Inventory for Supplemental Data

1. Supplemental Figure S1-S8 and the figure legends.
2. Supplemental experimental procedures.
3. Supplemental references

Supplemental Text and Figures

Figure S1

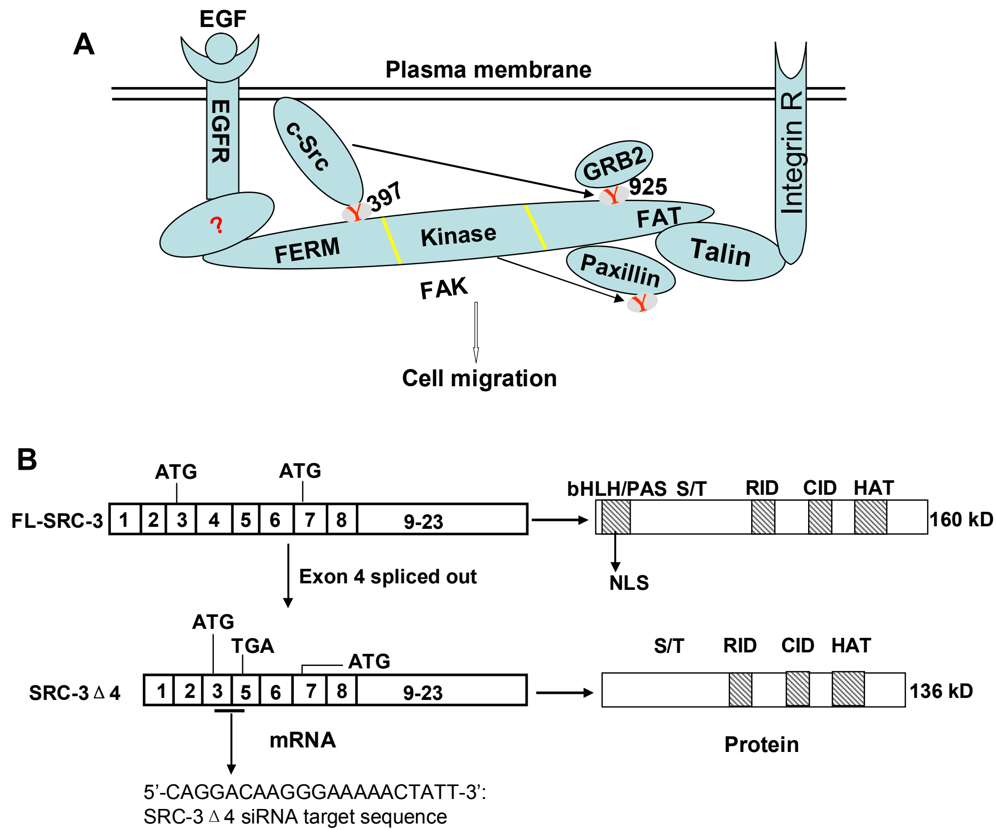


Figure S1 (A). FAK integrates integrin signaling and EGF signal pathway to regulate cell motility (Sieg et al., 2000). FAK protein consists of three regions: the N-terminal region harboring a FERM (band four point one, ezrin, radixin, and moesin) homology domain, the kinase domain, and the C-terminal region harboring a FAT (focal adhesion targeting) domain. The "?" mark represents the unknown factor that mediates the interaction between FAK and EGFR. See the text for details. **(B).** Structures of full-length SRC-3 (FL-SRC-3) and SRC-3Δ4 mRNAs and proteins. FL-SRC-3 mRNA consists of 23 exons with the translation start codon in exon 3. There is a potential internal translation start site in exon 7 that is in-frame with the primary start codon in exon 3. SRC-3Δ4 mRNA is an alterative splicing isoform with exon 4 deleted of the FL-SRC-3 mRNA. The deletion of exon 4 causes the open reading frame to shift and generate a termination codon (TGA) in exon 5. SRC-3Δ4 protein synthesis is initiated at the internal translation start codon in exon 7. FL-SRC-3 protein contains 5 functional domains: the basic helix-loop-helix/PAS domain (bHLH/PAS), the serine/threonine rich domain (S/T), the nuclear receptor interacting domain (RID), the CBP interacting domain (CID), and the histone acetyltransferase domain (HAT). In comparison with FL-SRC-3, SRC-3Δ4 protein lacks the N-terminal bHLH/PAS domain that harbors the nuclear localization signal (NLS). Note that this SRC-3 isoform was originally named SRC-3Δ3 (Reiter et al., 2001). Based on the current GenBank database, an extra non-coding exon is added to 5' of SRC-3 mRNA and exon 3 is accordingly changed to exon 4.

