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TITLE: Role of SRC-3delta4 in the Progression and Metastasis of Castration-Resistant Prostate Cancer

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14. ABSTRACT SRC-3Δ4, an N-terminus deletion isoform of steroid receptor coactivator (SRC-3), was shown to act as a signaling adaptor of EGF signaling. Its role in prostate cancer (PCa) progression is unclear. As such, we tested whether SRC-3Δ4 coactivates AR in an androgen-independent manner and promotes prostate cancer cell growth and invasiveness in response to EGF signaling. We have found that EGF stimulated the interaction of SRC-3Δ4 with AR, the recruitment of SRC-3Δ4 to the promoters of AR target genes, and AR target genes' transcription in androgen-depleted culture conditions. In addition, SRC-3Δ4 promotes androgen-independent prostate cancer cell growth and invasion, in which EGF-induced phosphorylation of SRC-3Δ4 plays a critical role. However, SRC-3Δ4 did not affect AR-mediated prostate tumor growth and progression in xenograft mouse models. Taken together, these results demonstrate that SRC-3Δ4 acts as a coactivator of AR and regulates the transcription of AR target genes and prostate cancer cell growth and invasion in response to EGF signaling in cultured cells.						
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1. INTRODUCTION

AR and its target genes are commonly expressed in and are believed to contribute to castration resistant prostate cancers (CRPC) (1, 2). Upregulation of growth factor signaling and alterations in AR coactivators are two major molecular mechanisms for castration-resistant tumor progression (3). Steroid receptor coactivator 3 (SRC-3) promotes prostate cancer cell growth and invasion and is important for tumor metastasis in animal models of prostate cancer (4-6). The importance of SRC-3 in castration-resistant prostate tumor progression, however, remains largely unknown. SRC-3 Δ 4, an N-terminus deletion isoform of SRC-3, was shown to act as a signaling adaptor of EGF signaling in activating FAK. Interestingly, our preliminary data showed that SRC-3 Δ 4 is upregulated in castration resistant PCa cells as compared to androgen-dependent PCa cells, and SRC-3 Δ 4 coactivates AR-mediated PSA-luciferase reporter activity in the absence of androgen. As such, we hypothesized that SRC-3 Δ 4 acts as a coactivator of AR in the nucleus and regulates the transcription of AR target genes in response to EGF signaling. To test this hypothesis, we have performed the following specific Aims as described in our Statement of Work: Aim 1/Task 1-determine whether SRC-3 Δ 4 coactivates AR in an androgen-independent manner in response to EGF signaling; Aim2/Task 2-determine the role of SRC-3 Δ 4 in androgen-independent prostate cancer cell growth and invasion; Aim 3/Task 3-determine the underlying molecular mechanisms by which SRC-3 Δ 4 coactivates AR transcriptional activity and promotes androgen-independent prostate cancer cell growth and invasion (Aim 3/Task 3); Aim4/Task 4-determine the role of SRC-3 Δ 4 in prostate tumor progression and metastasis in castrated nude mice.

2. KEYWORDS: SRC-3 Δ 4, AR, EGF, androgen-independence, gene promoter occupancy, gene transcription and prostate tumor growth and progression.

3. ACCOMPLISHMENTS

Task 1(Aim 1) was to test whether SRC-3 Δ 4 coactivates AR in an androgen-independent manner in response to EGF signaling.

Task 1a To determine whether SRC-3 Δ 4 interacts with AR and translocates to the nucleus upon EGF stimulation. In comparison with full-length SRC-3 protein, SRC-3 Δ 4 lacks the N-terminal bHLH-PAS region that contains the nuclear localization signal (NLS) (7). There is an intriguing possibility, however, that SRC-3 Δ 4 interacts with AR via its steroid receptor interacting domain and translocates to the nucleus together with AR. We analyzed the subcellular localization and the interaction of SRC-3 Δ 4 and AR by immunofluorescence

