E07-04-0337 Ryu

Supplemental Methods

Isolation of detergent-resistant fractions. Cells were rinsed in PBS and then scraped in 1% Triton X-100 (v/v), containing 10 mM Tris/pH 8.0, 0.15 M NaCl and protease inhibitors. After 20 min on ice, lysates were centrifuged at 15,000×*g* for 20 min at 4°C. Supernatants (non detergent-resistant membranes) and pellets (detergent-resistant membranes) were retained. Pellets were resuspended in Triton X-100 lysis buffer containing 60 mM β-octylglucoside.

In vitro binding analysis. To assay the binding of PAK to V12-Rac1 or mutant V12-Rac1-6Q, endogenous PAK was isolated by immunoprecipitation from 500μg of OVCAR-3 cell lysate using anti-PAK antibodies (SantaCruz, CA). Purified isoprenylated Rac1 was preloaded with GDPβS and GTPγS, and then incubated with immobilized PAK proteins for 2 hour at 4°C. Beads were sedimented and washed four times with 50 mM Tris-HCl/pH 7.6, containing 150 mM NaCl, 1% Triton X-100, and 0.5 mM $MgCl_2$. Bound Rac proteins were detected by western blotting.

Cell migration assay. Migration was assessed using modified Boyden chambers containing Transwell filters (8-μm pores; Corning) coated on the underside with 5 $\mu g/cm^2$ fibronectin. OVCAR-3 cells (2×10^5) were added to the upper chamber of a Transwell filter, with buffer containing or not containing PA in the lower chamber. After incubation for 8 h, cells remaining on the filter upper surface were removed with a cotton swab. Cells on the lower side were fixed with 4% formaldehyde and stained with

0.1% crystal violet. The dye was extracted into 1% sodium deoxycholate and quantified by measuring absorbance at 590 nm

Supplemental Figure Legends

Figure S1. PLD and β3-integrin coprecipitations with actin cytoskeleton depend on integrin engagement. OVCAR-3 cells were serum starved for 24 hr, detached from culture dishes, maintained in suspension for 3 hr, replated on FN or PLL for 20 min, and then harvested and fractionated in Triton X-insoluble pellets (cytoskeleton fraction) as described in "Supplemental Methods" Samples were analyzed by western blotting using anti-β3-integrin, anti-PLDs, caveolin-1, or actin antibodies.

Figure S2. PLD activity did not affect integrin-induced cellular tyrosine phosphorylation, FAK, or Src activation. (A) OVCAR-3 cells were serum starved for 24 hr, detached from culture dishes, maintained in suspension for 3 hr, and replated on FN or PLL with 1-butanol (0.2% v/v), t-butanol (0.2% v/v), or 1-butanol and PA (50 μM) for 20 min. Cells were then harvested and total tyrosine phosphorylation levels were analyzed by western blotting with anti-phospho tyrosine antibody. **(B)** Cells were treated as described in **(A)** and FAK and Src kinase activities were analyzed by western blotting with anti-phosphospecific FAK (pY397-FAK) or Src (pY416-Src) antibodies. Actin was used as a loading control for western blotting. The result shown is representative of three independent experiments.

Figure S3. The polybasic region of Rac1 did not affect the interaction between

Rac1 and PAK. Purified isoprenylated Flag-V12-Rac1 or Flag-V12-Rac1-6Q mutants were preincubated with GTPγs or GDPβs, and then incubated with immobilized purified PAK. Bound Rac1 proteins were detected by western blotting. The result shown is representative of three independent experiments.