Figure S2

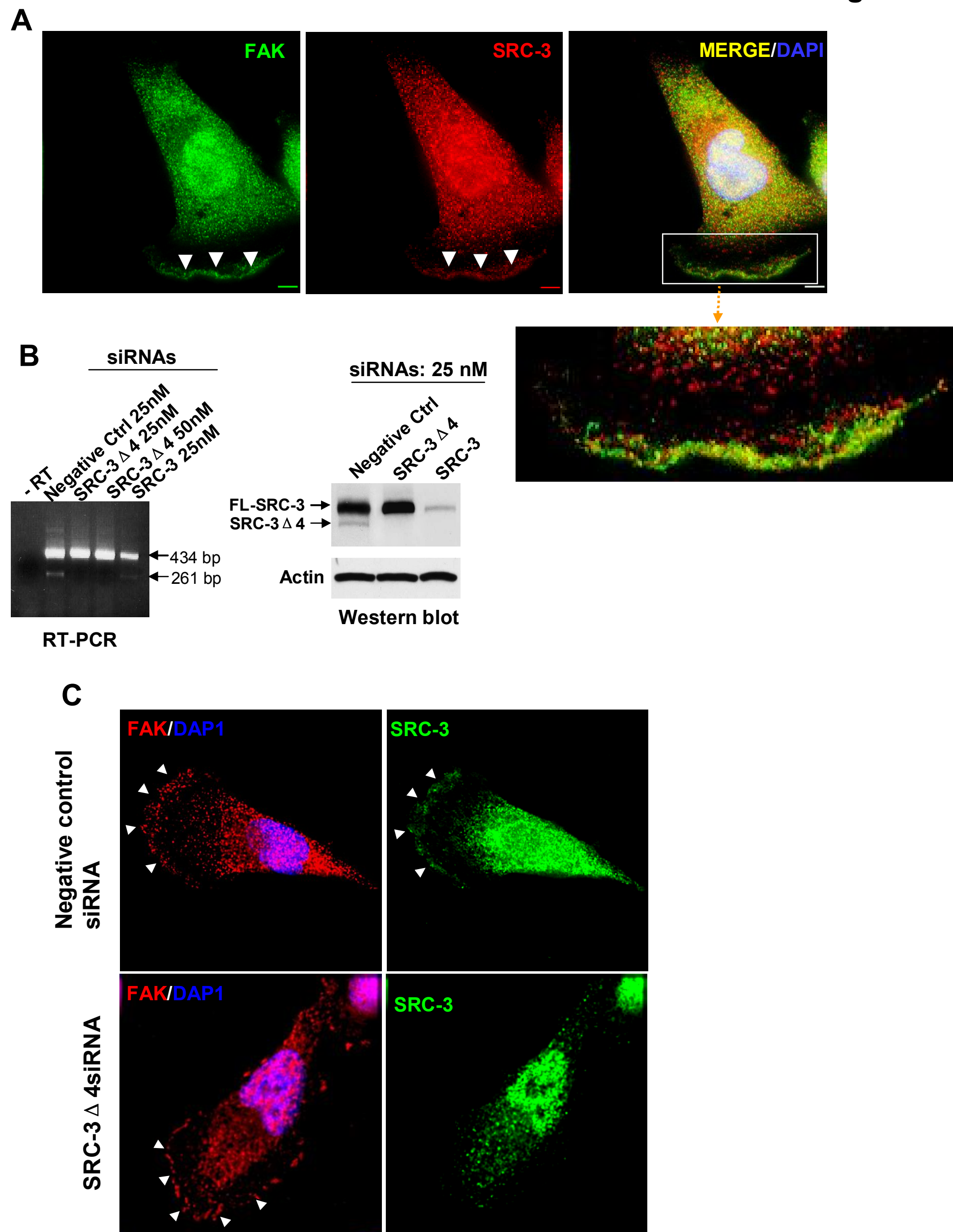
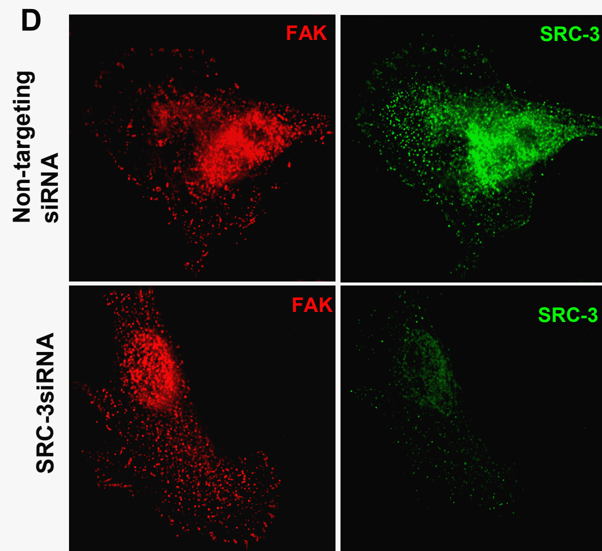
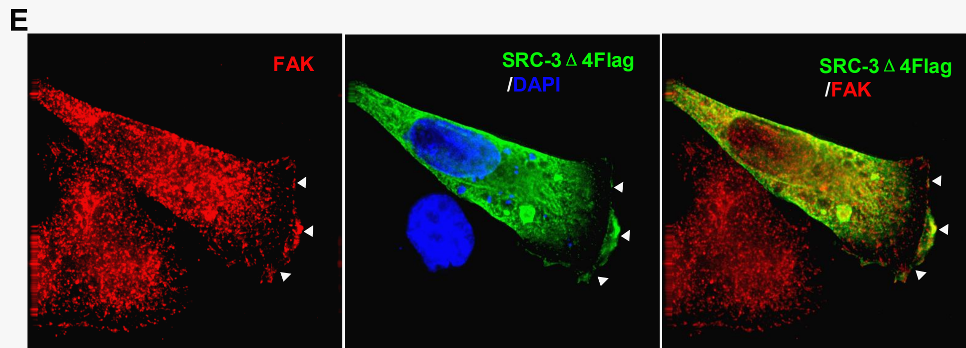