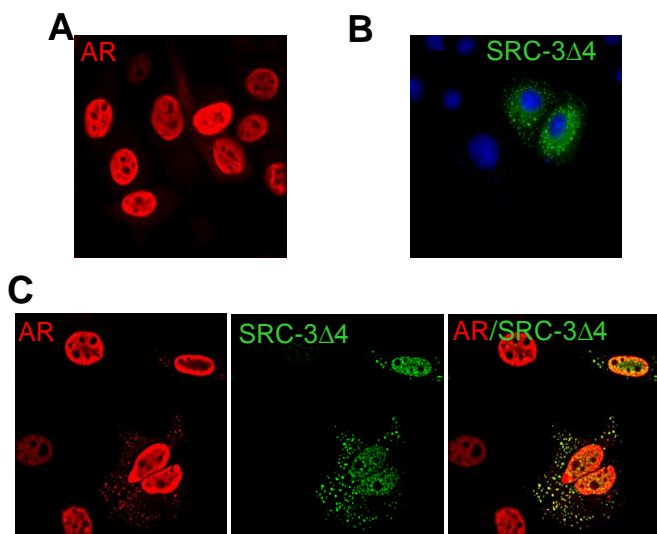


Figure 1 EGF stimulates SRC-3 Δ 4's interaction with AR and its nuclear localization. (A). Endogenous expression and cellular localization of AR in LNCaP-C4-2 cells growing in phenol-red free RPMI medium containing 5% charcoal-stripped fetal bovine serum (cs-FBS). (B). Cellular localization of SRC-3 Δ 4 in LNCaP-C4-2 cells transfected with a construct expressing SRC-3 Δ 4 bearing a Flag-tag at the C-terminus (SRC-3 Δ 4Flag). (C). LNCaP-C4-2 cells were cultured in phenol-red free RPMI medium containing 5% cs-FBS. Cells were transiently transfected with a construct expressing SRC-3 Δ 4Flag. 24 hours post-transfection, cells were serum-starved for 24 hours, and then were stimulated with 50 ng/ml EGF for 30 minutes. SRC-3 Δ 4Flag and endogenous AR proteins were detected by immunofluorescence using a Flag antibody (Sigma) and an AR antibody (Santa Cruz), respectively. Protein subcellular localization and co-localization were analyzed by deconvolution microscopy. The nucleus was stained with DAPI in blue color.

and co-immunoprecipitation (co-IP) in LNCaP-C4-2, a CRPC cell line. AR primarily localizes in the nucleus and a small fraction of AR is shown in the cytoplasm (Fig. 1A). As there is not a specific antibody available for differentiating endogenous SRC-3 Δ 4 from the full-length SRC-3, we transiently transfected SRC-3 Δ 4 with a Flag tag into LNCaP-C4-2 and detected SRC-3 Δ 4 using a Flag antibody. As shown in Fig. 1B, SRC-3 Δ 4 primarily localizes in the cytoplasm in the absence of EGF stimulation. In contrast, SRC-3 Δ 4 is shown to localize in the nucleus and co-localizes with AR (Fig. 1C) in response to EGF stimulation. Interestingly, SRC-3 Δ 4 and AR are also shown to co-localize in the cytoplasm (Fig. 1C), implying that EGF stimulates the interaction of SRC-3 Δ 4 with AR in the cytoplasm and its translocation with AR into the nucleus.

We confirmed the interaction between SRC-3 Δ 4 and AR by reciprocal co-IP using either a Flag antibody (against SRC-3 Δ 4) or an AR antibody. As shown in Fig. 2, EGF greatly stimulates the interaction of SRC-3 Δ 4 with AR under androgen-depleted culture conditions in LNCaP-C4-2 cells.

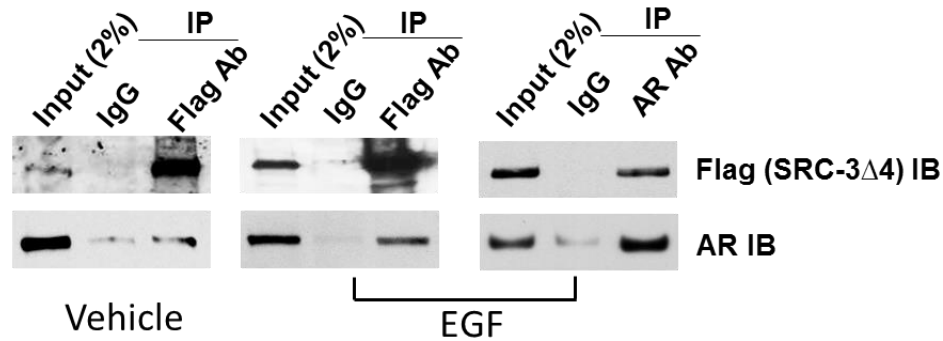
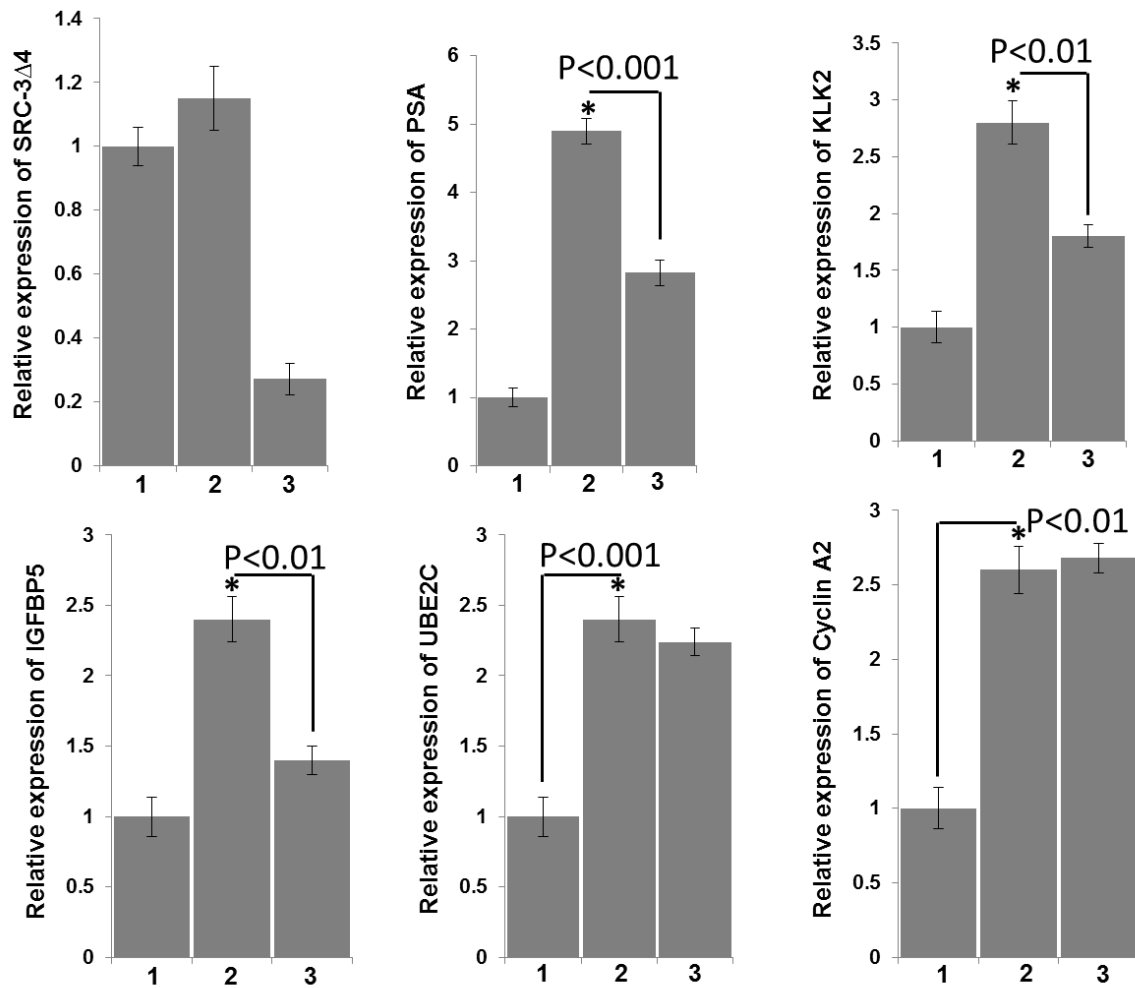


