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Supplemental Data

Atypical Protein Kinase C Regulates Dual Pathways for Degradation of the Oncogenic Coactivator SRC-3/AIB1

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Supplemental Experimental Procedures

Plasmids and Reagents

The V5 tagged PKCζ or PKCι mammalian expression vectors and their kinase dead mutants were kindly provided by Dr. Gordon B. Mills. The 20S C8 cDNA or PSMA7 cDNA was synthesized by RT-PCR from MCF-7 cell mRNA and was inserted into pGEX vector (Amersham Biosciences) for GST fusion protein. SRC-3 point mutants were generated by the site-directed mutagenesis kit (Stratagene). Sequences of the Ser/Thr to Ala mutants of SRC-3 in region 1031-1130 were shown in Fig. 4F. 2S/T-A: S1033/1042A; 4S/T-A: S1033/S1042/T1067/T1114A; 8S/T-A: S1033/S1042/S1048/ T1059/S1062/T1064/ T1067/T1114A. All SRC-3 deletion mutants were generated by double PCR strategy and the amplified fragments were inserted into the XbaI and SanDI sites of PCMV-flag-SRC-3 construct. The p21-SRC-3 fusion constructs were also generated by double PCR strategy and the amplified fragments were inserted into the HindIII and XhoI sites of PCR3.1 vector. pTER-shPKCζ was generated by inserting synthesized oligo into BglII and HindIII sites of pTER vector.

The anti-SRC-3 antibody was generated as described previously (Wu et al., 2002). Other antibodies were from various sources: anti-flag (Sigma), anti-β-actin (Sigma), anti-hemagglutini (HA, Roche), anti-ERα (Santa-Cruz), anti-ERβ (Santa-Cruz), anti-PR

(Santa-Cruz), anti-V5 and anti-SRC-1 (Bethyl), anti-p21 (BD Biosciences), anti-C8 and PSMA7 (Boston Biochem) and anti- PKCζ (upstate).

Kinase inhibitors LY294002, G06976 and GF109203x were from Calbiochem.

Cell Lines and Transfections

Flp-In 293(flag-SRC-3) cell line (integrated with ERα) was generated as described previously (Yi et al., 2005). Stable PKCζ knockdown MCF-7 cells were generated by being transfected with pTER-shPKCζ plasmid (shPKCζ sequence was derived from Dharmacon smartpool siRNA sequence) and were selected in 0.2mg/ml Geneticin and 0.5mg/ml zeocin (Invitrogen) containing DMEM medium. The colonies grown under antibiotics selection were mixed together as a pool. The conditions and procedures for plasmid, siRNA transfection and steroid hormone dependent transcription are the same as described previously (Yi et al., 2005). Smart pool siRNA and individual siRNAs (Dharmacon) were used to knock down PKCζ in MCF-7 cells. HeLa cells were transfected with expression plasmids containing constitutively active PKCζ, ERα and different SRC-3 mutants in the presence of estrogen unless otherwise specified. Cells were harvested and lysed two days after transfection. For PKCι rescue experiment, Flp-In 293 (flag-SRC-3) cells were transfected with 20 nM siPKCζ and siPKCι , or scrambled control siRNA for two days, and then transfected with ERα, V5-PKCι or the empty vector, and 10 nM siPKCζ and siPKCι. One day later, the expression of flag-SRC-3 was induced by 0.01 μg/ml tetracycline for 24 hrs. Cells were then harvested and the lysates were subjected to Western blot analysis. For kinase inhibitors, cells were treated different inhibitors for one day before harvested.

GST Pull-Down

E.coli expressed GST-C8, its deletion mutants or GST-PSMA7 was bound by 7 μl glutathione sepharose 4B (Amersham Biosciences) followed by the addition of 293T cell extracts. The procedure is essentially the same as described previously (Yi et al., 2005). For the interaction between GST-PSMA7 and HIF-1α or the interaction between GST-C8 and p21-SRC-3 fusion proteins, HA-HIF-1α and p21-SRC-3 fusion proteins were

transcribed and translated in vitro using TNT Quick coupled transcription/translation system (Promega). 45 μl of reaction mixture were used in the GST pull-down assay. The bound HA-HIF-1α and p21-SRC-3 fusion proteins were subjected to SDS-PAGE and detected by anti-HA, anti-p21 antibody, respectively.