Figure S4. PLD activity was required for integrin-mediated cell migration. (A) Cells were transfected as indicated with siRNA PLD1 or PLD2, or with luciferase as a control. One day after siRNA transfection, cells were transfected with empty vector or PLD 1 WT plus 2 WT (PLD1, 2^{WT}) or PLD 1 lipase inactive mutant plus 2 lipase inactive mutant (PLD1, 2^{KRM}), as indicated. After serum-starvation for 24 hr, cells were detached from culture dishes, maintained in suspension for 3 hr, and added to the upper chamber of a Boyden unit, which was fitted with a filter coated on its underside with FN. After 3hr, migration was measured. Cell migrations were quantified by normalizing against those of control luciferase and vector transfected cells. Error bars represent means ± SD (n=3). **(B)** OVCAR-3 cells were transfected with the indicated control luciferase or siRNA PLD1 and PLD2. One day after siRNA transfection, cells were transfected with control vector or V12-Rac1 or V12-Rac1-6Q, as indicated. After serum-starvation for 24 hr, cells were detached from culture dishes, and maintained in suspension for 3 hr. Cells were added to the upper chamber of a Boyden unit containing a filter coated on its underside with FN with/without PA containing buffer in its lower chamber. Migration levels were quantified 3hr later, migrated cell numbers were normalized versus control luciferase or vector transfected cells. Error bars represent means ± SD (n=3)

Figure S5. PLD activity inhibition by 1-buthanol treatment inhibits integrin-mediated PAK kinase activation. After serum-starvation for 24 hr, cells were detached from culture dishes, maintained in suspension for 3 hr, replated on FN with medium containing 1-butanol (0.2% v/v), t-butanol (0.2% v/v), or 1-butanol plus PA (50 μM) for 20 min. Cells were then harvested and incubated with immobilized anti-PAK1 immune complex and bead control. PAK proteins were immunoprecipitated and PAK kinase activities were assayed. Immunoprecipitated samples were separated on SDS-polyacrylamide gels, followed by gel staining, and MBP phosphorylations represent PAK kinase activity. The result shown is representative of three independent experiments.

Figure S6. Both PLD1 and PLD2 isozymes contribute to integrin-mediated cell spreading. (A) HEK293T cells were transfected as indicated with siRNA PLD1 or luciferase as a control. One day after siRNA transfection, cells were transfected with empty vector or PLD1 WT, and COS-7 cells were transfected with siRNA PLD2 or luciferase as controls. One day after siRNA transfection, cells were transfected with empty vector or PLD2 WT. Cells were then harvested, and PLD and β3-integrin expression levels were analyzed by western blotting using antibodies directed against PLDs or β3-integrin. Actin was used as a loading control for western blotting. The results shown are representative of three independent experiments. **(B)** Cells treated as in (A) were replated on FN for 20 min. After removing unbounded cells, cells were fixed and stained with TRITC-labeled phalloidin (n=3). Scale bar, 50 μm. **(C)** The graph shows numbers of spreading cells from (B). Error bars represent means ± SD

(n=3).”

