

EXTENDED EXPERIMENTAL PROCEDURES

Materials/Antibodies

JNK1 (554286) and JNK1/2 (554285) antibodies were from PharMingen. Phospho-JNK1/2 (9251), Phospho-Src-Tyr 416 (2101), MKK7 (4172), phospho-AKT-Ser473 (9271), phospho-MLK3 (2811) antibodies were from Cell Signaling. c-Src antibodies were from Santa Cruz Biotechnology (sc19), Cell Signaling (2110 and 2123) and a gift from T. Hunter, Salk Institute, La Jolla, CA (2-17). Antibodies to Yes were from Santa Cruz (sc-48396) and Cell Signaling (2734). Antibodies to flotillin-2 (sc-28320), Fyn (sc-16), AKT (sc-8312), MLK3 (sc-536), MKK4 (sc-964) LAMP-1 (sc-19992) and caveolin-1 (sc-894) were all from Santa Cruz Biotechnology. Other antibodies included p85 α (06-195 from Millipore/Upstate Biotechnology), calnexin (GTX109669 from GeneTex Inc.), flotillin-1 (3253 from Cell Signaling and 610820 from BD Bioscience). Palmitic acid, stearic acid, oleic acid, palmitoleic acid, eicosapentaenoic acid, insulin, lipopolysaccharide, enolase, and Optiprep were from Sigma Aldrich. PP2 and the Src cdc2 peptide substrate were from Biomol. PP3 was from Calbiochem and TNF ELISA was from R & D Systems. Percoll was from GE Healthcare Life Sciences.

Mice

Starting at 6 weeks of age, male C57BL/6 mice (Charles River Laboratories) housed in a specific pathogen-free facility were maintained on normal chow diet, or high fat diet (Bioserve, product S3282, 60% of calories are fat derived) for 16 weeks. All experimental procedures were approved by the Animal Subjects Committee at the UCSD according to US National Institutes of Health guidelines.

Cell Culture

c-Src-deficient fibroblasts, SYF^{-/-} fibroblasts and SYF+c-Src reconstituted cells were provided by D. Schlaepfer (UCSD Cancer Center, La Jolla, CA) and were maintained in DMEM low glucose with glutamine and sodium pyruvate (Invitrogen). J774A.1 and HEK293T cells were maintained in DMEM high glucose with glutamine and HEK293GP2 cells were maintained in DMEM high glucose with glutamine and sodium pyruvate.

cDNA Constructs and Retrovirus Production

pBABE-puro-Yes, Fyn-GFP and Src S3C/S6C cDNAs were from M. Frame (Beatson Institute for Cancer Research, Glasgow, UK). Wild-type, Y527F, G2A and G2A/Y527F Src cDNAs were from M. Resh (Sloan-Kettering Institute, New York, New York) and Y418F Src was from T. Hunter (Salk Institute, La Jolla, California). Fyn-GFP, wt Src, Y418F Src, S3C/S6C Src, Y527F Src, G2A Src and G2A/Y527F Src cDNAs were cloned into pBABE-puro. Retroviruses were produced by cotransfecting pBABE and pVSV-G plasmids into HEK293GP2 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), and retrovirus-containing supernatants were collected 36-72 hr post-transfection. SYF^{-/-} fibroblasts were infected with the retroviruses in the presence of polybrene (Sigma) and selected with puromycin to generate stably transduced cells. All cells were removed from transduction/puromycin selection medium for several days prior to treatment with FA.

FA Treatment

Low-endotoxin BSA was purchased from Equitech Bio (Kerrville, TX) and delipidated using activated charcoal (Chen, 1967). PA and SA were dissolved in ethanol at 70°C, and added to the BSA solution at 55°C and incubated for 30 min after sonication for 1 min, filtered using a 0.45 μ M filter and added to cells at a final concentration of 500 μ M/0.45% BSA. POA, OA and EPA were loaded onto BSA at 42°C for 15 min without sonication and filtered with a 0.45 μ M filter prior to adding to cells at 300 μ M.