Figure 2 EGF stimulates the interaction of SRC-3 Δ 4 with AR. LNCaP-C4-2 cells were cultured in phenol-red free RPMI medium containing 5% cs-FBS. Cells were transiently transfected with a construct expressing SRC-3 Δ 4Flag. 24 hours post-transfection, cells were serum-starved for 24 hours, and then were stimulated with 50 ng/ml EGF or vehicle for 30 minutes, then lysed. The interaction between SRC-3 Δ 4 and AR was analyzed by immunoprecipitation (IP) using a Flag antibody, an AR antibody, or a control IgG from total cell lysates, followed by Western blotting. Ab: antibody. IB: immunoblots.

Task 1b To determine whether SRC-3 Δ 4 regulates AR target gene expression upon EGF stimulation. After revealing an interaction and co-localization between SRC-3 Δ 4 and AR in the nucleus, we then tested whether SRC-3 Δ 4 regulates AR target gene expression upon EGF stimulation. The following genes have been shown to be regulated by AR and are important for androgen-independent or castration resistant prostate cancer growth and invasion and were analyzed in our experiment: PSA (8), kallikrein-related peptidase 2 (KLK2) (9), cyclin A2 (4), ubiquitin-conjugating enzyme E2C (UBE2C) (10), insulin-like growth factor binding protein 5 (IGFBP5) (11), and TMPRSS2-ERG fusion gene (12). As shown in Figure 3, knockdown of SRC-3 Δ 4 by siRNA significantly decreased EGF-stimulated expression of PSA, KLK2, and IGFBP5, but had no significant effect on UBE2C and cyclin A2, suggesting SRC-3 Δ 4 regulates a subset of AR target genes. To determine the role of SRC-3 Δ 4 on TMPRSS2-ERG fusion gene expression, SRC-3 Δ 4Flag was stably transduced into VCap cells (Fig. 4A). While knockdown of AR (Fig. 4B) greatly reduced the expression of TMPRSS2-ERG fusion gene, neither EGF stimulation nor SRC-3 Δ 4 overexpression significantly altered TMPRSS2-ERG fusion gene expression (Fig. 4C).

Task 1c To determine by chromatin immunoprecipitation (ChIP) assay whether EGF stimulates the recruitment of SRC-3 Δ 4 and AR to AR target genes. Steroid receptor coactivators regulate gene expression by binding to steroid receptors bound on the target gene promoter. To determine whether EGF stimulates the recruitment of SRC-3 Δ 4 to AR target genes, we generated an LNCaP-C4-2 cell pool stably expressing SRC-3 Δ 4Flag by lentiviral transduction (Fig. 5A). Occupancy of SRC-3 Δ 4 on the gene promoters were determined by ChIP assay using a Flag antibody. Interestingly, even in the absence of EGF stimulation, there is detectable

occupancy of SRC-3Δ4 on PSA gene promoter (Fig. 5C). Importantly, in comparison with the vehicle treatment (Fig. 5C), EGF stimulation greatly enhanced the occupancy of SRC-3Δ4 on PSA promoter (Fig. 5D).



