

METHODS AND MATERIALS

Reagents. Antibodies were obtained from the following sources: total Src, Src pTyr⁴¹⁸, Src pTyr⁵²⁹, Src non-pTyr⁵²⁹, and PKA/PKG substrate antibody (anti-RRXpS) from Cell Signaling; total ERK1, ERK1/2 phosphorylated on Tyr²⁰⁴ (clone E-4), SHP-1 (SC287), SHP-2 (SC7384), β_3 integrin (SC6627), α -tubulin, and β -actin from Santa Cruz Biotechnology; rabbit monoclonal antibodies for SHP-2 and β_3 integrin from Epitomics; PTP-1B from Upstate Biotechnology Inc., RPTP- α and caveolin-1 from BD Transduction Laboratories; PKGI from Calbiochem/EMD; PKGII from Abgent; and BrdU from Sigma.

The cGMP agonist 8-(4-chlorophenylthio)-guano-sine-3',5'-cyclic monophosphate (8-CPT-cGMP, referred to as cGMP) and the cGMP antagonist 8-(4-chlorophenylthio)- β -phenyl-1,N²-ethenoguanosine-3',5'-cyclic monophosphorothioate, Rp isomer (Rp-8-CPT-PET-cGMPS) were from Biolog. The NOS inhibitor *N*-nitro-L-arginine methyl ester (L-NAME), the soluble guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), and 1-propanamine,3-(2-hydroxy-2-nitroso-propylhydrazino) (PAPA-NONOate) were from Cayman. The Src kinase family inhibitor PP2 and its inactive cogener PP3 were from Calbiochem/EMD. Human fibrinogen and fibronectin were from Enzyme Research Ltd.

DNA Constructs. Vectors encoding variants of human PKGI, rat PKGII, and human β_3 integrin were described previously (1,2). Bacterial expression vectors encoding glutathione-S-transferase (GST)-tagged human SHP-1 and SHP-2 were from Addgene; truncated versions were generated by PCR. For mammalian SHP-1 expression vectors, SHP-1 variants were fused in-frame with the N-terminal peptide DYKDDDDKKG. Site-directed mutagenesis was performed using the QuickChange kit (Stratagene) as per the manufacturer's instructions. Venus vectors for bimolecular fluorescence complementation (BiFC) were provided by C.-D. Hu (Purdue University); α_{IIb} -VC integrin, containing the C-terminal half of Venus fused to the C-terminus of human α_{IIb} integrin, was described previously (3). The N-terminal half of Venus was inserted between amino acids 39 and 40 of PKGII to generate PKGII-VN. All PCR products were sequenced to assure absence of unwanted mutations.

siRNA Transfections and Viral Infections. Sequences targeted by siRNAs are summarized in Table S1; the oligoribonucleotides were produced by Qiagen. MC3T3 cells were plated at 1.3×10^5 cells per well of a 6-well dish or at 5×10^5 cells per glass slide, and were transfected 18 hours later (at ~40% confluency) with 100 pmol of siRNA and Lipofectamine 2000TM (Invitrogen) in 1 ml of 10% FBS-containing media.

Adenoviral vectors encoding β -galactosidase (LacZ), PKGI, PKGII, and SHP-1 variants were produced using the pAd/CMV/V5-DESTTM Gateway^R system (Invitrogen) (1,4). Lentiviral vectors

encoding human β_3 integrin variants were produced as described previously (3). At 24 hours after siRNA transfection, cells were infected in full growth medium at an MOI of ~ 10 ; 24 hours later cells were transferred to medium containing 0.1% FBS for an additional 24 hours until harvest.

Cell Fractionation. Cells were extracted in hypotonic buffer by Dounce homogenization, nuclei and cell debris were removed by centrifugation at 300 x g for 10 min, and the supernatant was subjected to centrifugation at 50,000 x g for 30 min to generate a “cytosolic” (supernatant) and a “membrane” (pellet) fraction. To isolate detergent-insoluble fractions, MC3T3 cells were plated on 150 mm dishes and infected with adenovirus encoding rat PKGII at an MOI of ~ 5 to increase PKGII expression by about 2-fold. Confluent cells were subjected to orbital flow for 5 min (120 rpm) or kept under static conditions. Triton-insoluble fractions were prepared as described (5). Lysates were concentrated to a minimal volume using trichloroacetic acid/acetone precipitation.