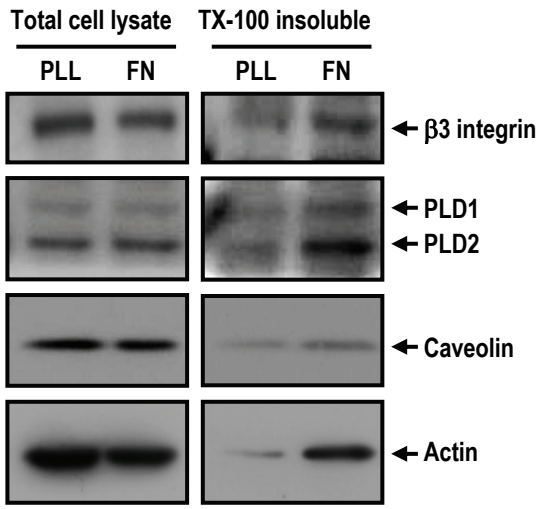
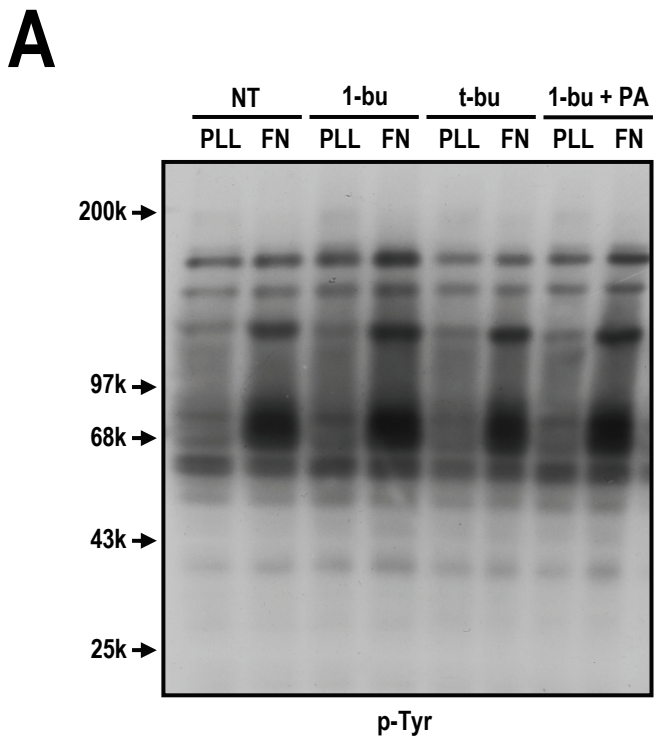


Fig. S1 Chae et al.



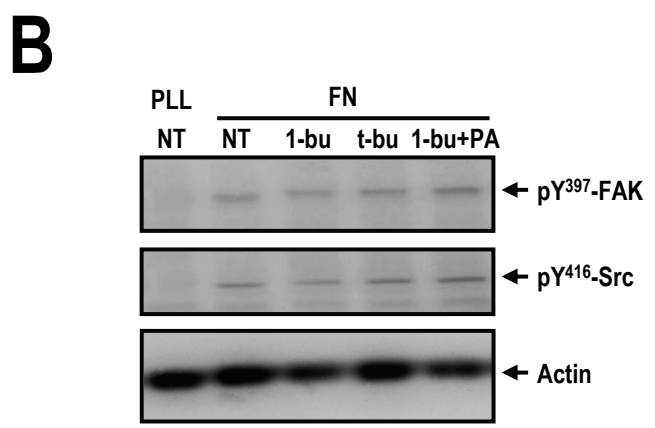
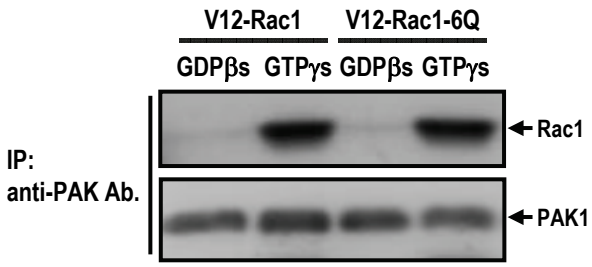


Fig. S2 Chae et al.