Labels on the X-axis: **1.** Ctrlsi-Vehicle, **2.** Ctrlsi-EGF, and **3.** SRC-3Δ4si-EGF

Figure 3 SRC-3Δ4 regulates the expression of a subset of AR target genes that are stimulated by EGF. LNCaP-C4-2 cells were cultured in phenol-red free RPMI medium containing 5% cs-FBS. Cells were transiently transfected with either a siRNA specifically targeting only SRC-3Δ4 (SRC-3Δ4si) or a non-targeting control siRNA (Ctrlsi). 24 hours post-transfection, cells were serum-starved for 24 hours, followed by the stimulation with 50 ng/ml EGF or vehicle for 24 hours. Expression of SRC-3Δ4, GAPDH, and AR target genes including PSA, KLK2, IGFBP5, Cyclin A2, and UBE2C was determined by RT-qPCR analysis. Data are presented using the comparative Ct method, in which GAPDH mRNA was used as the normalizer, and the normalized Ctrlsi-Vehicle treated samples were set to unity. Values represent the means ± SE of three separate experiments. “*” indicates a significant difference (Student’s *t* test).

To determine whether the recruitment of SRC-3Δ4 onto the PSA promoter is dependent on AR, we depleted AR by RNA interference (Fig. 5B). Depletion of AR virtually abolished the occupancy of SRC-3Δ4 on PSA gene promoter (Fig. 5C and 5D). Similar results were observed on the occupancy of SRC-3Δ4 on IGFBP5 gene promoter (Fig. 5F) except that there was no detectable recruitment of SRC-3Δ4 in the absence of EGF stimulation (Fig. 5E).

A ChIP-reChIP sequencing was proposed to identify novel genes that may be transactivated by AR/SRC-3Δ4 complex upon EGF stimulation. Although we have tried optimizing experimental conditions in a variety of ways, we still have technical difficulty with ChIP-reChIP sequentially using an AR antibody and a Flag antibody and have not yet successfully accomplished this assay. Despite this, we have successfully finished all other proposed experiments in this aim and the results fully support out hypothesis that SRC-3Δ4 acts as a coactivator of AR in the nucleus and regulates the transcription of a subset of AR target genes in response to EGF signaling.

