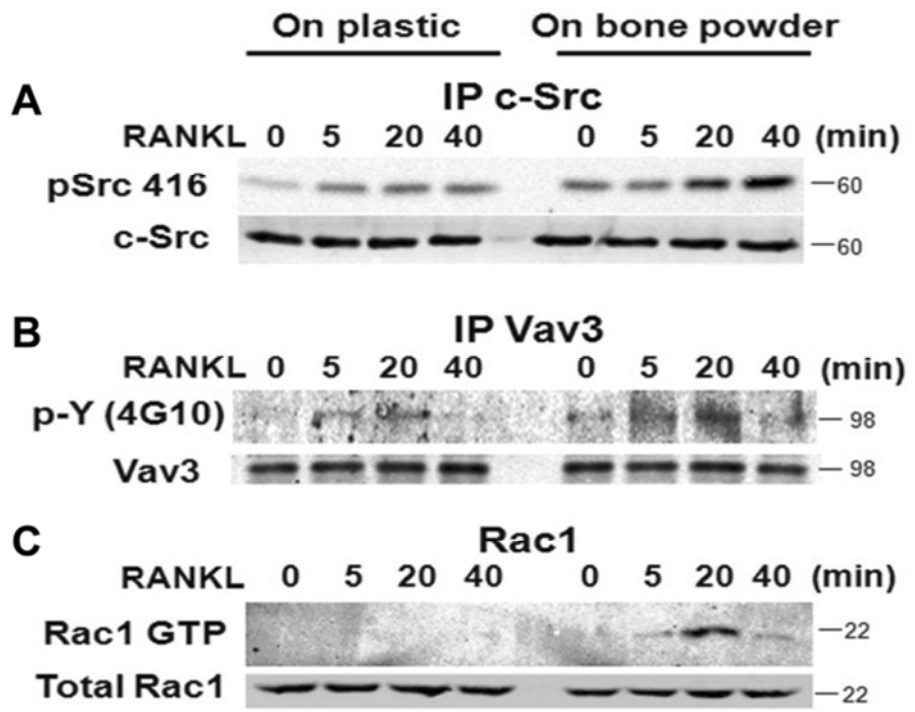

Figure S2. Bone powder enhances RANKL-mediated cytoskeleton-organizing molecule activation. WT spleen cells were cultured with RANKL and M-CSF for 5 days on plastic or bone powder. Serum- and cytokine-starved cells were exposed to RANKL (100 ng/ml) for 5, 20, 40 min. A) c-Src^{Y416} and c-Src were immunoblotted in c-Src immunoprecipitates. B) Phospho-tyrosine and Vav3 were immunoblotted in Vav3 immunoprecipitates. C) Rac1-GTP was assayed by pulldown assay and total Rac1 immunoblotted in lysates. Results are shown as representative of three independent experiments.