Immunoprecipitation and Western Blot Analyses. For Fig. 4H, MC3T3 cells were lysed in 50 mM Tris/HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol, 0.5% NP-40, and a protease inhibitor cocktail (Calbiochem/EMD). Lysates were cleared by centrifugation (16,000 x g for 15 min) and subjected to immunoprecipitation using a SHP-2-specific monoclonal antibody on protein G agarose beads. For Fig. 5C and fig. S7A, MC3T3 cells were infected with lentivirus expressing wild-type or mutant human β_3 integrin; cells were lysed as above, and cleared lysates were subjected to immunoprecipitation with a rabbit monoclonal antibody which recognizes human β_3 integrin more efficiently than murine β_3 integrin. Western blots were generated using the indicated antibodies from a different species. Western blots were developed with horseradish peroxidase-coupled secondary antibodies or Clean-Blot IP Detection ReagentTM (Thermo Scientific) and enhanced chemiluminescence detection (6). For fig. S7C, HEK 293T cells were cotransfected with DNA vectors encoding human β_3 integrin and rat PKGII using Lipofectamine 2000TM. For immunoprecipitation of PKGII, cells were lysed in 1% Triton X-100, 50 mM Tris/HCl pH 7.4, 500 mM NaCl, 5 mM sodium vanadate, and protease inhibitor cocktail to solubilize PKGII (1). For immunoprecipitation of β_3 integrin, cells were lysed in 0.1% SDS, 0.1% sodium deoxycholate, 100 mM Tris/HCl pH 7.4, 150 mM NaCl, 5 mM sodium vanadate, 2 mM PMSF and protease inhibitors, which partly solubilizes PKGII.

PTP Assays. PTP assays were performed in 50 mM Tris/HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol, 0.1% NP-40 using the C-terminal Src peptide TSTEPQ(pY)QPGENL as a substrate. Inorganic phosphate was measured with a modified malachite green/ ammonium molybdate method (7). Blank samples included HEK 293T cells transfected with empty vector (+/- PKGII) subjected to immunoprecipitation with anti-Flag antibody, and control IgG immunoprecipitates from MC3T3 cells.

Immunofluorescence Staining and BrdU Incorporation. Cells were fixed in 4% paraformaldehyde and permeabilized in 0.5% Triton-X-100. After blocking with 3% BSA, cells were stained using the indicated primary antibodies, and FITC- and Texas Red-conjugated secondary antibodies (Jackson ImmunoResearch). For triple staining, secondary antibodies conjugated to AlexaFluor 555 and 647 (Invitrogen), and a FITC-conjugated anti-vinculin antibody (Sigma-Aldrich) were used. For BrdU incorporation, cells received 200 μ M BrdU for 18 hours, were fixed, permeabilized, and incubated with 0.5 U/ μ l DNase I (Sigma-Aldrich) for 30 min at 37°C, prior to staining with anti-BrdU antibody (Sigma-Aldrich) and Hoechst 33342; cells were visualized with a Leica fluorescent microscope at 10-20x, and at least 300 cells were scored per condition.

BiFC assay. Cells were cotransfected with human α_{IIb} integrin-VC and β_3 integrin, and either wild-type PKGII-VN or PKGII (G2A)-VN. At 24 hours post transfection, cells were allowed to spread on fibrinogen-coated cover slips for 2 hours. After fixation and permeabilization, cells were stained for PKGII and human β_3 integrin using secondary antibodies conjugated to Alexa Fluor 555 and 647 (Invitrogen).