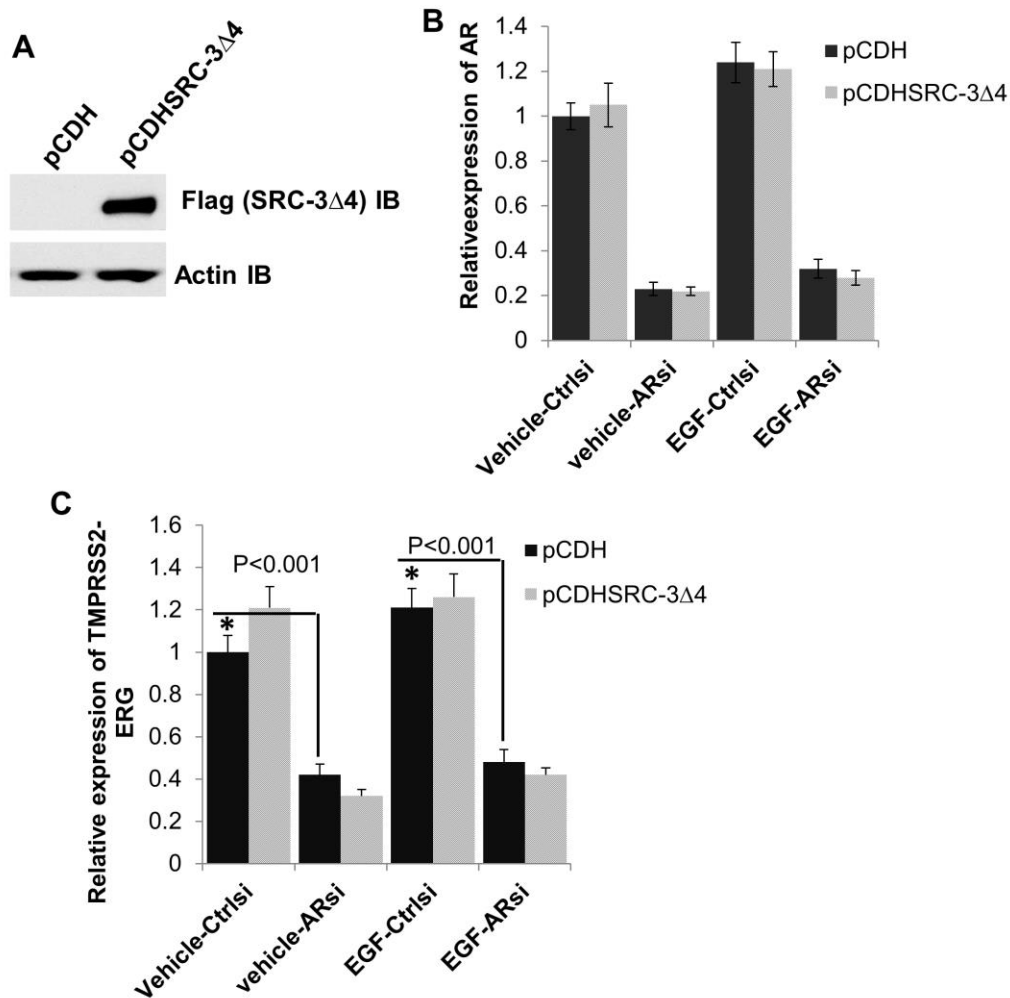


Figure 4 SRC-3Δ4 is not involved in the regulation of TMPRSS2-ERG by AR. (A). Generation of VCaP cell pool stably expressing SRC-3Δ4Flag. VCaP cells were transduced with lentiviruses expressing either SRC-3Δ4Flag or the empty vector pCDH. Two days post-transduction, cells were split and selected by puromycin (1 μg/ml). Expression of SRC-3Δ4Flag in the stable cell pools was analyzed by Western blotting using a Flag antibody. β-actin was probed as a loading control. (B) and (C). VCaP stable cell pools were cultured in phenol-red free RPMI medium containing 5% cs-FBS. Cells were transiently transfected with either a siRNA specifically targeting AR (ARsi) or a non-targeting control siRNA (Ctrl si). 24 hours post-transfection, cells were serum-starved for 24 hours, and then were stimulated with 50 ng/ml EGF or vehicle for 24 hours. Expression of AR (B), GAPDH, and TMPRSS2-ERG (C) was determined by RT-qPCR analysis. Data are presented using the comparative Ct method, in which GAPDH mRNA was used as the normalizer, and the normalized Vehicle-Ctrl si sample of pCDH cell pool was set to unity. Values represent the means ± SE of three separate experiments. “*” indicates a significant difference (Student’s *t* test).