Pulse-Chase Experiment

HeLa cells were transfected with flag-SRC-3, ERα and with or without active PKCζ. Cells were labeled with ^{35}S-Met/Cys as described previously (Yi et al., 2005). Flag-SRC-3 were immunoprecipitated from cell lysates by anti-flag antibody, boiled in 1X sample buffer and loaded to a SDS-PAGE gel.

In Vitro Kinase Assay

Purified recombinant SRC-3 from baculovirus was incubated with 75 mM MgCl2, 0.5 mM ATP, 1 μl γ-^{32}P-ATP, 1X protease inhibitor cocktail (Roche) and 100 ng PKCζ (upstate) in 8 mM Hepes, 0.2 mM EDTA containing solution in the absence or presence of purified ERα (Invitrogen) or 1 μM E2 for 30 min at 30°C. The reaction was stopped by the addition of 5X sample buffer and the kinase activity was detected by autoradiography.

Real-Time Quantitative PCR and RT-PCR

MCF-7 cell and HeLa cell total RNA was extracted using Tri-reagent (Molecular Research Center, Inc.). The pS2, c-myc, cyclin D1 mRNA and the cyclophilin mRNA (as an internal control) were quantitated by Taqman-based reverse transcriptase PCR using the AIB1 Prism 7700 sequence detection system (Applied Biosystems). The primers for the c-myc mRNA as follows: forward, 5'-TCCACAC ATCAGCACAACTACG, reverse, 5'-CACTGTCCAACTTGACCCTCTTG, probe, 5'-FAM-CCTCCACTCGGAAGGACT-TAMRA. The primers for the cyclin D1 mRNA were as follows: forward, 5'-GCTGCTCCTGGTGAACAAGC, reverse, 5'-AAGTGTTCAATGAAATCGTGCG, probe, 5'-FAM-TCATTGCGGCCAGGTT CC-

TAMRA. The primers for the pS2 and cyclophilin mRNA were described previously (Yi et al., 2005).

The RT-PCR analysis was performed using the Access RT-PCR kit (Promega). The primers for the flag-SRC-3 were described previously (Yi et al., 2005).

Experimental Repetitions

All experiments were repeated at least 3 times and statistics are provided in legends where appropriate. Gel bands and Western blots are normalized against controls (e.g., β-actin), and quantifications (by Scion Image) are noted in Figures.

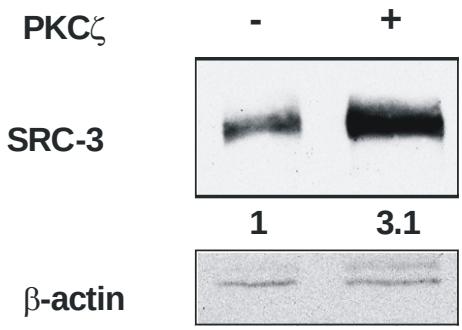
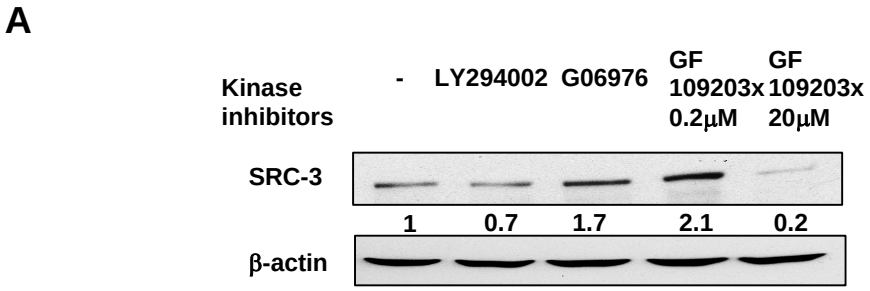


Figure S1. PKCζ Increases SRC-3 Protein Level
293T cells were transfected with flag-SRC-3 and ERα in the absence and presence of active PKCζ. Shown is Western blot analysis using anti-SRC-3 antibody.