Lentiviral Delivery of shRNA

shRNAs were cloned into the pLSLP lentiviral vector. Hairpin sequences were as follows: Src: 5'-GGTGCCAAATCCCCATCA-3' and Fyn: 5'-CAATTACGTGGCTCCAGTT-3'. pLSLP constructs were cotransfected with pVSV-G and pCMVdelta8.2 packaging vectors into HEK293T cells using Lipofectamine 2000 and viral supernatants were collected at 36 and 72 hr post-transfection. Cells were infected with the lentivirus in the presence of polybrene and selected in puromycin before analysis.

Immunoprecipitation/Kinase Assays

JNK kinase assay was carried out as described (Solinas et al., 2006). For c-Src kinase assay, c-Src was immunoprecipitated from membrane fractions, washed, and subjected to in vitro kinase assay using either acid-activated enolase, or the cdc2 (6-20) peptide as substrates. For enolase kinase assay, 50 μ g enolase was resuspended in 50 mM HEPES-NaOH buffer, pH 7.0, 1 mM DTT, 1 mM MgCl₂, and incubated at 0°C for 60 min, mixed with an equal volume of glycerol and stored at -80°C. Just before use, enolase was mixed with an equal volume of 100 mM acetic acid and incubated at 30°C for 10 min. 0.5 μ l enolase was used per reaction and incubated at 30°C for 7-15 min before being resolved by SDS-PAGE, transferred to PVDF membrane (Millipore), and exposed to a phosphorimager screen. For the kinase assay using cdc2 peptide substrate, immunoprecipitated c-Src was incubated with the peptide at 30°C for 5-10 min, spotted onto p81 whatman paper and washed three times with 0.75% phosphoric acid and once with acetone before ³²P-ATP incorporation was measured with a scintillation counter.

Lipid Raft Isolation

For detergent-dependent lipid raft isolation, methods were as described (Lingwood and Simons, 2007). Cells were collected in TNE buffer (150 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl, pH 7.4), sheared with a 25 gauge needle, and then treated with 1% Triton X-100 at 4°C for 30 min. Lysates were then loaded onto a 40%–30%–0% step gradient using Optiprep and centrifuged for 2 hr at 260000 x g. Four fractions were collected from the top of the gradient, concentrated using trichloroacetic acid(TCA)/acetone precipitation and subjected to SDS-PAGE separation. For lipid raft analysis of mouse tissue, BAT and WAT were homogenized, and subjected to two mid-speed centrifugations to pellet tissue debris and to separate the lipids from the whole cell lysate. The protein concentration was determined and equal amounts of protein were subjected to Triton X-100 solubilization. Detergent-independent lipid rafts were isolated as described (Ostrom and Insel, 2006). Briefly, cells were lysed in basic 500 mM Na₂CO₃ buffer and membranes were mechanically disrupted by Dounce homogenization and sonication before being loaded onto 45%–35%–5% step sucrose gradients in 2-(N-morpholino)ethanesulfonic acid (MES)-buffered saline (25 mM MES, 150 mM NaCl, pH 6.0)/250 mM Na₂CO₃ and centrifuged at 260,000 x g for 16 hr. Fractions were collected from the top of the gradient and concentrated and analyzed as above.

Real-Time PCR

Procedures were as described (Solinas et al., 2007). Primers used were mouse CHOPf 5'-TTCTGCTTTCAGGTGTGGTG-3', mouse CHOPr 5'-CGGAACCTGAGGAGAGAGTG-3'; mouse spliced XBP1f 5'-GAGTCCGCAGCAGGTG-3', mouse spliced XBP1r 5'-GTGT CAGAGTCCATGGGA-3'.

Subcellular Distribution of ³H-Labeled FA

Cells were incubated with 200 μM [9,10-³H] PA (Perkin-Elmer) at a specific activity of 47.5 Ci/mmol, combined with 300 μM PA loaded onto BSA for 2 hr, or 110 nM [9,10-³H] OA at a specific activity of 45.5 Ci/mmol and 300 μM cold OA loaded onto BSA for 2 hr. Lipid rafts were isolated using Triton X-100 and radioactivity in each fraction was measured using a scintillation counter and normalized to protein content.

Immunoblotting

Procedures were as described (Solinas et al., 2006).

Glucose Tolerance Test

Male C57BL6 mice were maintained on high fat diet for 16 weeks. Three hrs prior to the beginning of the experiment, mice were dosed with vehicle or 30 mg/kg dasatinib (ChemieTek, Indianapolis, IN) in 1% citric acid by oral gavage. For glucose tolerance test, mice were injected with glucose (1.5 g/kg, i.p.) after 6 hr of fasting. Blood glucose was measured using glucose meter (LifeScan).