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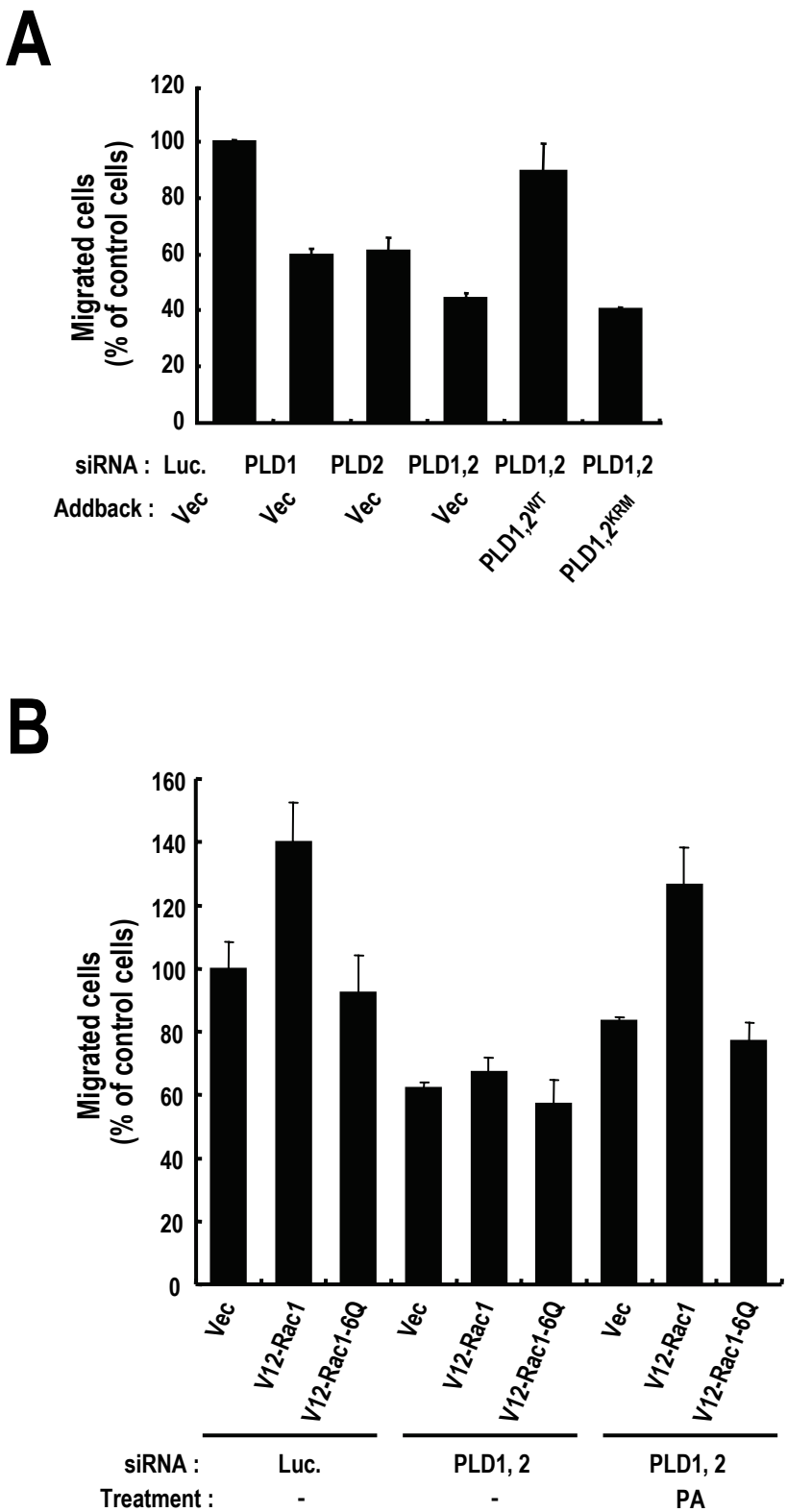