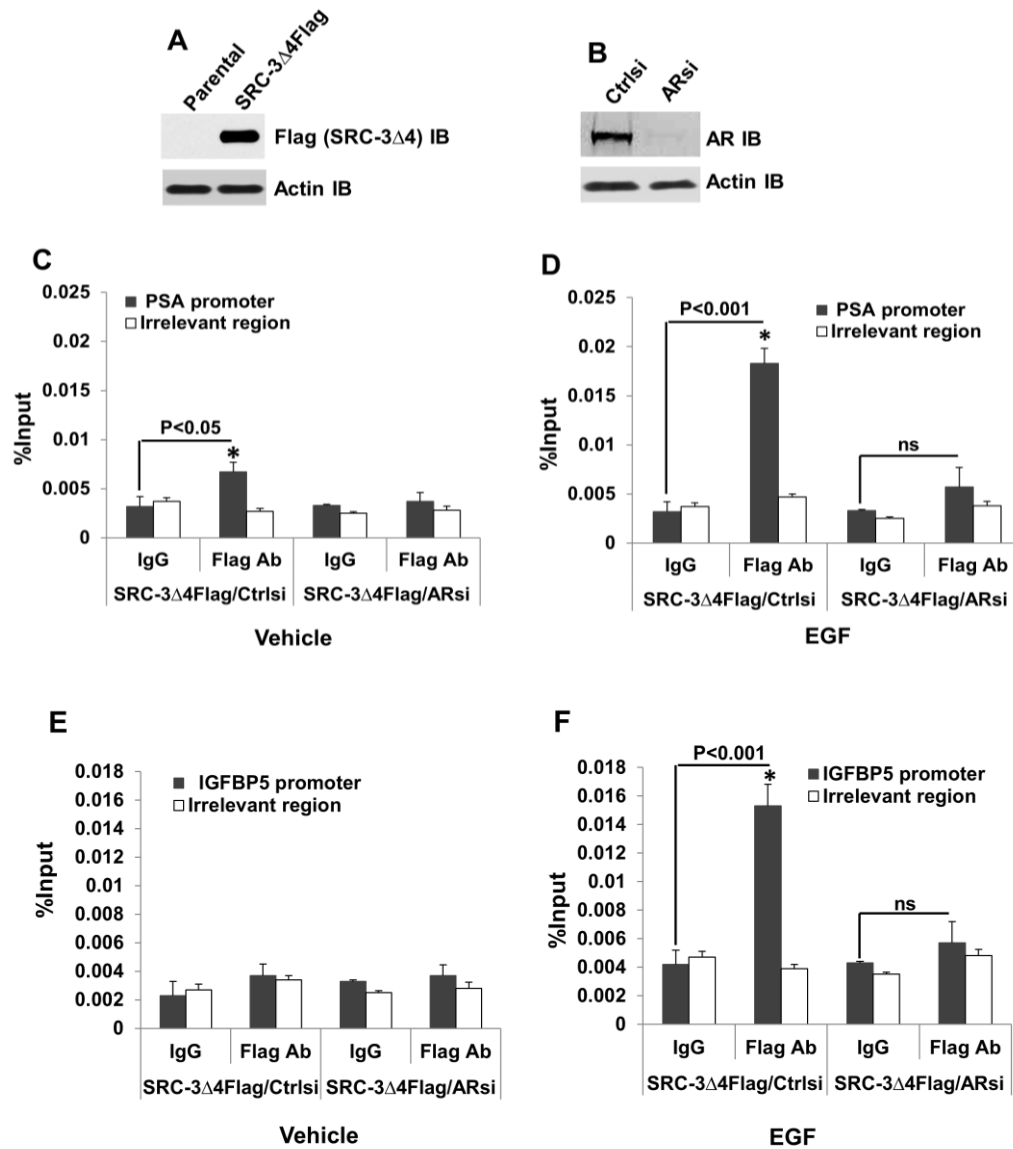


Figure 5 EGF stimulates SRC-3 Δ 4's occupancy on the AR target genes' promoters in an AR-dependent manner. **(A)**. Generation of LNCaP-C4-2 cell pool stably expressing SRC-3 Δ 4Flag. LNCaP-C4-2 cells were transduced with lentiviruses expressing SRC-3 Δ 4Flag. LNCaP-C4-2 cells were split and selected by puromycin (1 μ g/ml). Expression of SRC-3 Δ 4Flag in the stable cell pools was analyzed by Western blotting using a Flag antibody. β -actin was probed as a loading control. **(B)**. Transient knockdown of AR in LNCaP-C4-2 cell pool stably expressing SRC-3 Δ 4Flag. Cells were cultured in phenol-red free RPMI medium containing 5% cs-FBS. Cells were transiently transfected with either a siRNA specifically targeting AR (ARsi) or a non-targeting control siRNA (Ctrl si). 24 hours post-transfection, cells were serum-starved for 24 hours, and then were stimulated with 50 ng/ml EGF or vehicle for 24 hours. Knockdown of AR was confirmed by Western blotting using an AR antibody. **(C) and (D)**. SRC-3 Δ 4's occupancy on PSA gene promoter. LNCaP-C4-2 cells stably expressing SRC-3 Δ 4Flag were transiently transfected with either a siRNA specifically targeting AR (ARsi) or a non-targeting control siRNA (Ctrl si) as shown in **(B)**. ChIP assays then were performed using either a Flag Ab or mouse IgG. SRC-3 Δ 4Flag protein occupancy on PSA promoter region in response to vehicle **(C)** or EGF **(D)** stimulation was analyzed by quantitative real-time PCR and presented as the percentage of sheared chromatin input. An irrelevant region (1800 bp downstream of transcription start site) was served as a negative control. **(E) and (F)**. ChIP analysis of SRC-3 Δ 4's occupancy on IGFBP5 gene promoter in response to vehicle **(E)** or EGF **(F)**. Values represent the means \pm SE of three separate experiments. "*" indicates a significant difference (Student's *t* test), and "ns" means no significant difference.

Task 2 (Aim 2) was to determine the role of SRC-3 Δ 4 in androgen-independent prostate cancer cell growth and invasion. EGF signaling activates AR and stimulates prostate cancer cell growth and invasion (8, 13). Our findings in Aim 1 suggest that in response to EGF stimulation, SRC-3 Δ 4 regulates the expression of AR target genes including PSA, KLK2, and IGFBP5. These genes are important for castration-resistant prostate cancer cell growth and/or invasion. To determine the role of SRC-3 Δ 4 in castration-resistant prostate cancer cell growth and invasion, we have conducted the following experiments.

Task 2a To determine the role of SRC-3 Δ 4 in regulating the growth and invasion of androgen-independent prostate cancer cell lines CWR22Rv1 and LNCaP-C4-2. We depleted endogenous SRC-3 Δ 4 in LNCaP-C4-2 (Fig. 6A) and CWR22Rv1 (Fig. 6D) cells by stably expressing a shRNA specifically targeting SRC-3 Δ 4. The effects of SRC-3 Δ 4 depletion on the growth and invasion of CWR22Rv1 and LNCaP-C4-2 cells were tested by MTS cell growth assay and matrigel-transwell cell invasion assay, respectively. Stable knockdown of SRC-3 Δ 4 significantly inhibited EGF-induced growth (Fig. 6B) and invasion (Fig. 6C) of LNCaP-C4-2 cells, so did in CWR22Rv1 cells (Fig. 6E and 6F).