Immunofluorescence

Cells grown on poly-L-lysine-coated glass-bottomed petri dishes were fixed in 4% formaldehyde and blocked with PBS + 2% normal donkey serum (NDS) + 0.1% saponin for 10 min. Primary and secondary antibody incubations (1:100-1:400 dilutions) were carried out in PBS + 2% NDS for one hr at 4°C. Cells were imaged on the Leica SPE-2 confocal imaging system with the help of the UCSD National Center for Microscopy and Imaging Research (NCMIR).

Lysosome Enrichment

Cells were collected in freshly prepared homogenization medium (HM) (0.25 M sucrose, 1 mM Na₂EDTA, 10 mM HEPES) pH 7 and disrupted in a Dounce homogenizer. Two ml post-nuclear lysates + 120 μl 10% BSA were layered over 666 μl Percoll, and centrifuged at 78000 g (max) for 35 min. Two upper fractions were collected for comparison (fractions 1 and 2), and the lysosome band was carefully collected from the remaining solution while collecting as little of the surrounding material as possible. The lysosome band was resuspended in a volume of HM buffer equal to fractions 1 and 2. The three fractions were solubilized with Triton X-100, and the remaining Percoll was removed by high speed centrifugation (109000 g (max)) for 2 hr at 4°C. The lysates were then analyzed by immunoblotting.

For lipid raft analysis of the lysosome-enriched band, an equal volume of lysosomes resuspended in HM and TNE buffer were solubilized with Triton X-100 at 4°C and loaded on an Optiprep density gradient as described above. Fractions were collected, concentrated and analyzed by immunoblotting.

SUPPLEMENTAL REFERENCES

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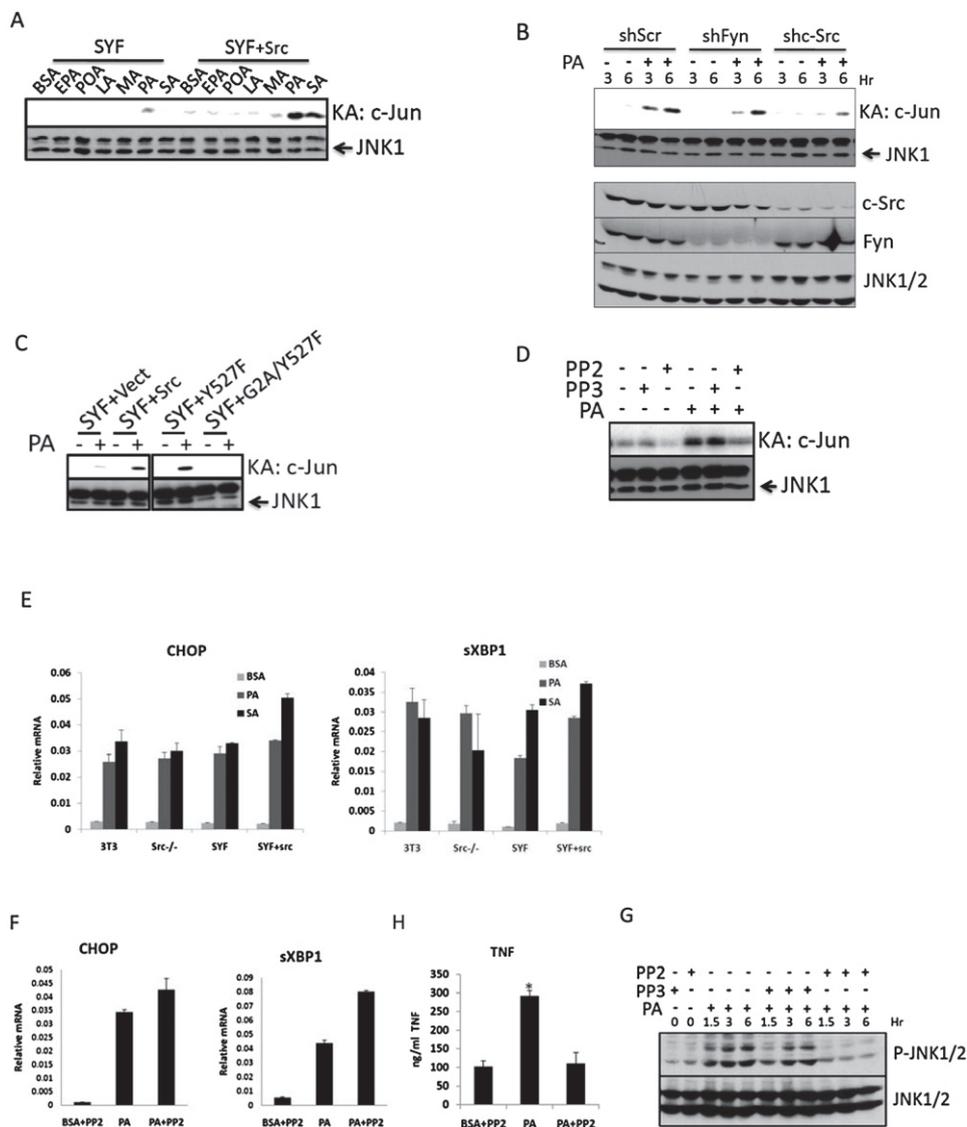