Figure S2





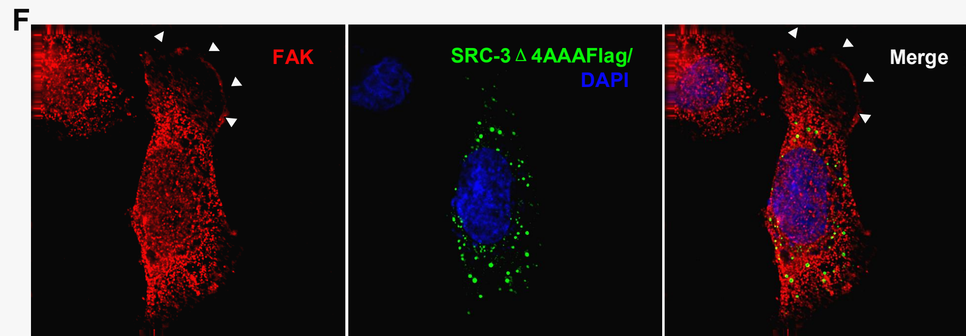


Figure S2

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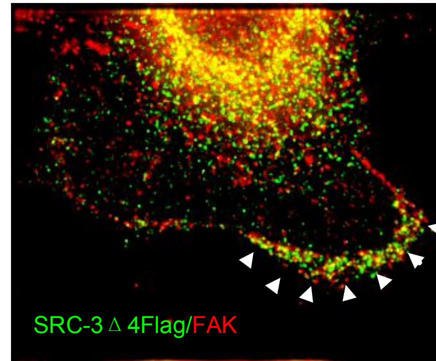
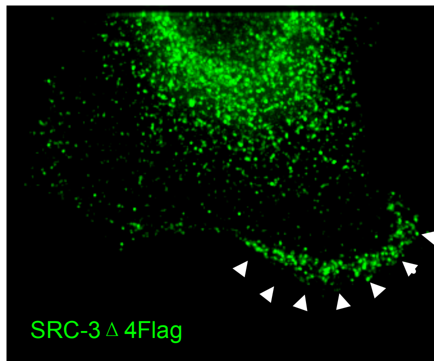




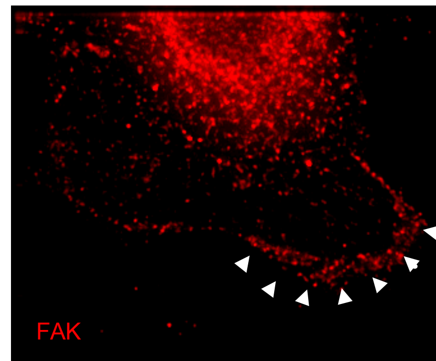


Figure S2 SRC-3Δ4 localizes in the lamellipodia of MDA-MB231 cells geographically with FAK. **(A).** Immunofluorescent staining of endogenous FAK (Green) and SRC-3 (Red) proteins in MDA-MB231 cells. Partial overlapping of immunofluorescent signals between SRC-3Δ4 and FAK is reflected by the yellow immunofluorescence resulting from the merge of the images. Arrowheads indicate the lamellipodia. Bar: 10μM **(B).** Validation by RT-PCR and Western blotting of the siRNA specifically targeting SRC-3Δ4. SRC-3Δ4 siRNA is generated by targeting the junction sequence between exon 3 and exon 5 in SRC-3Δ4 mRNA (Fig. S1B). SRC-3 siRNA SMART pool (Dharmacon) that targets both FL-SRC-3 mRNA and SRC-3Δ4 mRNA was used as a positive control. The silencer negative control #1 (Negative Ctrl) purchased from Ambion was used as a negative control for SRC-3Δ4 siRNA. The 434 bp and 261 bp PCR products were amplified from FL-SRC-3 mRNA and SRC-3Δ4 mRNA, respectively. "-RT" denotes the control without the addition of reverse transcriptase in RT-PCR. **(C).** Immunofluorescent staining of endogenous FAK and total SRC-3 proteins in MDA-MB231 cells treated with either negative control siRNA or SRC-3Δ4 siRNA. Arrowheads indicate the lamellipodia. **(D)**. Immunofluorescent staining of endogenous FAK and total SRC-3 proteins in MDA-MB231 cells treated with either non-targeting siRNA or SRC-3 siRNA targeting both FL-SRC-3 and SRC-3Δ4 mRNAs. **(E) and (F).** Immunofluorescent staining of endogenous FAK using a FAK antibody and exogenously expressed SRC-3Δ4Flag (**E**) or SRC-3Δ4 with simultaneous mutations of T56, S659, and S676 to alanines (SRC-3Δ4AAAFlag, **F**) using a Flag antibody in MDA-MB231 cells. Arrowheads indicate the lamellipodia. **(G)**. High resolution images showing the lamellipodia localization of exogenously expressed SRC-3Δ4 protein and its partial overlapping with FAK in the lamellipodia.