SUPPLEMENTAL FIGURES

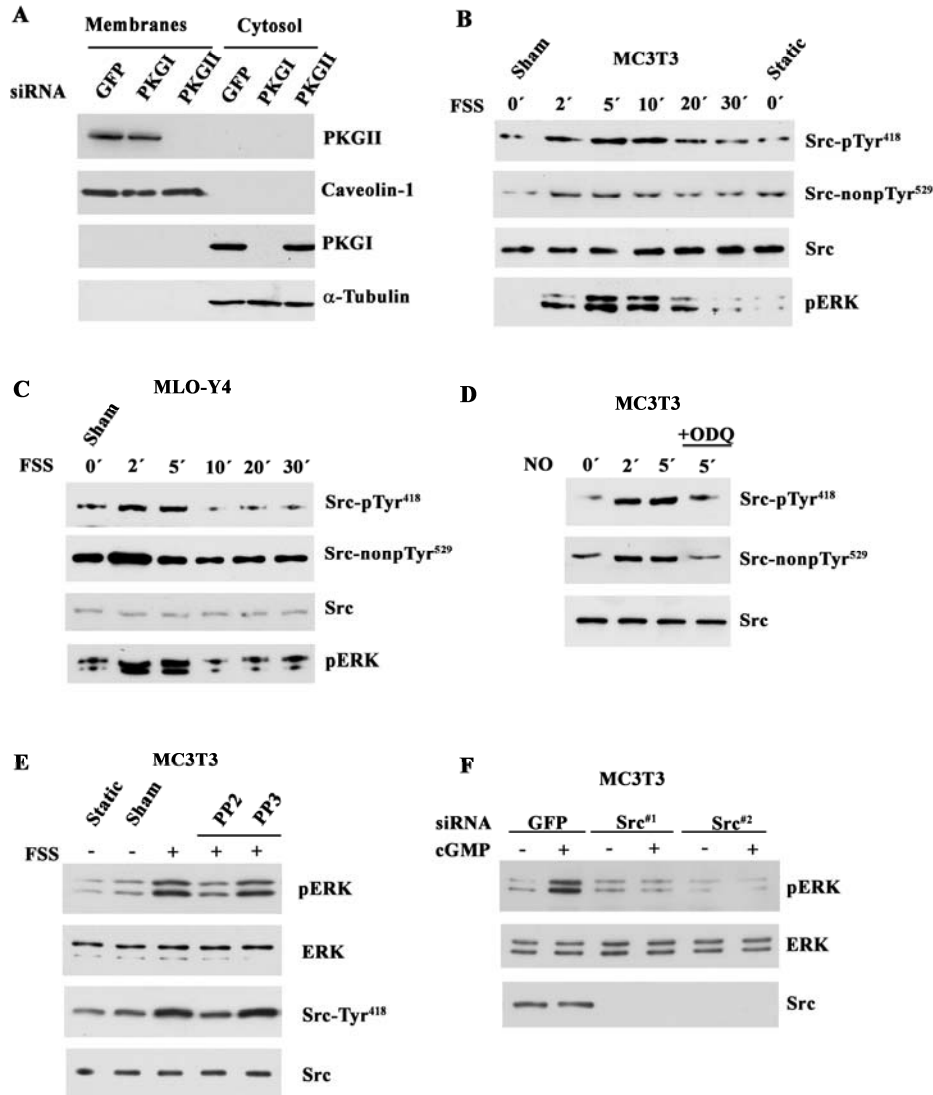


Figure S1: siRNA knockdown of PKGI or PKGII; effects of fluid shear stress and cGMP on Src and ERK phosphorylation in MLO-Y4 and MC3T3 cells. (A) MC3T3 cells were transfected with siRNAs targeting GFP (control), PKGI, or PKGII, and cell membranes (top two panels) or cytosolic extracts (bottom two panels) were analyzed by Western blotting for the presence of PKGII and caveolin-1, or PKGI and α -tubulin, respectively. (B and C) Serum-starved MC3T3 osteoblast-like cells (B) and MLO-Y4 osteocyte-like cells (C, provided by Dr. L. Bonewald) were sham-treated or exposed to fluid shear stress (FSS; 12 dynes/cm²) for the indicated times; some cells were kept static without being mounted into the flow chamber (B, last lane). Cell lysates were analyzed by Western blotting with antibodies specific for Src phosphorylated on Tyr⁴¹⁸ (Src-pTyr⁴¹⁸), Src with unphosphorylated Tyr⁵²⁹ (Src-nonpTyr⁵²⁹), total Src (Src), and ERK1/2 phosphorylated on Tyr²⁰⁴ (pERK). (D) MC3T3 cells were treated with the NO donor PAPA-NONOate (10 μ M) for the indicated times; some cells were pre-treated for 1 hour with the soluble guanylate cyclase inhibitor ODQ (10 μ M). (E) MC3T3 cells were treated as in (B); some cells were pre-treated for 1 hour with the Src family kinase inhibitor PP2 (10 μ M) or the inactive analog PP3 (10 μ M), prior to stimulation with fluid shear stress for 5 min. (F) MC3T3 cells were transfected with siRNAs targeting GFP or two different sequences in Src; after serum starvation for 24 hours, some cells were treated with 100 μ M 8-CPT-cGMP (cGMP) for 5 min. Western blots shown in A to F are representative of at least two independent experiments.