Figure S1. Src Is Required for JNK Activation by PA, Related to Figure 1

(A) SYF and SYF+Src cells were for 6 hr treated with various saturated and unsaturated FA loaded onto BSA. Cell lysates were prepared and JNK activity was measured with GST-c-Jun (1-79) as a substrate.

(B) HEK293T cells were transduced with lentiviruses carrying shRNAs specific for c-Src or Fyn or a scrambled (Scr) sequence as a control, and selected in puromycin-containing medium. Cells were then treated with PA for 3 or 6 hr as indicated and JNK activity was measured as above. Knockdown of c-Src and Fyn was examined by immunoblotting.

(C) SYF^{-/-} cells were reconstituted with empty vector, wt c-Src, c-Src(Y527F) and c-Src (Y527F/G2A) were analyzed for PA-induced JNK activation as in Figure 1D. Please note that all samples were run on the same gel and exposed for the same length of time.

(D) SYF+Src cells were pretreated with the Src kinase inhibitor PP2 or the control compound PP3 for 2.5 hr before treatment with BSA or BSA loaded with PA, and measurement of JNK activity as above.

(E) NIH 3T3, Src^{-/-}, and SYF+Src cells were treated for 6 hr with either BSA alone or BSA loaded with PA or SA. Induction of CHOP mRNA or XBP1 mRNA splicing was analyzed by Q-RT-PCR. Results are averages of at least three independent experiments ± s.d.

(F) J774A.1 macrophages were pretreated with 15 μM PP2 for 2.5 hr before treatment with PA and ER stress markers were measured as above.

(G and H) Pretreatment with PP2, but not with the control compound PP3, prevented PA-induced JNK activation (G) and TNF production (H) in J774A.1 macrophages. Cells were treated with the inhibitors for 2.5 hr before incubation with PA as indicated. In (H), TNF was measured after a 2 hr incubation.