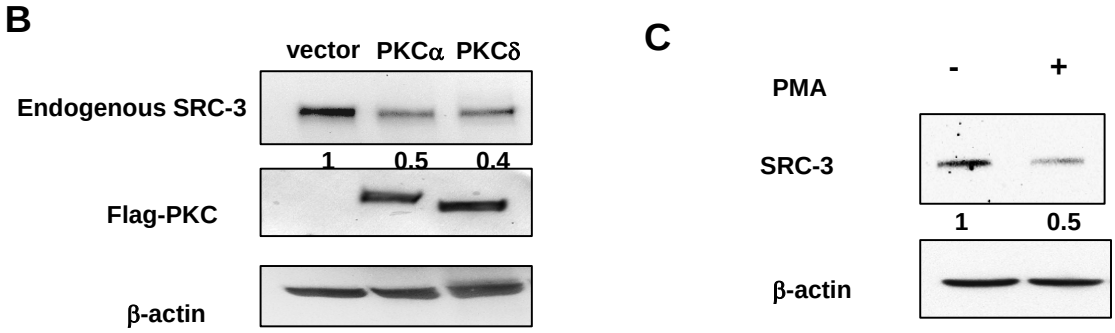


Figure S2
(A) Effects of different kinase inhibitors on endogenous SRC-3 levels in MCF-7 cells. At low concentrations (0.2 μM) the GF109203x compound inhibits conventional and novel PKCs, whereas at higher concentrations (20 μM) it inhibits all PKCs including the atypical PKCs (the IC50 for aPKC is 6-10 μM)(Mao et al., 2000; Uberall et al., 1999). G06976 has been used as an inhibitor for conventional PKC (Martiny-Baron et al., 1993). LY294002 inhibits PI3K activity.
(B) The effects of conventional and novel PKC (PKCα and PKCδ, respectively) on SRC-3 level in HeLa cells.

(C) The effect of PMA (activator for conventional and novel PKC) on SRC-3 level in HeLa cells. Cells were treated with 50 ng/ml PMA overnight before harvested.

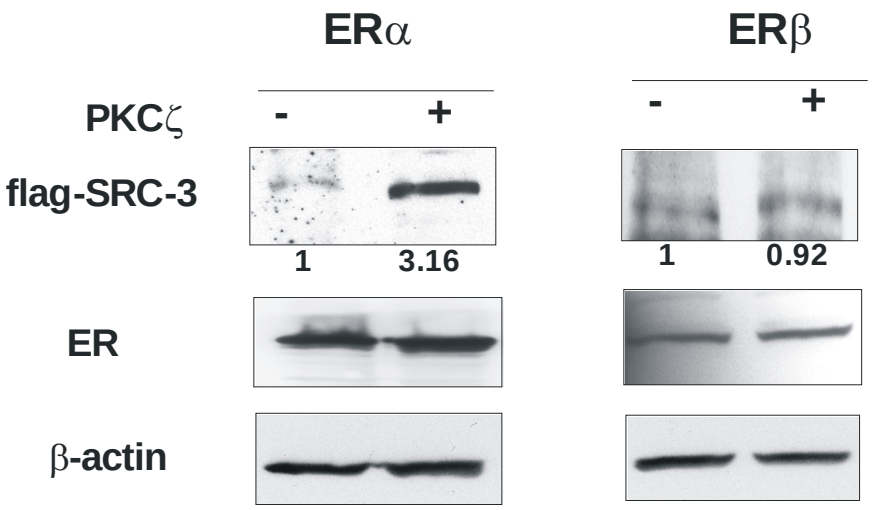


Figure S3. ERβ Is Not Able to Support the Stabilization Effect of PKCζ on SRC-3 in Contrast to ERα