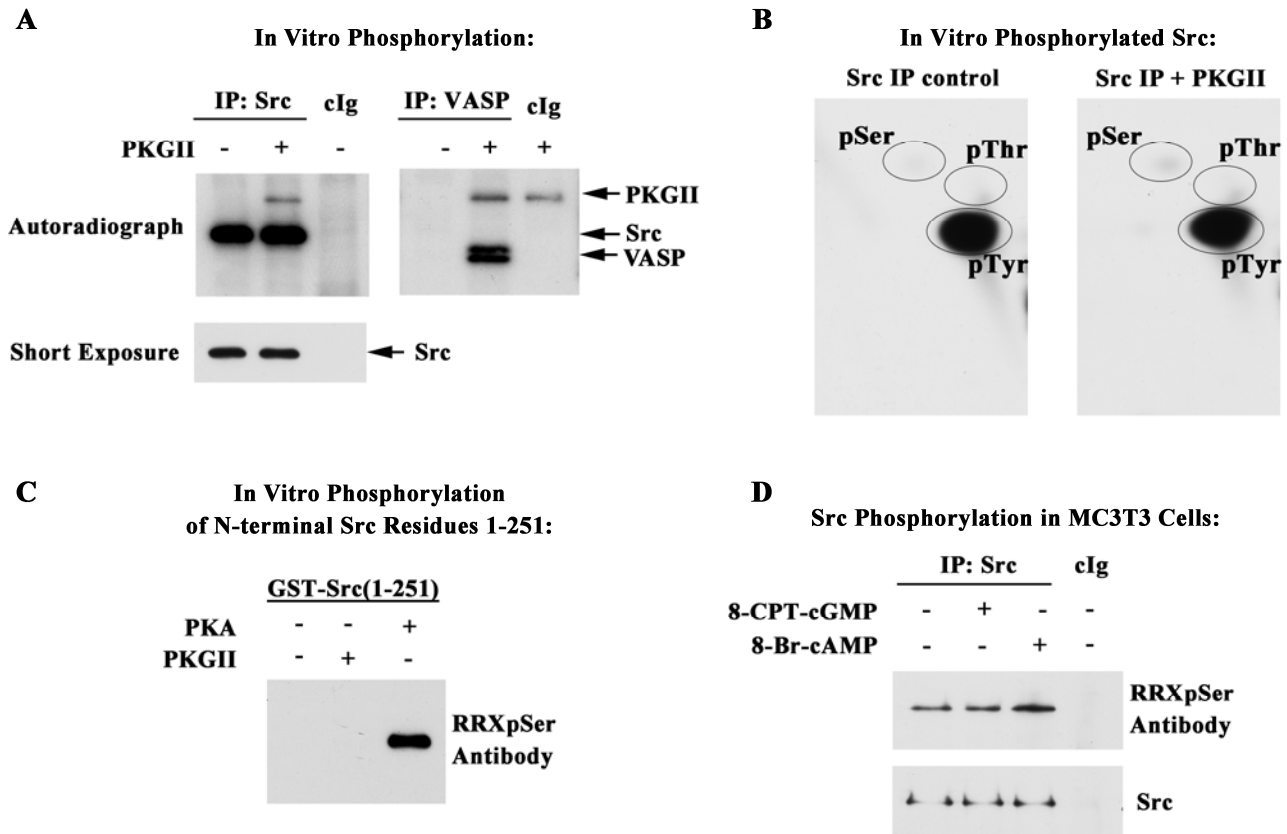


Figure S2: PKGII does not directly phosphorylate Src. (A) Src and VASP were immunoprecipitated from HEK 293T cells that were transfected with expression vectors encoding murine Src or human VASP, respectively. Washed immunoprecipitates were incubated for 15 min at 37°C in kinase buffer containing 100 μ M [γ - 32 P] $_{4}$ ATP and 10 mM MgCl $_2$ in the absence or presence of 100 ng purified PKGII and 10 μ M 8-CPT-cGMP; proteins were analyzed by SDS-PAGE/autoradiography (representative of three experiments). (B) Bands corresponding to in vitro phosphorylated Src in (A) were excised and subjected to acid hydrolysis followed by phospho-amino acid analysis using two-dimensional high voltage electrophoresis. Spots corresponding to ninhydrin-stained phospho-amino acid standards are circled (representative of two experiments). (C) GST-tagged Src (1-251), containing the first 251 amino acids of murine Src, was purified from bacteria and incubated for 15 min at 37°C with kinase buffer containing 100 μ M ATP and 10 mM MgCl $_2$ in the absence or presence of purified PKGII and cGMP or the catalytic subunit of PKA. Proteins were analyzed by Western blotting using a PKA/PKG substrate antibody which recognizes the sequence RRXpS (representative of two experiments). (D) Serum-deprived MC3T3 cells were treated for 5 min with 100 μ M 8-CPT-cGMP (cGMP) or 1 mM 8-Br-cAMP (cAMP) as indicated. Cell lysates were subjected to immunoprecipitation with anti-Src antibody or control IgG, and immunoprecipitates were analyzed using the PKA/PKG substrate antibody described in (C) or rabbit anti-Src antibody (representative of two experiments).