Figure S3

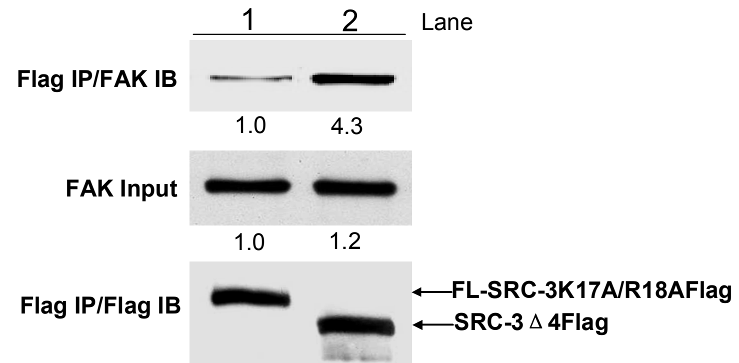


Figure S3 Compared to SRC-3Δ4, SRC-3K17A/R18A mutant has greatly decreased interaction with FAK. 293T cells were transfected with either a plasmid encoding SRC-3Δ4Flag or SRC-3Δ4K17A/R18AFlag, together with FAK plasmid. IP was performed using anti-Flag conjugated agarose beads. The input is 3% of the amount of total cell lysate for IP. Numbers below the Western blots represent the relative intensity of the protein bands. The band intensity in Lane 1 of each blot is set as “1.0”.

Figure S4

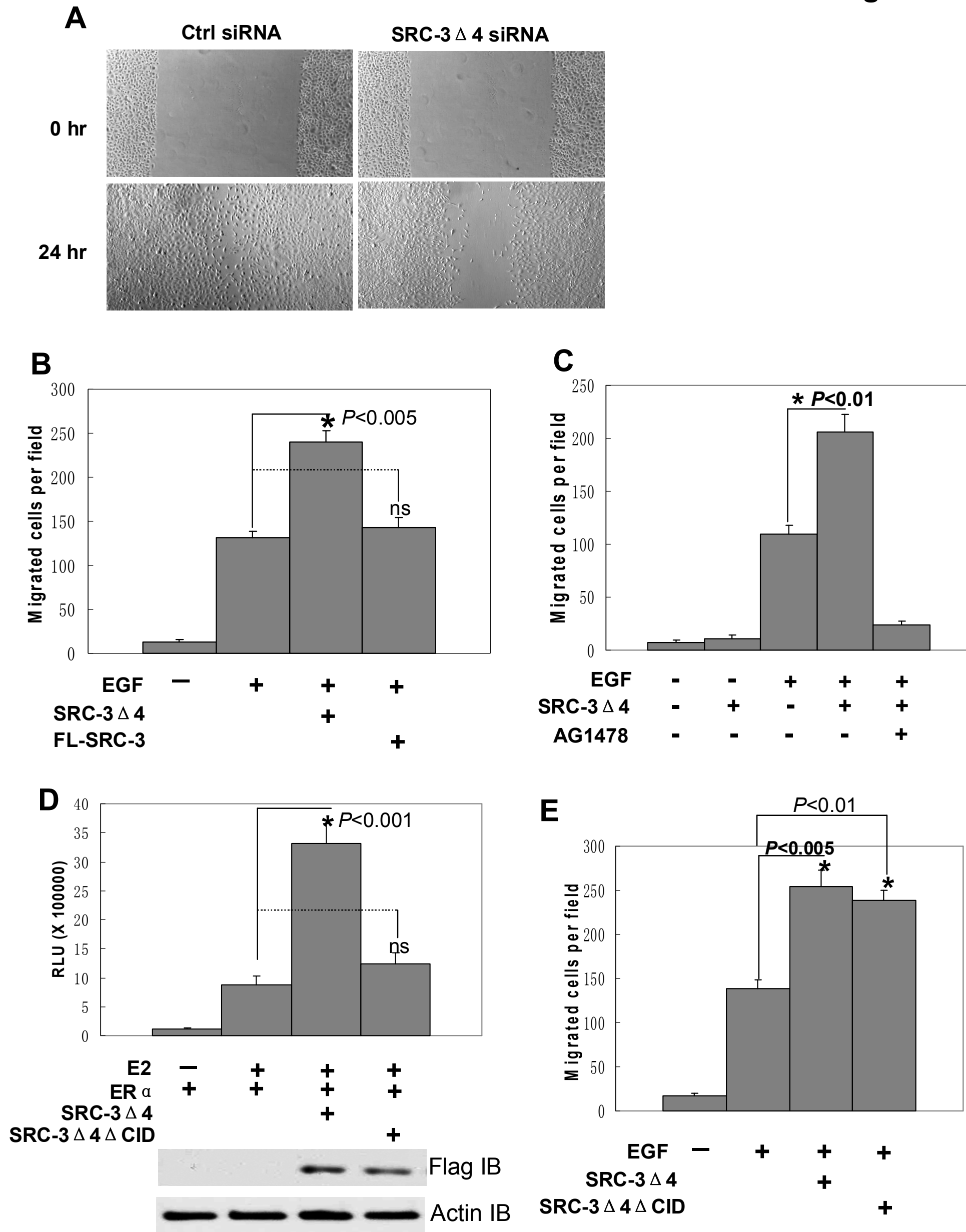
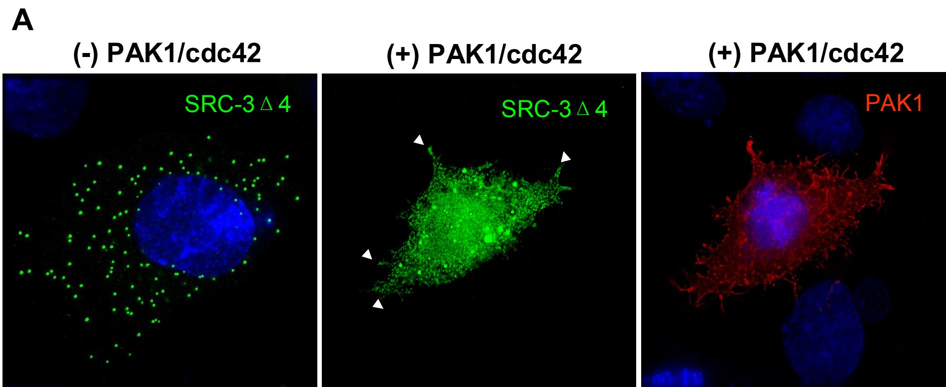