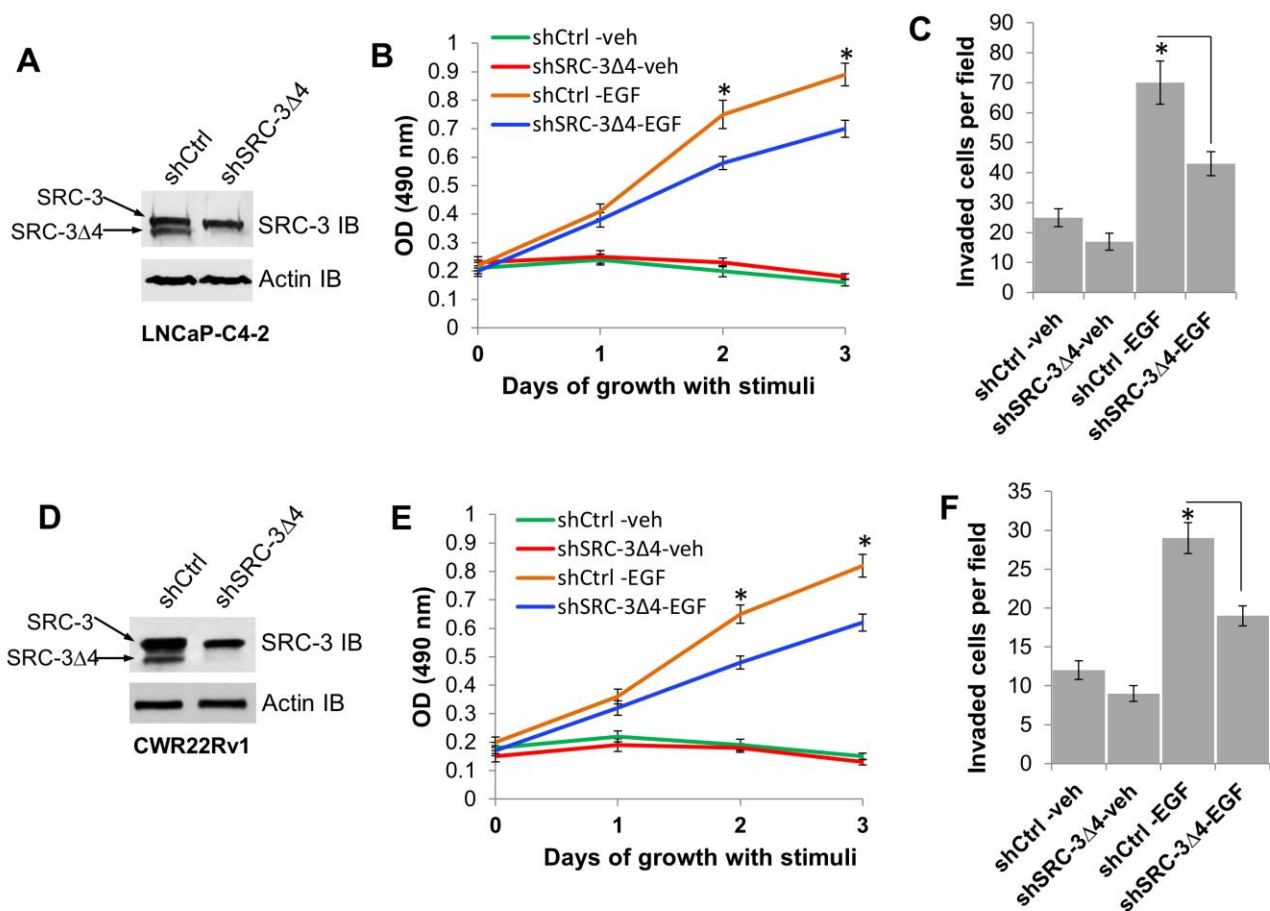


Figure 6 Knockdown of SRC-3Δ4 significantly inhibited EGF-induced growth and invasion of androgen-independent prostate cancer cells. (A) and (D): generation of LNCaP-C4-2 and CWR22Rv1 cell pools with stable knockdown of SRC-3Δ4. LNCaP-C4-2 and CWR22Rv1 were transduced with lentiviruses expressing either a shRNA specifically targeting SRC-3Δ4 (shSRC-3Δ4) or a scrambled shRNA sequence (shCtrl). Two days post-transduction, cells were split and selected by puromycin (1 μg/ml). Expression of SRC-3Δ4 in the stable cell pools was analyzed by Western blotting using a SRC-3 antibody that recognizes both SRC-3 and SRC-3Δ4. β-actin was probed as a loading control. (B) and (E): MTS growth assays of LNCaP-C4-2 (B) and CWR22Rv1 (E) stable cell pools. Cells were plated into 96-well plate with serum-free RPMI media. 24 hours later (Day 0 with stimulation), cell numbers in one set of cells were measured by MTS assay and presented as OD (490 nm); other sets of cells were stimulated with either 25 ng/ml EGF or vehicle (veh, 1 x PBS) for different time periods. (C) and (F): transwell-matrigel invasion assay of LNCaP-C4-2 (C) and CWR22Rv1 (F) stable cell pools. Cells were plated into the inserts (upper chamber) with serum-free RPMI media. Cell invasion was stimulated by adding EGF (25 ng/ml) to the lower chamber, 1 x PBS as vehicle (veh) control. Values represent the means ± SE of three separate experiments. “*” indicates a significant difference (P<0.01, Student’s *t* test).

Task 2b To determine whether overexpression of SRC-3Δ4 in androgen-dependent prostate cancer cells promotes androgen-independent growth and invasion in response to EGF stimulation. To confirm the function of SRC-3Δ4 in promoting androgen-independent prostate cancer growth and invasion, we generated pools of cells stably overexpressing SRC-3Δ4 or a lentiviral vector control (Fig. 7A and 7D). Consistent with the findings shown in Fig. 6, overexpression of SRC-3Δ4 greatly increased EGF-induced growth and invasion of both LNCaP (Fig. 7B and 7C) and CWR22 (Fig. 7E and 7F) cell lines under hormone-free culture conditions. Importantly, the effect of SRC-3Δ4 overexpression in promoting EGF-induced CWR22 cell invasion was greatly inhibited when cells were treated with a siRNA against AR (siAR, Fig. 7G), suggesting that the function of SRC-3Δ4 in promoting prostate cancer cell invasion is dependent on AR.