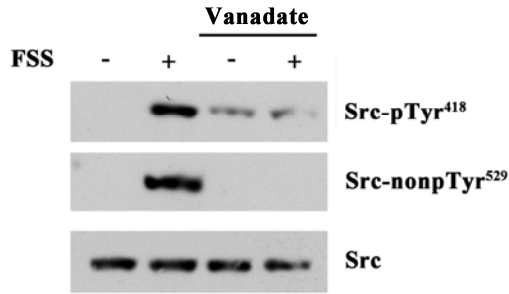
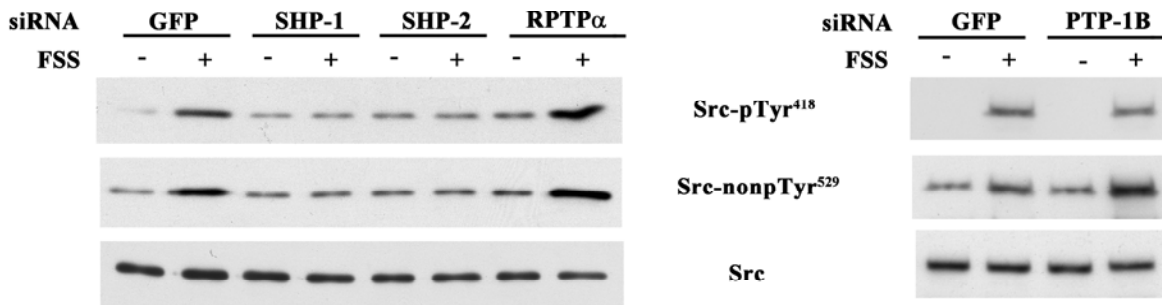
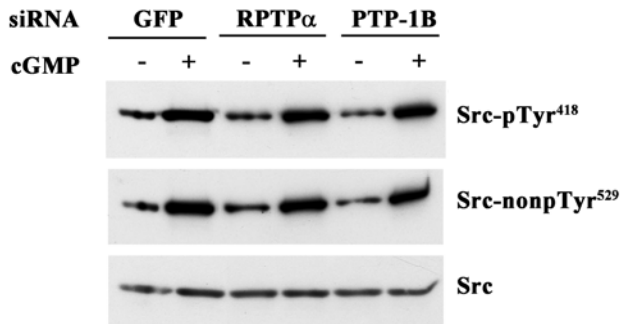
A**B****C**

Figure S3: Effect of vanadate and phosphatase-specific siRNAs on Src phosphorylation and dephosphorylation. (A) MC3T3 cells were pre-treated for 1 hour with 10 μ M vanadate prior to stimulation with fluid shear stress (FSS; 12 dynes/cm²) for 5 min. Src phosphorylation was assessed as in fig. S1B (representative of three experiments). (B and C) MC3T3 cells were transfected with the indicated siRNAs, serum-starved, and exposed to fluid shear stress (B) or treated with 100 μ M 8-CPT-cGMP (cGMP; C) for 5 min (representative of three experiments).