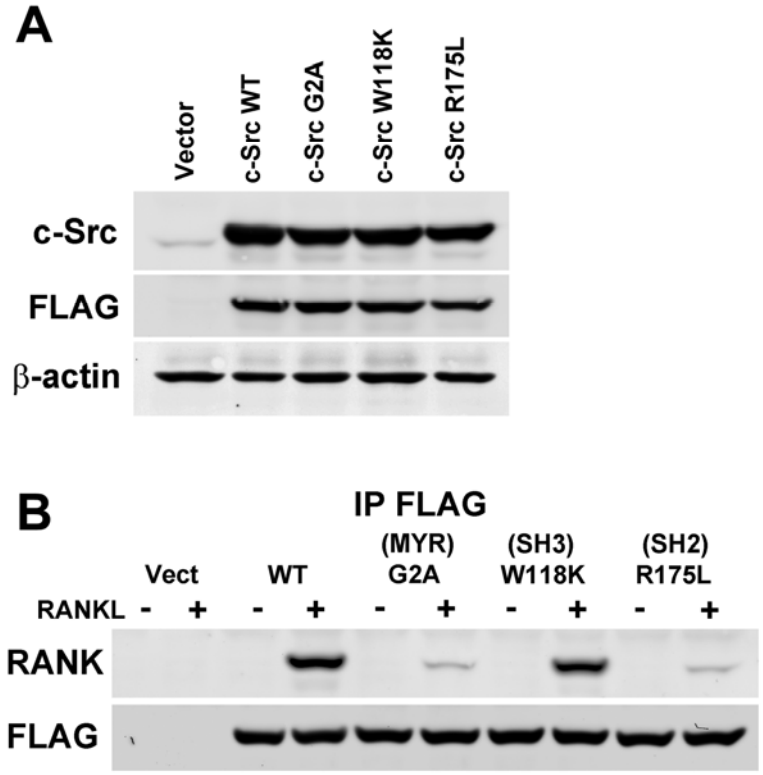

Figure S3. c-Src N-terminal myristoylated region and SH2 domain mediate RANK association. (A) Lysates of c-Src-/-. splenic macrophages, retrovirally transduced with empty vector or FLAG-tagged WT c-Src or inactivating Myr (c-Src^{G2A}), SH3 (c-Src^{W118K} or SH2 (c-Src^{R175L}) domain point mutants were immunoblotted with anti-FLAG or anti-c-Src mAb. (B) Cytokine-starved c-Src-/- pre-osteoclasts, retrovirally transduced with FLAG-tagged WT or point mutant c-Src constructs, were maintained +/- RANKL for 30'. FLAG immunoprecipitates were immunoblotted for RANK or FLAG. Results were representative of three independent experiments.