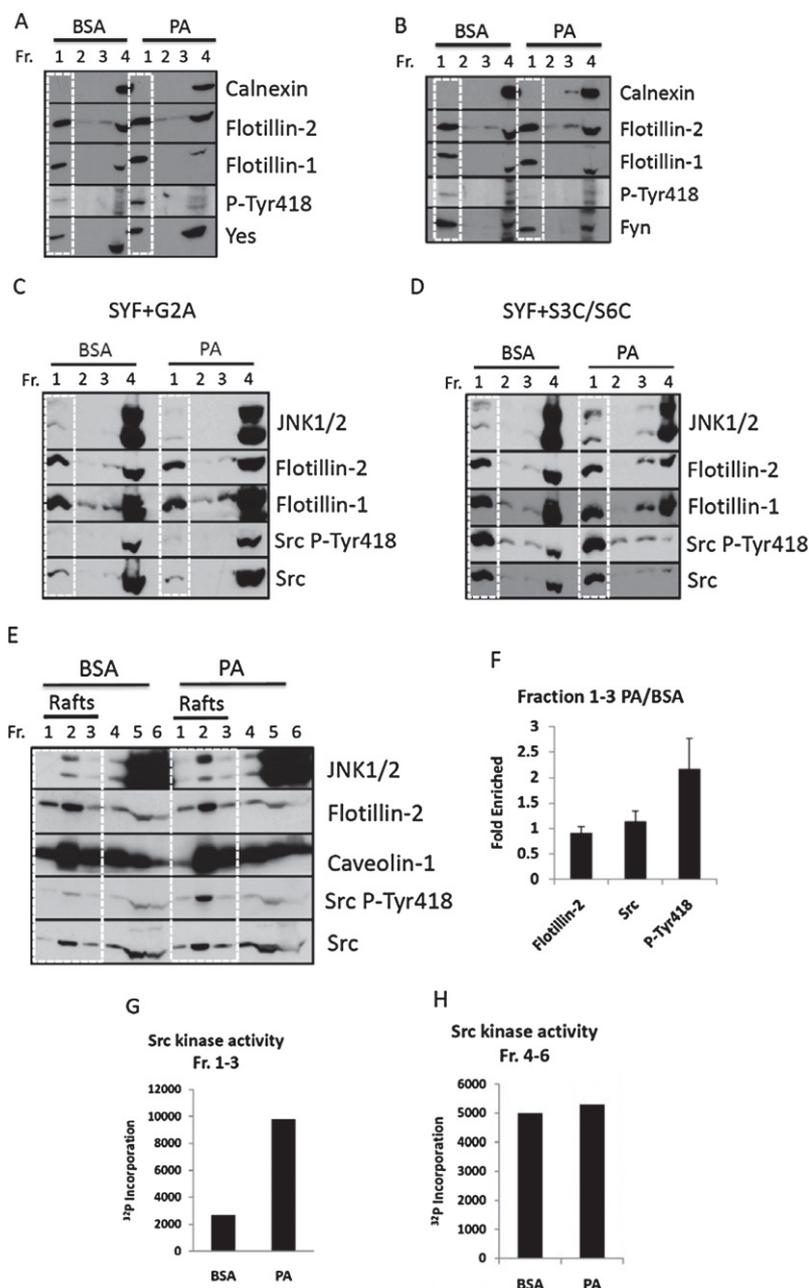


Figure S2. Activated c-Src Is Enriched within DRM/Lipid Rafts after PA Treatment, Related to Figure 2

(A and B) *SYF*^{-/-} cells reconstituted with Yes (A) or Fyn (B) were treated for 2 hr with PA and their membranes solubilized and fractionated as in Figure 2. The different fractions were analyzed for presence of the indicated proteins by immunoblotting.

(C and D) *SYF*^{-/-} cells reconstituted with c-Src G2A (C) or S3C/S6C (D) mutants were treated for 3 hr with PA and their membranes were analyzed as above.

(E and F) Lipid rafts were isolated using a detergent-independent procedure. (E) SYF+Src cells were treated for 2 hr with BSA or BSA loaded with PA and cell lysates were prepared using basic Na₂CO₃ buffer. Cell membranes were disrupted by Dounce homogenization and sonication before separation of lipid rafts by sucrose density gradient centrifugation. Fractions were collected from the top of the gradients, such that lipid rafts are in fractions 1-3. Immunoblotting reveals that Tyr418 phosphorylated c-Src and JNK1/2 are enriched in fractions 1-3 that contain caveolin-1 and flotillin-2. (F) Results from three independent experiments were quantitated to examine PA-induced enrichment of the indicated proteins within the raft fractions (Fr. 1-3).

(G and H) c-Src was immunoprecipitated from pooled fractions 1-3 (G) or pooled fractions 4-6 (H) and its activity was measured using a Cdc2 peptide as a substrate. Active c-Src was enriched in the lipid raft fractions from PA-treated cells, but not in the soluble fractions 4-6.