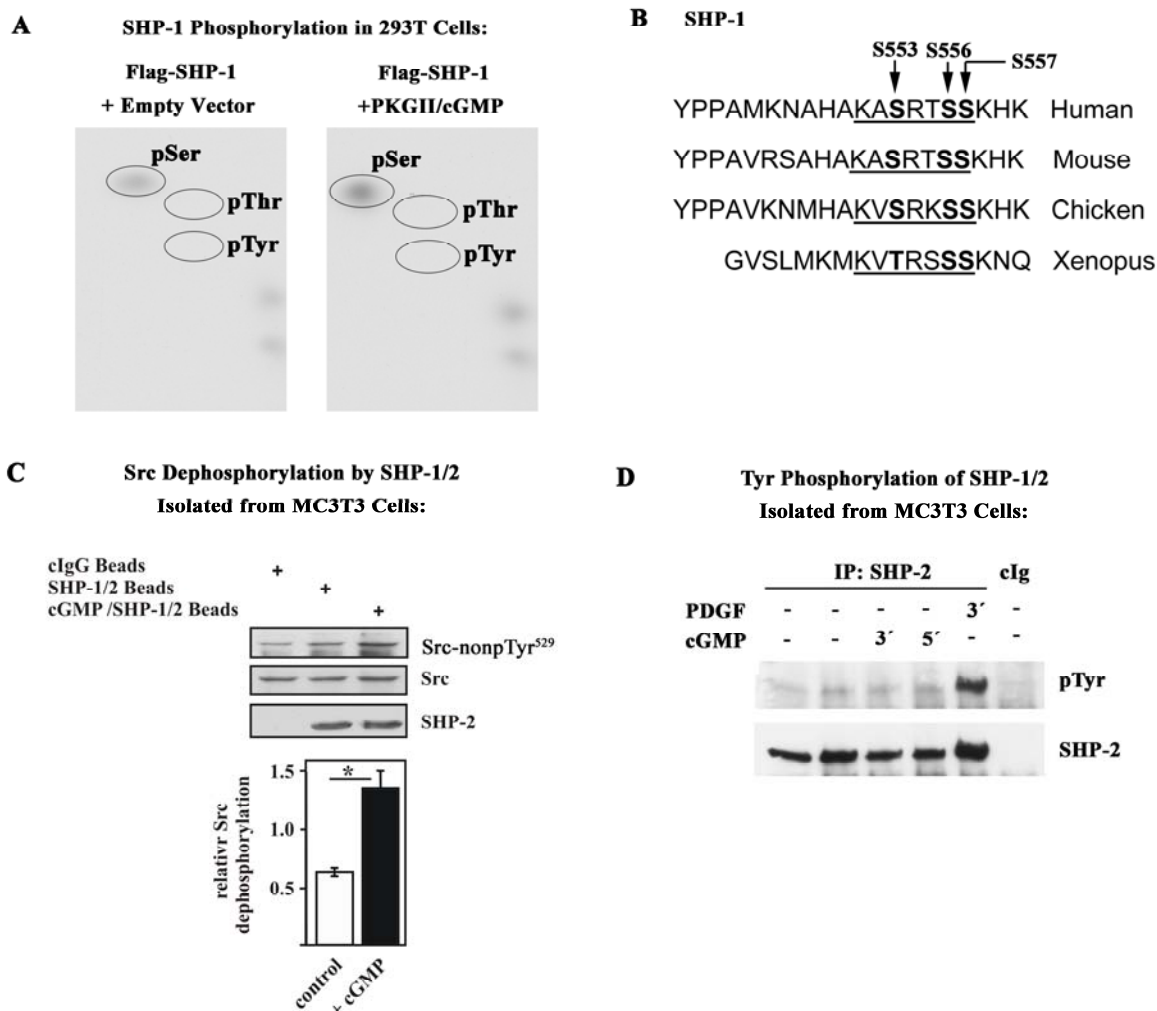


Figure S4: Analysis of SHP-1 phosphorylation and function in MC3T3 cells. (A) HEK 293T cells were cotransfected with Flag-tagged SHP-1 and empty vector or PKGII, and labeled with $^{32}\text{PO}_4$. Some cells were treated with 100 μM 8-CPT-cGMP (cGMP), and SHP-1 was isolated by immunoprecipitation as described in Fig. 4D. Bands corresponding to SHP-1 were excised, and subjected to phospho-amino acid analysis as described in fig. S2B. Migration of phospho-amino acid standards is indicated (representative of two experiments). (B) SHP-1 sequences from human, mouse, chicken, and *Xenopus* were aligned using BLAST (8), and a putative PKG consensus sequence is underlined. The indicated serine residues were mutated to alanines to generate SHP-1 AAA. (C) Serum-deprived MC3T3 cells were stimulated with 100 μM cGMP for 5 min or left untreated; cell lysates were subjected to immunoprecipitation using a SHP-2-specific antibody or control IgG as described in Fig. 4H. To examine SHP-mediated dephosphorylation of full-length Src, the immunoprecipitates containing SHP-1 and -2 (Fig. 4H) were incubated with MC3T3 cell lysates for 20 min at 37°C. The lysate and bead mixture was analyzed by Western blotting using an antibody specific for Src-nonpTyr⁵²⁹, total Src, and SHP-2. The bar graph summarizes three experiments (mean \pm SEM, $p < 0.05$ for the comparison of immunoprecipitates obtained from control compared to cGMP-treated cells). (D) MC3T3 cells were mock-treated or treated with 100 μM cGMP for 3 or 5 min; some cells received 30 ng/ml platelet-derived growth factor (PDGF) for 3 min. Cell lysates were subjected to immunoprecipitation as in Fig. 4H, and immunoprecipitates were analyzed by Western blotting using a phospho-Tyr-specific antibody. Note that Tyr-phosphorylated SHP-2 in PDGF-treated cells migrates with a slightly higher apparent molecular weight (representative of two experiments).