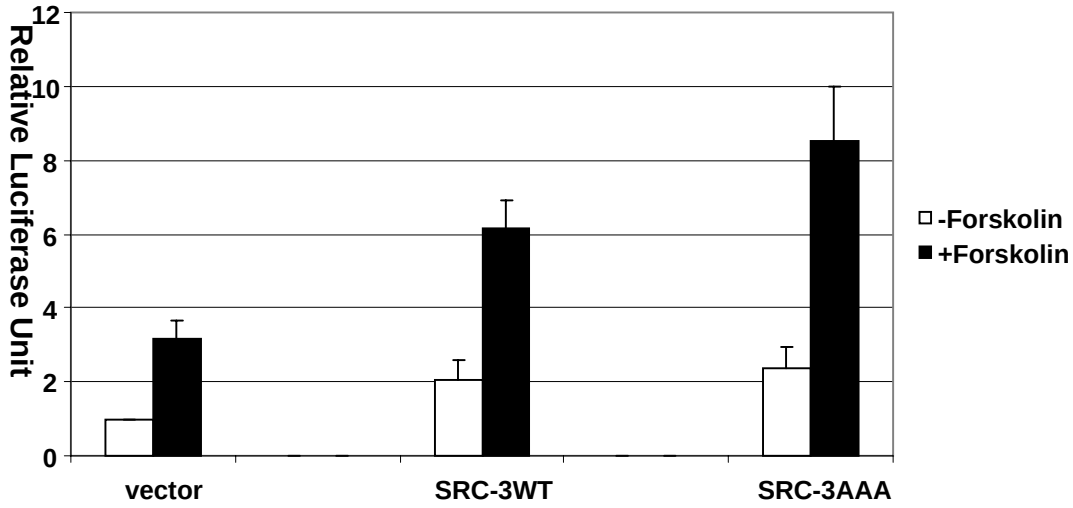


Figure S4. SRC-3AAA Mutant Is Functional in Activating CREB-Mediated Transcription

HeLa cells were transfected with CRE-luc and SRC-3 containing expression vectors or the empty vector. Two days after transfection cells were treated with 10 μM Forskolin for 5 hrs before harvested.

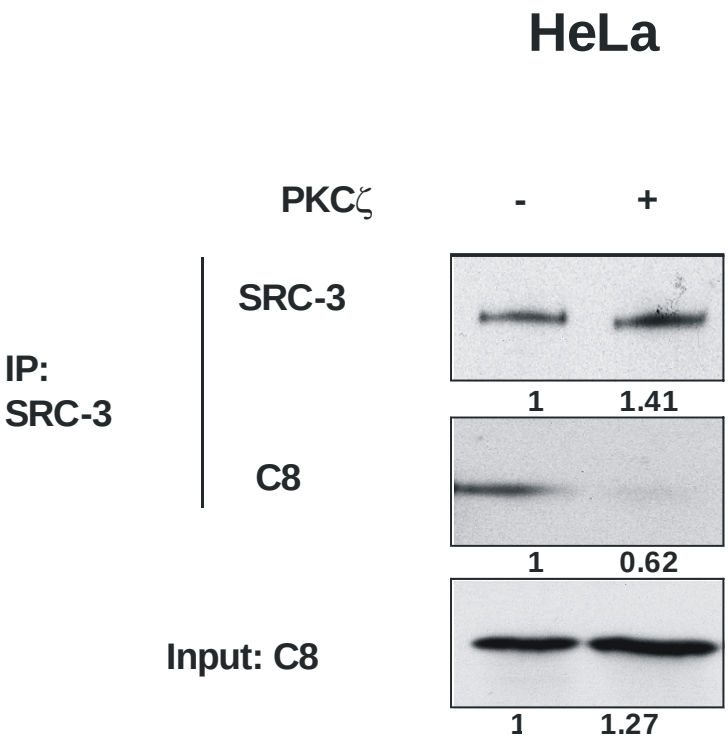


Figure S5. PKCζ Decreases the Interaction between Endogenous SRC-3 and C8 in HeLa Cells

HeLa cells were transfected with expression vector containing active PKCζ or the empty vector. Cells were treated with 10 μM MG132 for 16 hrs before lysed in order to keep the same amount of input SRC-3 and C8. Co-immunoprecipitation experiment was performed using anti-SRC-3 antibody and the amount of C8 bound to SRC-3 was detected by Western blot analysis using anti-C8 antibody.