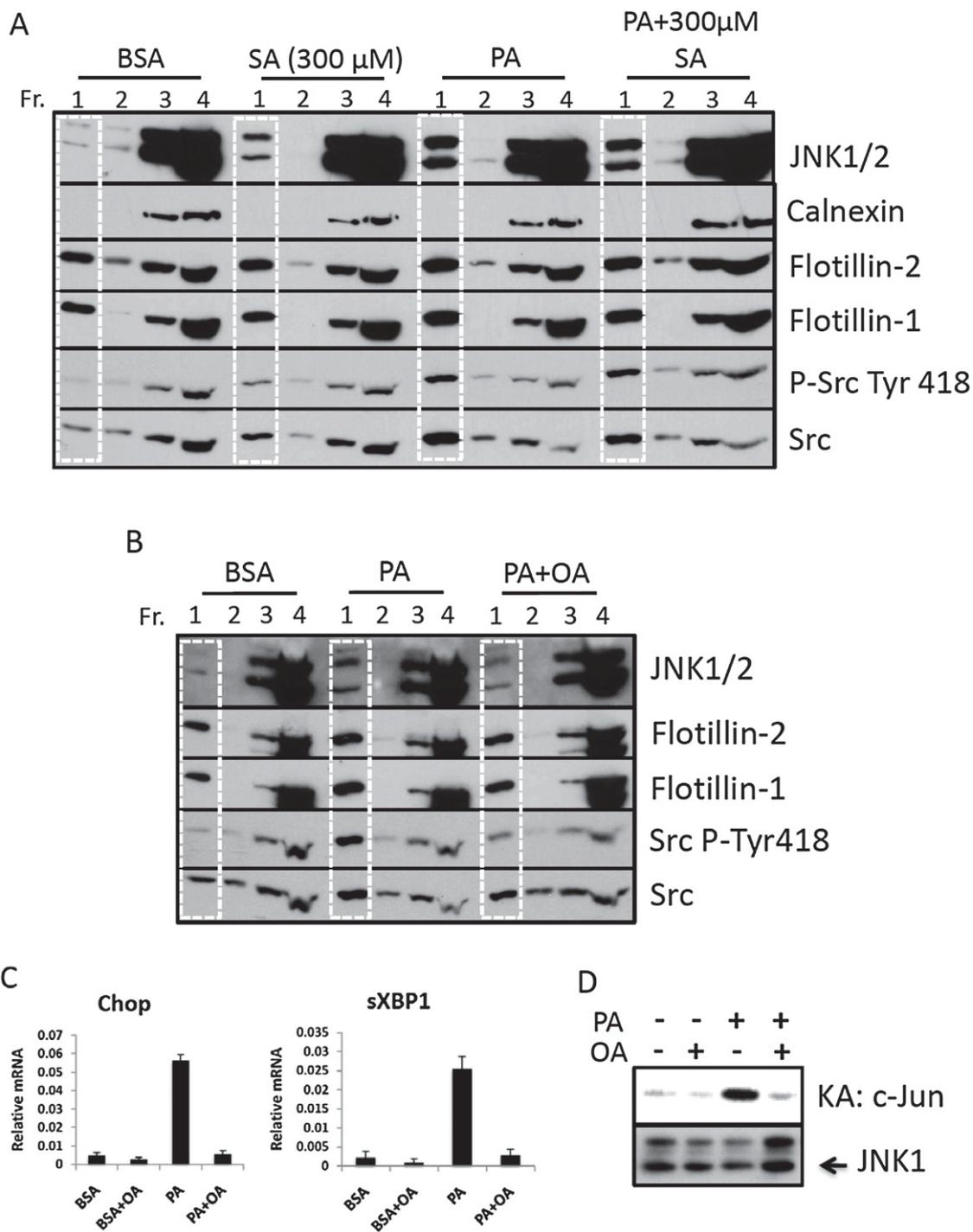


Figure S3. Effect of Pretreatment of SA and OA on PA-Induced Src Redistribution and JNK Activation, Related to Figures 3 and 4

(A) SYF+Src cells were pretreated with 300 μ M SA for 15 min prior to a 2 hr treatment with PA, and membranes were solubilized and fractionated on Optiprep gradients as described in Figure 2.

(B–D) SYF+Src cells were pretreated with 300 μ M OA for 15 min prior to a 2 hr treatment with PA, and cells were collected and their membranes were analyzed as above (B); and ER stress markers were analyzed as above by Q-RT-PCR (C) and JNK activation (D).