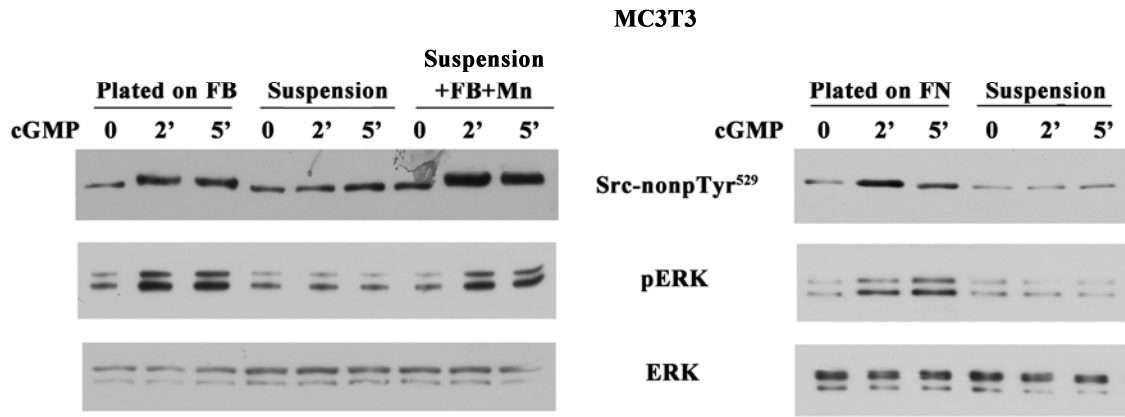


Figure S5: cGMP activation of Src requires ligation of β_3 integrins. MC3T3 cells were allowed to adhere to fibrinogen-coated dishes (FB, left panel) or fibronectin-coated dishes (FN, right panel); some cells were kept in suspension (on bovine serum albumin-coated dishes) or received soluble fibrinogen and MnCl_2 as described for hPOBs in Fig. 5E. After 1 hour, cells were stimulated with $100 \mu\text{M}$ 8-CPT-cGMP (cGMP) for the indicated times, and Src and ERK phosphorylation was assessed as in fig. S1B (representative of two experiments).

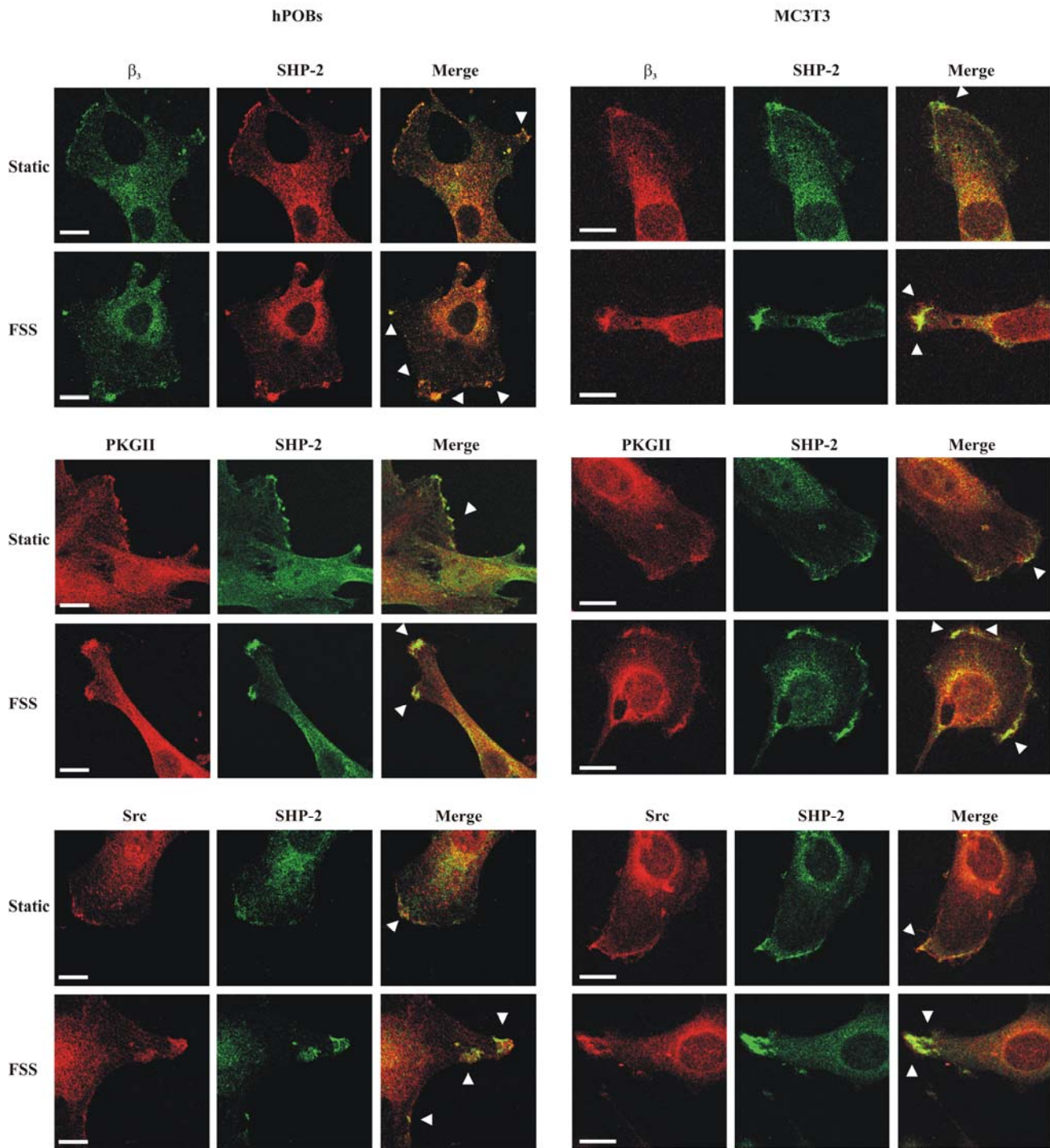


Figure S6: Colocalization of β_3 integrin, PKGII, and Src with SHP-2-containing membrane complexes. hPOBs (left) and MC3T3 cells (right) were infected with PKGII adenovirus to increase PKGII expression by about 2-fold; cells were held static or exposed to orbital fluid shear stress (FSS; 120 rpm) for 5 min, and PKGII, SHP-2, Src, and β_3 integrin were detected by immunofluorescence staining using rabbit antibodies specific for PKGII, Src, or β_3 integrin, and mouse antibodies for SHP-2. Representative confocal images of three experiments are shown; the bar represents 5 μm .