Figure S4 SRC-3Δ4 promotes cancer cell migration. **(A).** Wound-healing assay in MDA-MB231 cells transfected with either the silencer negative control siRNA (CtrlsiRNA) or SRC-3Δ4 siRNA. 48 hrs post-transfection, cultures were scratched with a sterile pipette tip and photographed by a phase-contrast microscope at the time of scratching and 24 hrs later. **(B)**. Transwell cell migration assay in HeLa cells transfected with SRC-3Δ4 or FL-SRC-3. 24 hrs post-transfection, cells were serum-starved overnight. EGF (50 ng/ml) was added to the media in bottom chamber during migration process. Values are means ± s.e of three separate experiments. ns: no significance. **(C)**. HeLa cells were transfected with SRC-3Δ4 or pSG5 vector control. Cell migration assay was performed as in (B). For AG1478 treatment, AG1478 was added to the media in the transwell during migration process. Values are means ± s.e of four separate experiments. **(D)**. Deletion of the p300/CBP interacting domain (CID, aa 833-899) in SRC-3Δ4 abolishes its transcriptional co-activity on ERα-regulated ERE-luciferase gene expression. HeLa cells were singly- or co-transfected with the constructs as indicated in the figure. Luciferase acitivity is represented as relative luciferase units (RLU). Values are means ± s.e of three separate experiments.The equivalent expression levels of SRC-3Δ4 and SRC-3Δ4ΔCID were shown by western blotting using a Flag antibody. **(E)**. SRC-3Δ4ΔCID retains the capability of promoting EGF-stimulated cell migration. HeLa cells were transfected with SRC-3Δ4, SRC-3Δ4ΔCID, or pSG5 vector control. Transwell cell migration assay was performed as in (B). Values are means ± s.e of four separate experiments.

Figure S5



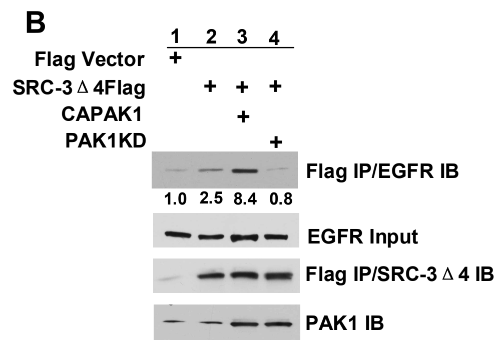


Figure S5 PAK1 promotes SRC-3Δ4 localization to the filopodia and its interaction with EGFR in HeLa cells. **(A).** HeLa cells were transfected with SRC-3Δ4Flag alone or together with PAK1 and cdc42. SRC-3Δ4 (Green) and PAK1 (Red) proteins were detected by immunofluorescence using a Flag Ab and a PAK-1 antibody, respectively. Arrowheads indicate the filopodia. **(B).** HeLa cells were transfected with SRC-3Δ4Flag alone, or together with either constitutively-active PAK1 (CAPAK1) or a PAK1 kinase-dead mutant (PAK1KD). The interaction between SRC-3Δ4Flag and endogenous EGFR was determined by co-IP using anti-Flag beads. Numbers below the Western blots represent the relative intensity of the protein bands by setting the band intensity in Lane 1 as "1.0".