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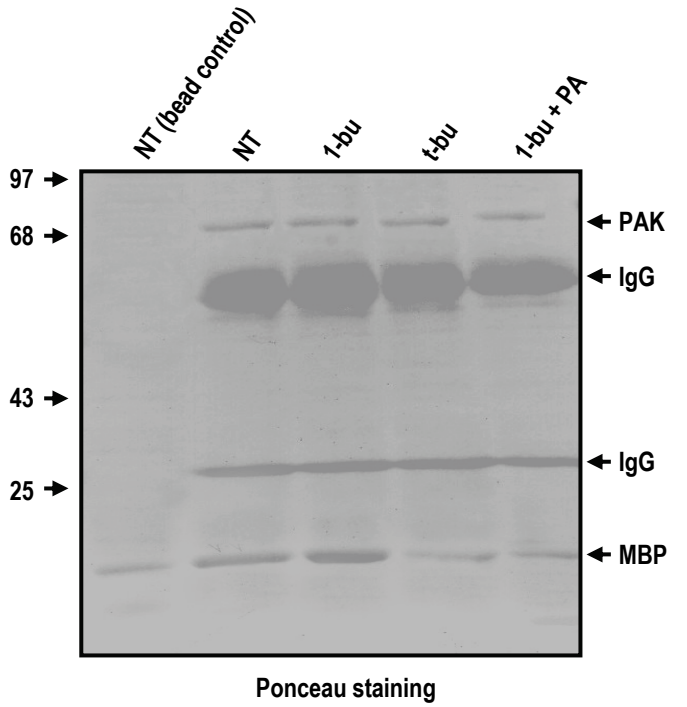


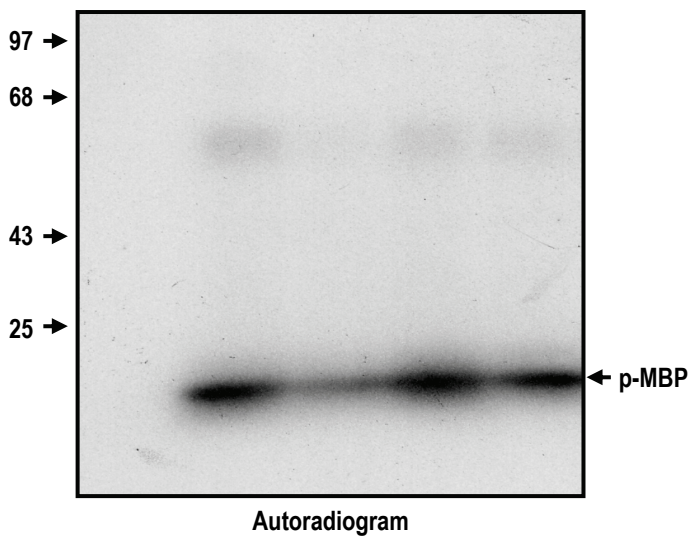
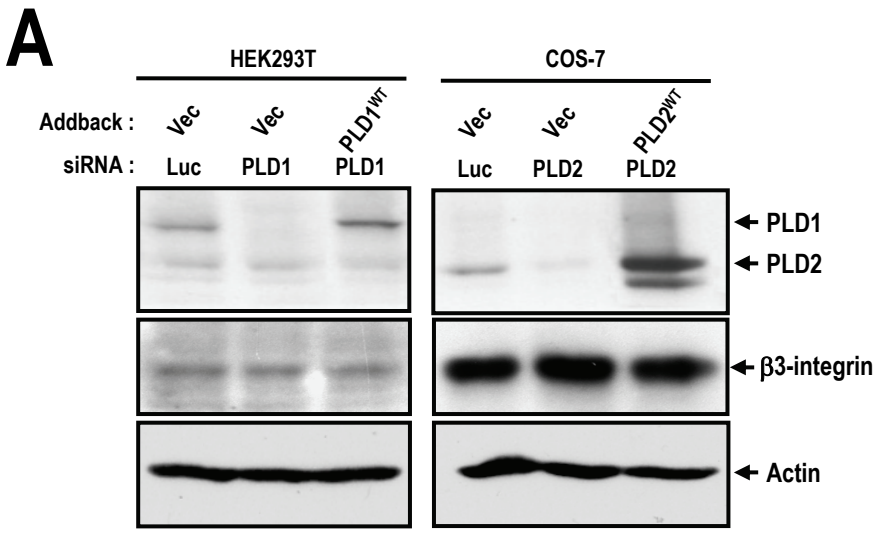
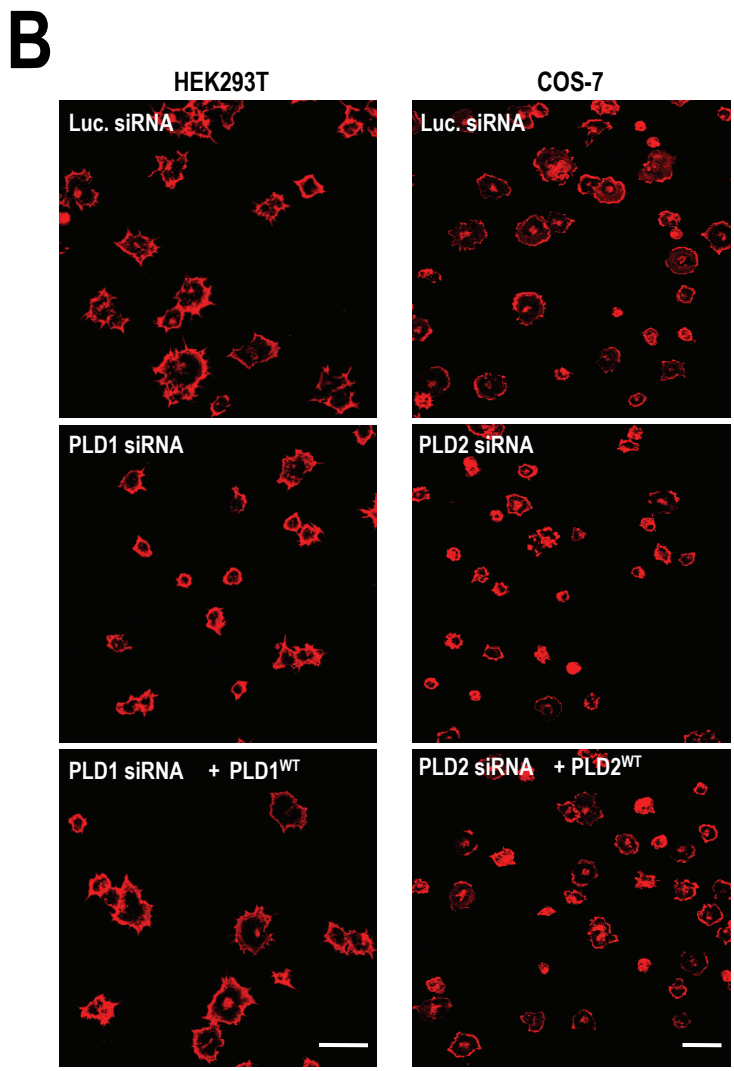