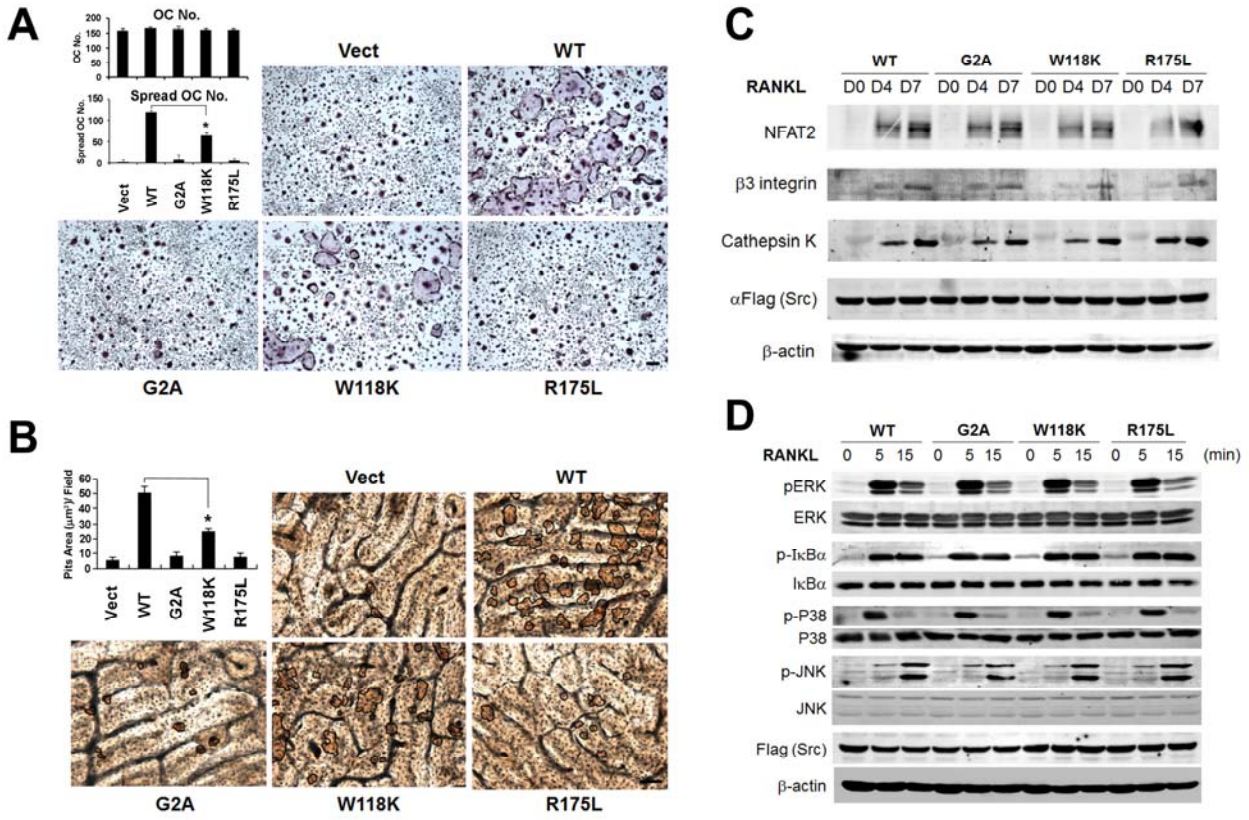

Figure S4. c-Src N-terminal myristoylated region and SH2 domain principally organize the osteoclast cytoskeleton but do not regulate differentiation. (A) Spleen cells of Src-/- mice, transduced with c-Src domain inactivating point mutants were cultured with M-CSF and RANKL for 7 days after which the cells were stained for TRAP activity. Total number of osteoclasts and those spread were counted. *p<0.05). Scale bar, 200 μm. The data are shown as the mean ± SD of triplicate samples. (B) Transduced osteoclasts, generated on bone, were removed after 5 days. Resorptive lacunae were stained by lectin (brown reaction product outlined) and pit area determined. *p<0.05). Scale bar 200 μm. The data are shown as the mean ± SD of triplicate samples. (C) c-Src-/- splenic macrophages, transduced with either empty vector or WT or point mutant c-Src were cultured in RANKL and M-CSF, with time. Osteoclast differentiation markers were determined by immunoblot. Actin serves as loading control. (D) c-

Src-/- splenic macrophage, transduced as in C, were cultured in RANKL and M-CSF for 3 days. The cells were cytokine-starved and exposed to RANKL, with time. Signaling molecules were identified by immunoblotting. Actin serves as loading control. Results are representative of three independent experiments.