Figure S6

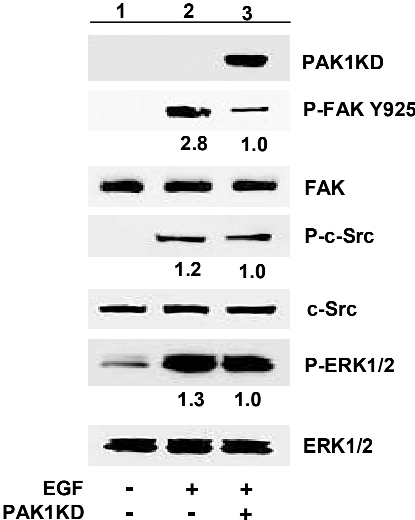
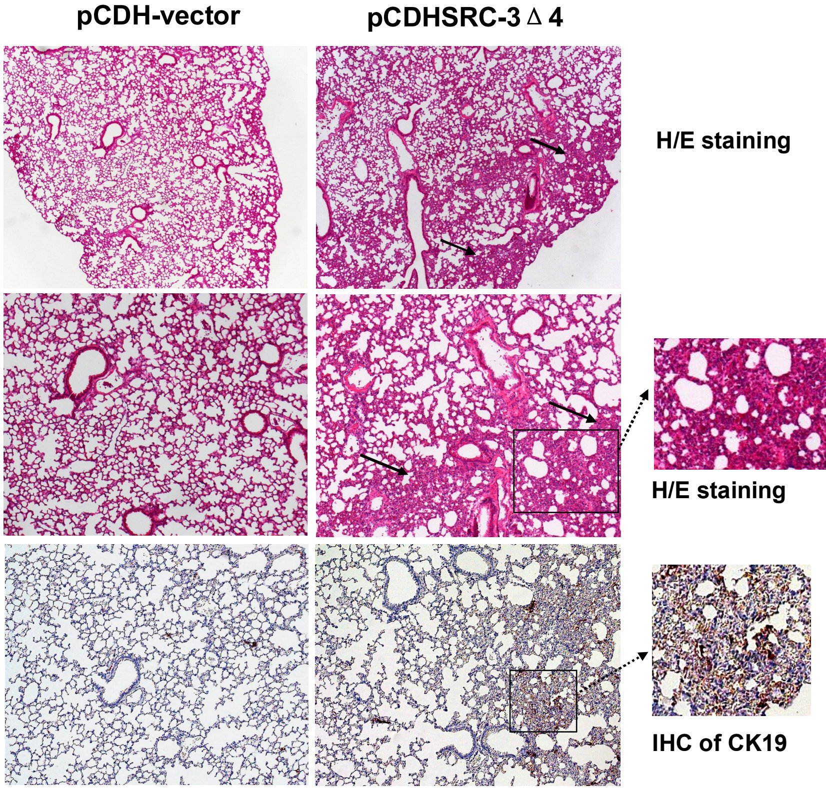


Figure S6 PAK1KD inhibits EGF-induced FAK phosphorylation at Y925. MDA-MB231 cells were transfected with PAK1KD or pCDNA3 vector control. Cells were serum starved overnight, then stimulated with EGF for 15 min. EGF-induced phosphorylation of FAK, c-Src, and ERK1/2 were analyzed by Western blotting. Numbers below the Western blots represent the relative intensity of the protein bands. The band intensity in Lane 3 of each blot is set as “1.0”.

Figure S7



Lungs

Figure S7 Histological analysis of tumor metastasis in lung. Tumor metastasis in lung was analyzed by H/E staining and confirmed by IHC of CK19. Magnification: 25X (top images) and 100X (the middle and bottom images). Arrows indicate the regions with tumor foci.

Figure S8

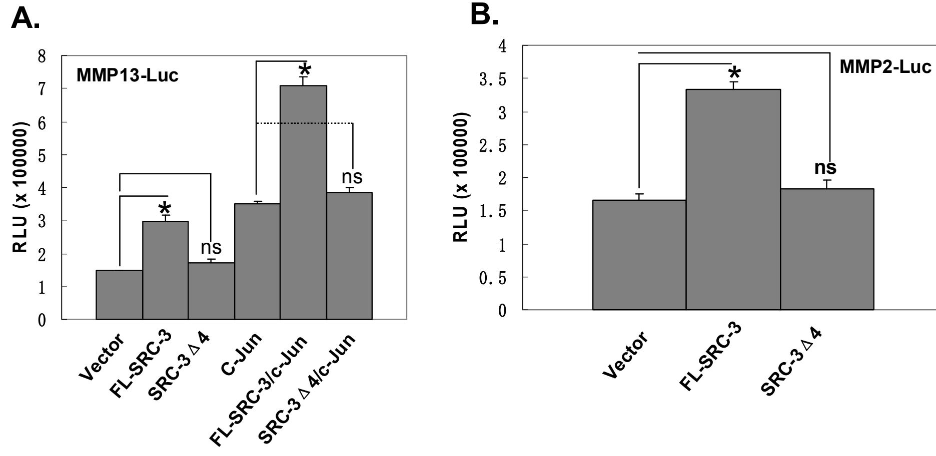


Figure S8 SRC-3Δ4 does not co-activate MMP2- and MMP13-luciferase activity. HeLa cells were singly- or co-transfected with the constructs as indicated in the figure. Luciferase acitivity is represented as relative luciferase units (RLU) on the Y-axis. Values are means ± s.e of three separate experiments. "*" indicates significant difference ($p<0.005$, student's *t* test). ns: no significance.