Fig. S5 Chae et al.





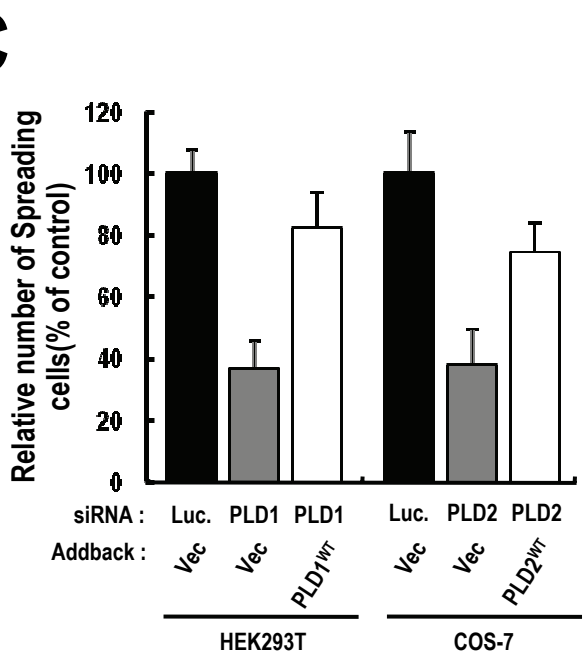


Fig. S6 Chae et al.