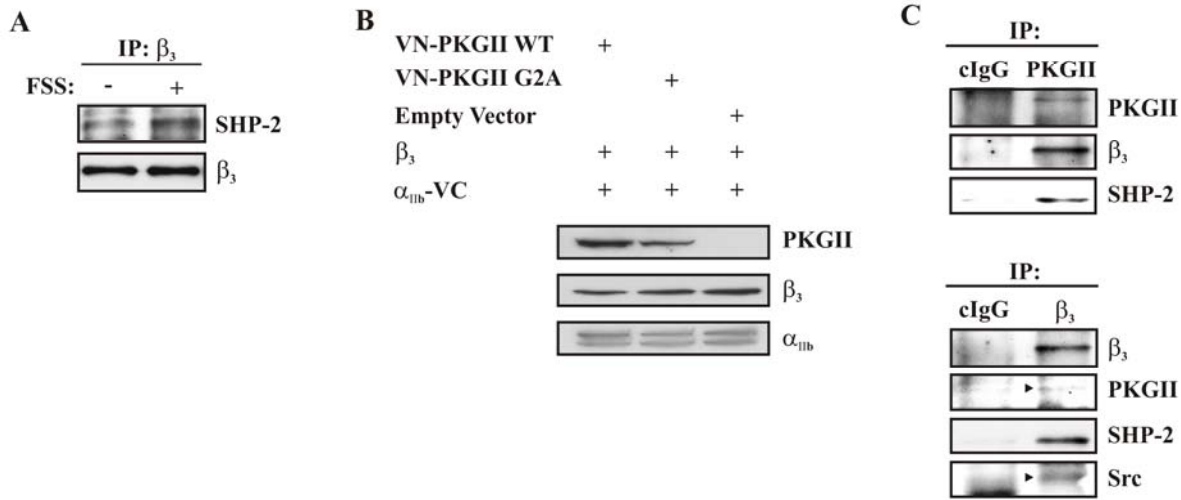


Figure S7: Interactions between PKGII, SHP-2, Src, and β_3 integrin (A) MC3T3 cells infected with lentivirus expressing human β_3 integrin were exposed to orbital fluid shear stress (FSS; 120 rpm) for 5 min or held static. β_3 integrin was immunoprecipitated, and precipitates were analyzed by Western blotting for the presence of SHP-2 (representative of two experiments). (B) MC3T3 cells were transfected with vectors encoding VN-PKGII and α_{11b} -VC chimeras, and human β_3 integrin, as indicated, and cell lysates were analyzed by Western blotting for the presence of PKGII-fusion proteins, β_3 integrin, and α_{11b} integrin. (C) Coimmunoprecipitation of β_3 integrin and SHP-2 with PKGII, and of PKGII, SHP-2, and Src with β_3 integrin (representative of two experiments). Note that under conditions allowing coimmunoprecipitation, the amount of solubilized PKGII is low.

Gene:	siRNA Target Sequence (5'-3')	Comments
PKGI	CCGGACAUUUAAAGACAGCAA	Target sequence in the C-terminus of PKG I α and β
PKGII^{#1}	CTGCTTGGAAGTGGAACTA	
PKGII^{#2}	CCGGGTTTCTTGGGTAGTCAA	Mismatched with rat
β_3 Integrin^{#1}	CCGCTTCAATGAAGAAGTGAA	
β_3 Integrin^{#2}	CACGGTGAGCTTTAGTATCGA	Mismatched with human
SHP-1^{#1}	CTGGACATTTCTTGTGCGTGA	
SHP-1^{#2}	CTGGATCAGATCAACCAGCGA	Mismatched with human
SHP-2^{#1}	CAGAAGCACAGTACCGGTTTA	
SHP-2^{#2}	AAGGACATGAATATACCAATA	Mismatched with human
PTP-1B	TCGGATTAAATTGCACCAGGA	
RPTP-α	TTCACGGATGCCATAACAGAA	
GFP	AAGCTGACCCTGAAGTTCATC	
Src^{#1}	TTCCCTTGTGTCCATATTTAA	
Src^{#2}	CCCAGACTTGTTGTACATATT	

Table S1. siRNA target sequences.

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