Supplemental experimental procedures

Expression plasmids

The expression plasmid of full-length SRC-3 with a c-terminal Flag tag (pSG5SRC-3Flag) was described previously (Yi et al., 2008). pSG5SRC-3Δ4Flag was cloned by the following procedures. First, cDNA was synthesized from total mRNA purified from MDA-MB231 cells. Second, RT-PCR was performed using exon 2 upstream primer (5'-GACTGGTTAGCCAGTTGCTG-3') and exon 6 downstream primer (5'-ATGTTTCCGTCTCGATTCACC-3'). Third, the 261 bp RT-PCR product was cloned into pCRII-TOPO vector (Invitrogen) following the manufacturer's instructions. The absence of exon 4 in the amplicon was confirmed by sequencing. Last, pSG5SRC-3Δ4Flag was generated by replacing the Bam H I/Hpa I fragment of SRC-3 cDNA in pSG5SRC-3Flag with the corresponding SRC-3Δ4 fragment released from pCRII-TOPO construct. Flag-tagged SRC-3K17A/R18A was generated as described previously (Li et al., 2007). The expression constructs of full-length FAK (pcDNA3.1-HA-FAKWT), the N-terminal FAK (pCS-Myc-FAK-NT(1-402)), and the c-terminal FAK (pcDAN3.1-HA-FRNK) were kindly provided by Dr. David Schlaepfer at University of California, San Diego, and were described previously (Hauck et al., 2001). pcDNA3EGFR was kindly provided by Dr. Mien-Chie Hung at MD Anderson Cancer center. The constructs of GST-SRC-3Δ4NT(1-250), GST-SRC-3Δ4RID(450-700), GST-SRC-3Δ4CID(701-921) were generated by PCR-amplifying the corresponding region using pSG5SRC-3Δ4Flag as template, followed by subcloning the BamH I/Xho I-digested PCR products into BamH I/Xho I-cut pGEX-5X-1 (GE Healthcare). Similarly, Flag-SRC-3Δ4NT and Flag-SRC-3Δ4RID were generated by subcloning the BamH I/Xho I-digested PCR products into BamH I/Xho I-cut pCMVTag2B (Stratagene). pCMVTag2BSRC-3ΔRID was generated by PCR using pCMVTag2BSRC-3 as template and the

following two primers: the forward primer (5'- GGAAGATCTAGTGCTTTC CCCATGTTACCAAAGCAAC-3') and the reverse primer 5'-GGA AGATCTGTCACTGAGGTGATCTCTGCTATTTGD-3'. The PCR product was then cut with Bgl II and ligated. SRC-3ΔRID fragment was cut off from pCMVTag2BSRC-3ΔRID by Hpa I/Xho I digestion and replaced the corresponding SRC-3Δ4 fragment in pSG5SRC-3Δ4 to generate pSG5SRC-3Δ4ΔRID. GFP-RID was generated by PCR-amplifying RID region using pSG5SRC-3Δ4Flag as template, followed by subcloning the Sal I/Kpn I-digested PCR product into Sal I/Kpn I site of pEGFP-C1 (Clontech). The expression constructs of PAK1 kinase-dead mutant (PAK1K299R) and constitutive-active PAK1 (PAK1T423E) were described previously (Adam et al., 2000; Vadlamudi et al., 2000). pCDHSRC-3Δ4Flag was generated by inserting SRC-3Δ4Flag fragment released from pSG5SRC-3Δ4Flag by Apa I/NheI digestion (blunt-ending Apa I site with Klenow emzyme) into pCDH-CMV-MCS-EF1-Puro (System Biosciences) digested with Xba I/Nhe I (blunt-ending Xba I site with Klenow enzyme). All of the SRC-3Δ4 constructs with point mutation(s) were generated by site-directed mutagenesis using the Quick-change kit (Stratagene, La Jolla, CA) and the sequences of the resulting mutants were verified by sequencing.

Cell culture and transfection

Cell lines MDA-MB231, HeLa, and HEK293T were purchased from American Type Culture Collection (ATCC). SRC-3-null mouse embryonic fibroblast (MEF SRC-$3^{-/-}$) cells were generated as described previously (Wu et al., 2004). Cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% FBS. All the culture media and supplements were purchased from Invitrogen. Cells were transfected with Fugene HD reagent (Roche) following the manufacturer's instructions. Unless specifically indicated, cells were harvested for

various analyses 48 hrs post-transfection.

Immunoprecipitation and Western blotting

For immunoprecipitation (IP) of endogenous proteins, 3 x 10^7 of MDA-MB231 cells (from two T150 plates with 95% confluency) were lysed with EBC lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% NP-40, 1 mM PMSF, 1 mM Complete protease inhibitors (Roche Diagnostics), 10 mM NaF, 1 mM sodium orthovanadate, and 1 mM Phosphatase Inhibitor Cocktail I (Sigma)). The total protein lysate was evenly distributed into two portions for IPs using either each specific antibody or the corresponding IgG control. For immunoprecipitation of exogenously expressed SRC-3Δ4Flag and its associated proteins, 1 x 10^7 of HeLa cells were transiently transfected with 6μg of SRC-3Δ4Flag plasmid. The cells were lysed with EBC lysis buffer and 1mg of protein supernatant was used for each IP. The supernatant was precleared with 40 μl protein A/G agarose beads for 1 hr at 4ºC with constant rotation. The samples were then incubated with desired antibody for 2 hr, followed by the addition of 40 μl protein A/G agarose beads for additional 1 hr. The beads were washed three times (5 min/wash) with lysis buffer. Proteins were boiled off the beads in 2x Laemmili sample buffer and resolved on 4–15% SDS-PAGE gels (BioRad). 3% of the amount of protein supernatant for IP was loaded as the input control. Western blotting was performed by first blocking nitrocellulose membranes with 5% nonfat milk in PBS-T buffer for 30min, followed by overnight incubation with primary antibody at 4ºC and 1 hr incubation with appropriate secondary antibody at room temperature. The Western blot was visualized by chemiluminescence (Amersham) and quantified by Image J software (NIH, US). Antibodies used for IP were: anti-SRC-3 (Santa Cruz), anti-FAK (Santa Cruz), anti-Flag (Sigma), and anti-HA (BD Biosciences). Primary antibodies used in western blotting were: anti-SRC-3 (Wu et al., 2004), anti-Flag (Sigma), anti-FAK (BD Biosciences), anti-phospho-Y925 (Cell Signaling) and