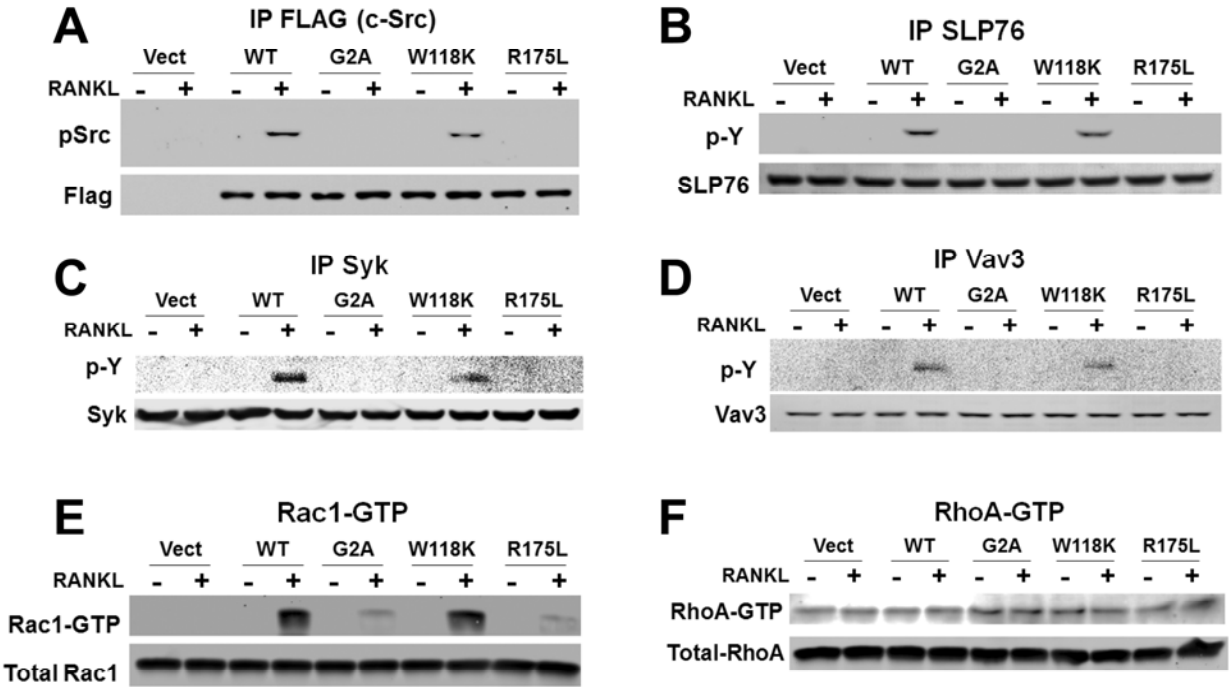

Figure S5. RANKL activates cytoskeleton-organizing molecules principally via c-Src MYR and SH2 domains. c-Src-/-spleen cells, transduced with either FLAG-tagged WT or point mutant c-Src were cultured with RANKL and M-CSF for 5 days on bone powder. Serum- and cytokine-starved cells were exposed to RANKL (100 ng/ml) for 20 min. (A) Phosphorylated c-Src^{Y416} and FLAG, in FLAG immunoprecipitates, (B) phosphotyrosine and SLP-76 in SLP-76 immunoprecipitates, (C) phosphotyrosine and Syk in Syk immunoprecipitates and, (D) phosphotyrosine and Vav3 in Vav3 immunoprecipitates were determined by immunoblot. (E) Rac1-GTP and (F) RhoA-GTP were detected by pulldown assay and total GTPase, by immunoblot. The experiments were repeated twice.

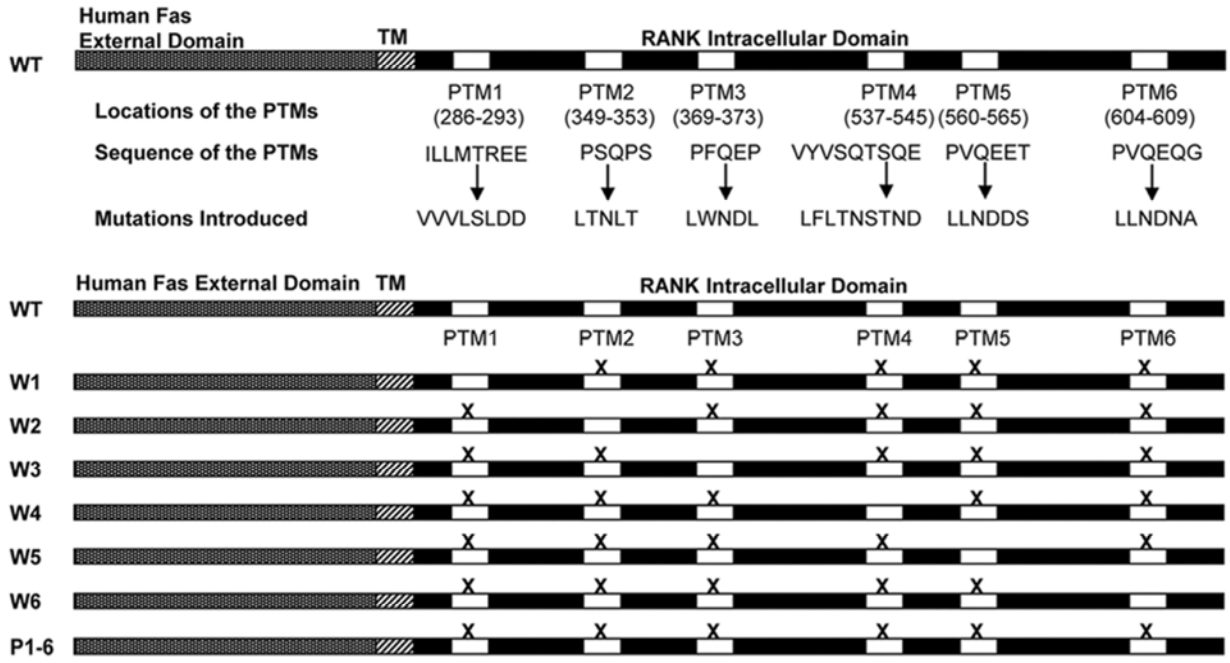

Figure S6. Schematic diagram of hFas/RANK cytoplasmic domain mutants, designated W1, W2, W3, W4, W5, and W6, in which all but one PTM is deleted. hFas/RANK-WT and hFas/RANK-P1-6 serve as respective positive and negative controls.