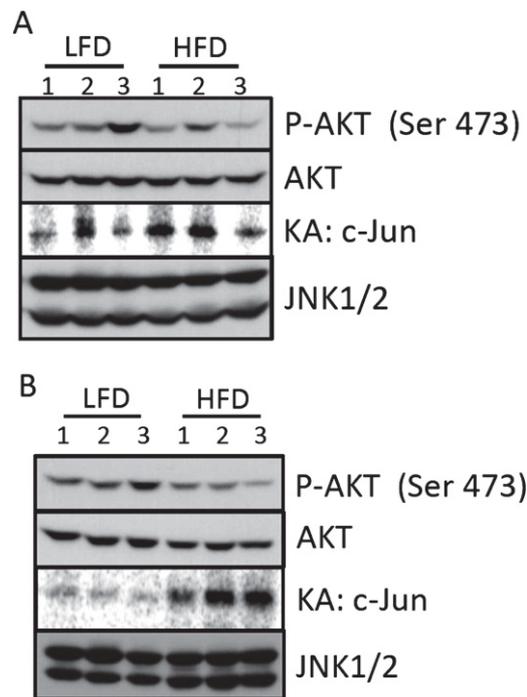


Figure S4. Analysis of JNK and AKT in Adipose Tissues, Related to Figure 5

(A and B) Male C57BL/6 mice (same as those used in Figure 5) were maintained on high fat diet (HFD) or normal chow (LFD) for 16 weeks. Lysates of isolated BAT (A) or WAT (B) were immunoblotted for AKT and Ser473-phosphorylated AKT, whereas JNK activity was measured by a kinase assay.

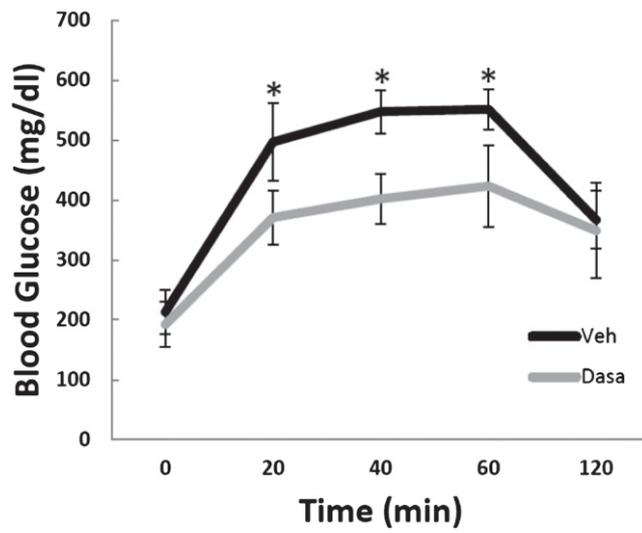


Figure S5. Dasatinib Improves Glucose Control in Obese Mice, Related to Figure 5

Male C57BL/6 mice were maintained on high fat diet for 16 weeks. Mice were fasted for 6 hr before the beginning of the experiment, and were treated with 30 mg/kg dasatinib in 1% citric acid or vehicle 3 hr prior to the beginning of the glucose tolerance test. Mice were injected with 1.5 g/kg glucose i.p. and blood glucose was measured at the indicated time points. Results are averages \pm s.d. (n = 5 per group), *p < 0.05.

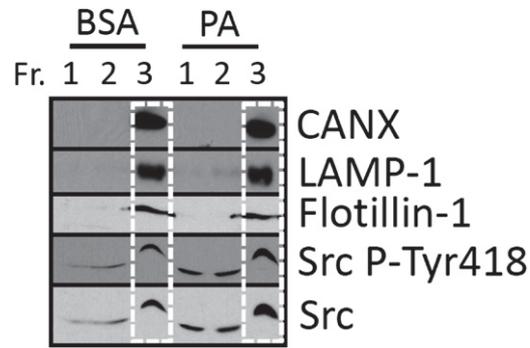


Figure S6. Enrichment of Activated c-Src in Lysosomal Membranes after PA Treatment, Related to Figure 6

SYF+Src cells were treated for 2.5 hr with or without PA. Cells were then collected and disrupted by Dounce homogenation, and post-nuclear lysates were separated on a Percoll gradient. Two upper fractions (1 and 2) were collected for comparison, and the lysosome band (Fr. 3) was carefully collected and re-suspended in a volume of buffer equal to Fr. 1 and 2. The three fractions were solubilized with Triton X-100, the remaining Percoll was removed by high speed centrifugation, and the presence of the indicated proteins examined by immunoblotting.