anti-phospho-Y397 (Biosource International) of FAK, anti-PAK1 (abcam), anti-c-Src (Santa Cruz), anti-phospho-c-Src (Cell Signaling), anti-β-actin (Chemicon International), anti-GFP (Santa cruz), anti-HA, anti-EGFR (Santa Cruz), anti-ERK1/2 (Cell signaling), and anti-phospho-ERK1/2 (Promega).

***In vitro* protein-protein pull-down assay**

HA-FAK or HA-EGFR protein was expressed in 293T cells and purified using anti-HA conjugated agarose beads according to the manufacturer's instructions. The N-terminal region of SRC-Δ4 (Δ4NT, aa 1-250), the receptor interaction domain-containing region (Δ4RID, aa 450-700), and the CBP interacting domain-containing region (Δ4CID, aa 701-921) were expressed as GST-fusion proteins in bacteria. Purified GST-SRC-3Δ4 fragment fusion proteins were incubated with HA-FAK or HA-EGFR protein immobilized on agarose beads with constant rotation in cold room for 2 hrs. Beads were spun down and washed five times (5 min per wash). Proteins were then eluted off the beads with 2 x Laemmili sample buffer. The Western blot was probed with GST antibody.

Wound-healing assay MDA-MB231 cells were transfected with either the silencer negative control siRNA or SRC-3Δ4 siRNA. 72 hrs post-transfection when cells reached 95% confluency, a straight scratch was made in the center of the plate with a sterile pipette tip and photographed by phase-contrast microscope at the time of scratching and 24 hrs later.

Luciferase reporter assay

Luciferase reporter assay was performed as described previously (Yan et al., 2008). Luciferase activity was determined with the Promega luciferase assay kit.

***In vitro* phosphorylation assay**

In vitro phosphorylation assay was carried out in 50 mM HEPES, 10 mM $MgCl_2$, 2 mM $MnCl_2$,

0.5 mM dithiothreitol, and 5 mM β-glycero-phosphate. Each reaction contains 30 ng of purified PAK1 kinase (Calbiochem), 800 ng of purified GST-SRC-Δ4 fragment fusion protein, 5 μCi ^{32}P-ATP (Perkin Elmer), 25 μM cold ATP in a total volume of 30 μl. The reaction was carried out at 30 °C for 30 min and then stopped by adding 10 μl of 4 X SDS sample buffer. Proteins were resolved by SDS-PAGE gel and visualized by autoradiography.

PAK1 phosphorylation assay in 293T cells

293T cells were transfected with SRC-3Δ4Flag alone, or co-transfected with CAPAK1 and SRC-3Δ4Flag or its mutants with mutation (s) in the potential PAK1 phosphorylation site(s). 36 hrs post-transfection, cells were washed once with phosphophate-free DMEM (Invitrogen) and incubated in phosphophate-free DMEM containing 5% FBS for 2 hrs. Cells were then metabolically labeled with ^{32}P-orthophosphoric acid (Perkin Elmer, Boston, MA) in phosphate-free DMEM containing 10% FBS for 5 hrs. Cell were lysed and SRC-3Δ4 proteins were immunoprecipitated using anti-Flag M2 agarose beads. Samples were resolved by SDS-PAGE gel and the protein phosphorylations were determined by autoradiography.

Generation of MDA-MB231 cell line stably overexpressing SRC-3Δ4 **using lentiviral transduction**

Production of pseudolentiviral particles and generation of MDA-MB231 cell line with stable overexpression of SRC-3Δ4 by lentiviral transduction were performed by following the manufacturer's instructions. Pseudolentiviruses were produced in 293TN cells (System Biosciences) by co-transfecting lentiviral expression construct pCDHSRC-3Δ4Flag and pPACK packaging plasmid mix (System Biosciences, Cat. # LV500A-1). Pseudoviral particles were harvested 48 hrs post-transfection. MDA-MB231 cells were transduced with prepared virus with the addition of polybrene (5μg/ml). Two days post-transduction, cells were split and selected by

puromycin (1 μg/ml).

MTT cell proliferation

Cell proliferation assay was performed using CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT) kit (Promega) by following the manufacturer's instructions.

Immunohistological staining

Tissue sections were deparaffinized and rehydrated in graded ethanol for immunohistological staining by following the general procedures as described previously (Long et al., 2003). Samples were incubated with anti-mammaglobin A (Abcam) or anti-CK19 (Abcam) at the dilution of 1:1000 overnight at 4°C. Samples were then incubated with biotinylated secondary antibody solution for 1 hr at room temp, followed by incubation with VECTASTAIN ABC reagent (Vector Laboratories) for 30 min. Signals were developed with 3, 3′-diaminobenzidine (DAB) substrate kit (Vector Laboratories). The sections were counterstained with hematoxylin.

Supplemental References

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