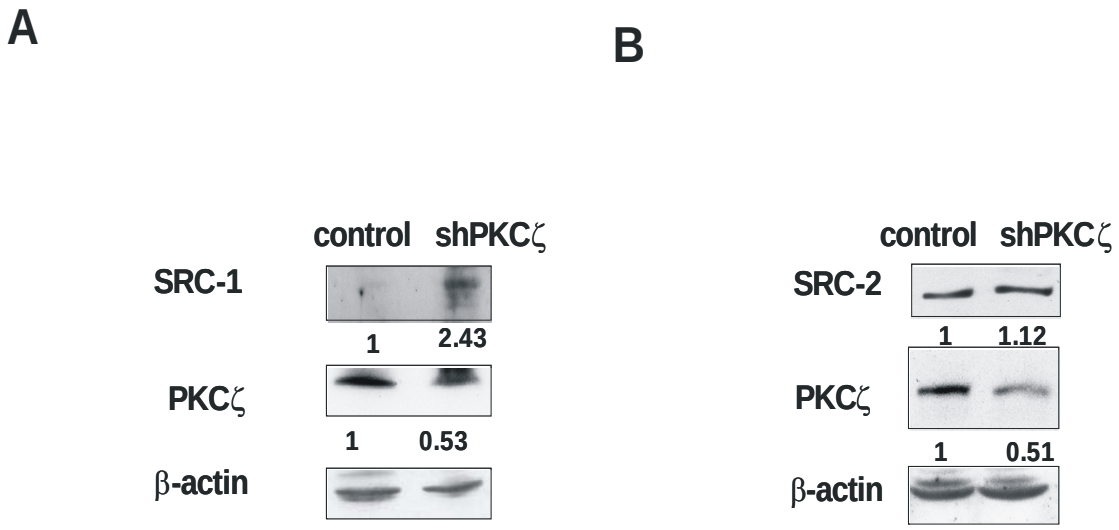


Figure S6.
The effects of PKCζ knock-down on the protein levels of SRC-1 **(A)** and SRC-2 **(B)**.

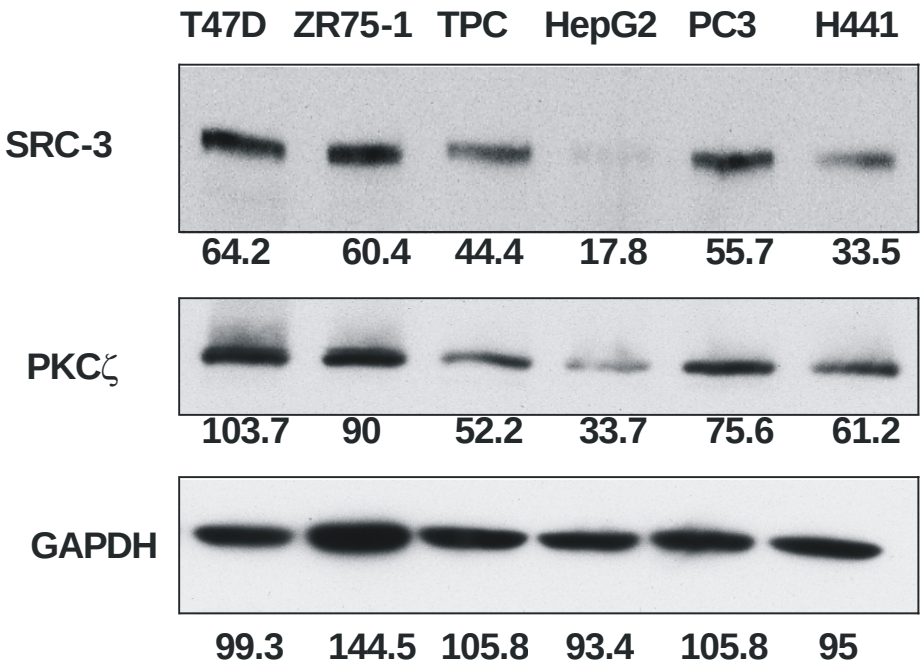


Figure S7.
The correlation of endogenous SRC-3 and PKCζ protein levels in several different cancer cell lines (T47D and ZR75-1: breast cancer cell lines; TPC: thyroid cancer cell line; HepG2:Hepatocellular liver carcinoma cell line; PC3: prostate cancer cell line; H441: lung adenocarcinoma epithelial cell line).

Supplemental References

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