Supporting Materials and Methods

Plasmid Construction. To create the pLJ-YAAA and pLJ-FAAA mutants, double-stranded blunt-*Xba*I oligos as indicated below were subcloned into *Stu*I-*Xba*I-digested pGMT vector (YAAA: 5'-agt acg ccg ccg ccg aag atc tgt at-3'; FAAA: 5'-agt ttg ccg ccg ccg aag atc tgt at-3'). The *Bam*HI-*Xho*I fragments from each resulting plasmid were subcloned into *BamH*I-*Sal*I-digested pLJ vector. Site-directed mutagenesis was performed (Stratagene) to create a point mutation for M253A by using the primers 5'-cct att ctg tcg cga gga gcc actcct at-3' and 5'-agt ggc tcc tcg cga cag aat agg tcg gg-3'. For the add-back mutants, double-stranded oligos were subcloned into a unique restriction site, *Esp*I (1) (EEEEEYMPM: 5'-tga gcg aag aag aag aag aat aca tgc caa tgt-3'; EEEEEFMPM: 5'-tga gcg aag aag aag aag aat tca tgc caa tgt-3'; EEEEEYAAA: 5'-tga gcg aag aag aag aag aat acg ccg ccg cct-3'). Insertion into the *Esp*I site resulted in an additional serine at the beginning and a leucine at the end of the insertion, respectively.

Cells and Tissue Culture. The Bosc 23 viral packaging cell line (2) was grown in DMEM with 10% FBS supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen) in 10% CO_2. BALBc/3T3 fibroblasts (clone A31) were grown in DMEM with 10% CS, supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin in 10% CO_2.

Retroviral Infections and Establishment of BALBc/3T3 Clones. Bosc 23 cells were transfected with 10 μg of DNA constructs by using the BES method as described (3). Infections with viral supernatants, drug selection, and isolation of G418-resistant colonies were carried out as described (4).

Antibodies, Immunoprecipitations, and Immunoblotting. Rabbit anti-ShcA antibody (Upstate Biotechnology) and rabbit anti-MT antibody 18-8 (5) were used for immunoprecipitations. Mouse anti-ShcA antibody (BD Transduction Laboratories),

mouse anti-MT antibody F4 (5), rabbit anti-phospho-Akt antibody (Cell Signaling Technologies), and rabbit anti-Akt antibody (Cell Signaling Technologies) were used for immunoblotting. Rabbit anti-p85 antibody R32 (K. Auger and T.M.R., unpublished work) was used for immunoprecipitations and immunoblotting. Confluent 100- or 150-mm dishes of cells were incubated in DMEM with 0.1% CS overnight. Cells were rinsed twice with 1× PBS solution, lysed in NP-40 lysis buffer (20 mM Tris, pH 8.0/137 mM NaCl/1% Nonidet P-40/10% glycerol/ 5 mM EDTA) with inhibitors (25 mM β-glycerophosphate, 1 mM Na_3VO_4, 0.03 units/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 100 mM PMSF) for 30 min and harvested. Protein concentrations were normalized by using the Bradford assay (Bio-Rad) and used in Western blots or further used for immunoprecipitation. Approximately 1 mg of total protein was used for each immunoprecipitated sample. A 50-μl slurry of protein A agarose beads was used for each sample along with 1–2 μl of appropriate antibody. Protein A agarose beads were blocked with 3% BSA for 90 min for Figs. 2 and 4*C*. Samples were incubated for 3–4 h and washed three times with NP-40 lysis buffer. SDS loading buffer was added to each sample, and they were resolved by SDS/PAGE for analysis.

Lipid Kinase Assays. Lipid kinase assays were performed as described (6). Briefly, after immunoprecipitation with an anti-MT antibody 18-8, beads were washed with 1% NP-40 in PBS twice, 0.5 M LiCl in 0.1 M Tris twice, TNE buffer (10 mM Tris, pH 8.0/100 mM NaCl/1 mM EDTA) once and kinase buffer (20 mM Hepes, pH 7.5/5 mM $MgCl_2$) once. Sonicated lipid mixture of phosphatidylserine, phosphatidylinositol, and phosphtidylinositiol-4,5-bisphosphate were added to washed beads and incubated at room temperature for 3 min. Thirty-five microliters of ATP mixture (kinase buffer with 10 μCi of [γ-^{32}P]ATP and 10 μM cold ATP) was then added to each sample. The kinase reaction was incubated at room temperature for 10 min and stopped with 75 μl of 1 M HCl and 180 μl of a 1:1 mixture of methanol and chloroform. The organic layer was extracted, and the organic extracts were spotted on an oxalate-treated TLC Silica gel 60 plate. (On some occasions the organic layer was stored at –80ºC overnight before spotting.) The plate was then developed in a chamber containing *n*-propanol/2 M acetic acid (65:35). The products

were visualized by autoradiography or phosphorimaging. Only phosphtidylinositiol-3,4,5-trisphosphate was shown in the figures.

Focus Formation Assays. Cells were plated at 7.5×10^5 cells per 100-mm dish, and the next day cells were infected with titered retroviral supernatants for 4–6 h in the presence of 8 μg/ml polybrene. Infected cells were split into three plates the next day. For focus formation, media were changed every 3–4 days with DMEM containing 7% CS for ≈3 weeks without drug selection; for colony formation, the cells were selected in 500 μg/ml G418, and media were changed every 3–4 days. The focus formation assay and one each of the G418 drug selected plates were fixed and stained with either 0.2% crystal violet or 2% methylene blue in 50% ethanol.

Soft Agar Assays. Approximately 4,000–6,000 G418-selected, early-passage cells were mixed with 0.3% agarose in DMEM with 10% CS and plated into six-well dishes containing 0.8% agarose in DMEM with 10% CS. Cells were fed every 5–7 days with 1 ml of DMEM with 10% CS without agarose, and allowed to grow for 3–5 weeks before being scored for growth.

1. Druker, B. J., Sibert, L. & Roberts, T. M. (1992) *J. Virol.* **66,** 5770–5776.

2. Pear, W. S., Nolan, G. P., Scott, M. L. & Baltimore, D. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8392–8396.

3. Chen, C. A. & Okayama, H. (1988) *BioTechniques* **6,** 632–638.

4. Cherington, V., Morgan, B., Spiegelman, B. M. & Roberts, T. M. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 4307–4311.

5. Pallas, D. C., Schley, C., Mahoney, M., Harlow, E., Schaffhausen, B. S. & Roberts,T. M. (1986) *J. Virol.* **60,** 1075–1084.

6. Auger, K. R., Wang, J., Narsimhan, R. P., Holcombe, T. & Roberts, T. M. (2000) *Biochem. Biophys. Res. Commun.* **272,** 822–829.