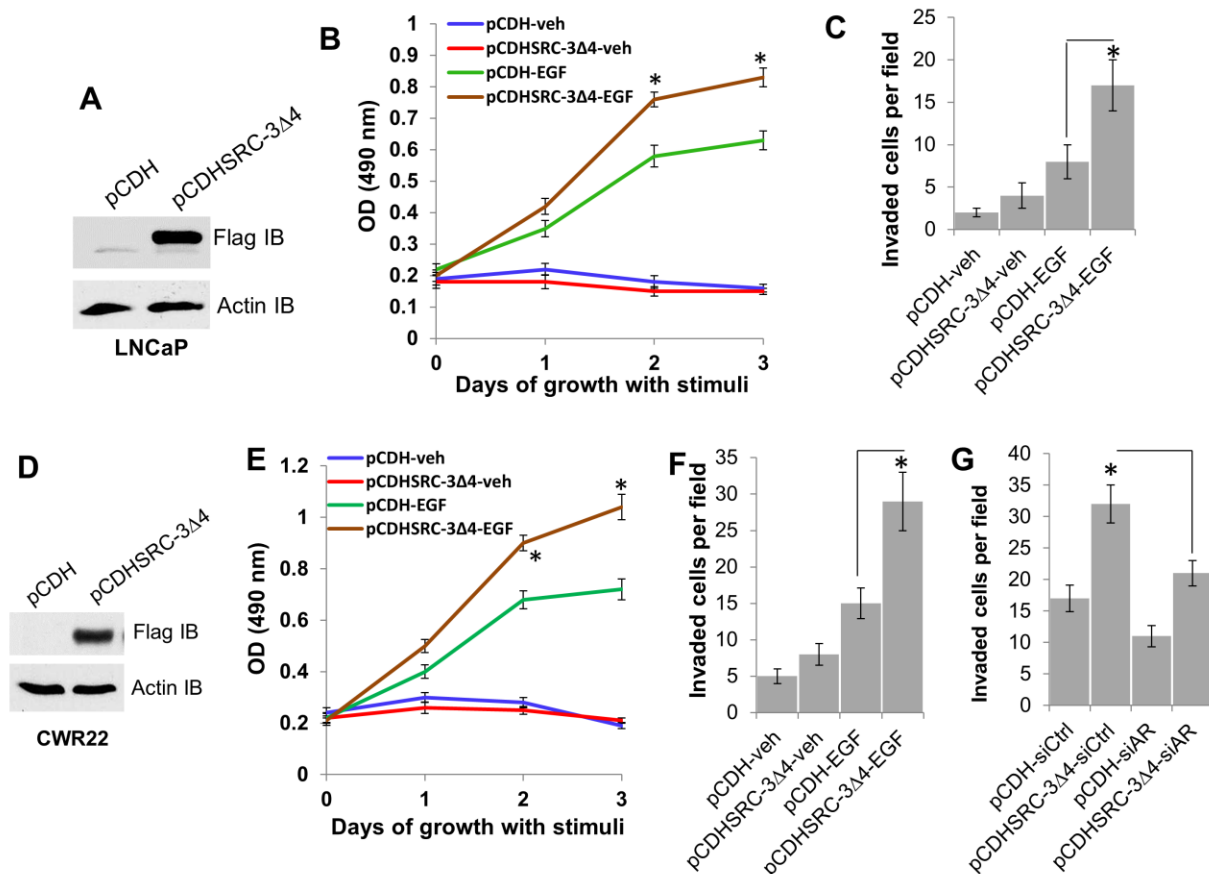


Figure 7 Overexpression of SRC-3Δ4 promoted androgen-independent growth and invasion of androgen-dependent prostate cancer cells with the stimulation of EGF. (A) and (D): generation of LNCaP and CWR22 cell pools with stable overexpression of SRC-3Δ4Flag or a control vector pCDH by lentiviral transduction, followed by puromycin selection. Exogenous expression of SRC-3Δ4 in the stable cell pools was analyzed by Western blotting using a Flag antibody. β-actin was probed as a loading control. (B) and (E): MTS growth assays of LNCaP (B) and CWR22 (E) stable cell pools. Cells were plated into 96-well plate with serum-free RPMI media. 24 hours later (Day 0 with stimulation), cell numbers in one set of cells were measured by MTS assay and presented as OD (490 nm); other sets of cells were stimulated with either 25 ng/ml EGF or vehicle (veh, 1 x PBS) for different time periods. (C) and (F): transwell-matrigel invasion assay of LNCaP (C) and CWR22 (F) stable cell pools. Cells were plated into the inserts (upper chamber) with serum-free RPMI media. Cell invasion was stimulated by adding EGF (25 ng/ml) to the lower chamber, 1 x PBS as vehicle (veh) control. (G): transwell-matrigel invasion assay of CWR22 stable cell pools treated with either a siRNA specifically targeting AR or a non-targeting control siRNA (siCtrl). Values represent the means ± SE of three separate experiments. “*” indicates a significant difference (P<0.01, Student’s *t* test).

Task 3 (Aim 3) To determine the underlying molecular mechanisms by which SRC-3Δ4 coactivates AR transcriptional activity and promotes androgen-independent prostate cancer cell growth and invasion. An EGF-induced tyrosine phosphorylation site (Y1357) on SRC-3 was identified, and this phosphorylation is important for SRC-3’s function in coactivating transcription factors and in promoting breast cancer cell growth (14). As SRC-3Δ4 was shown to directly interact with EGFR upon EGF stimulation (7), EGFR may phosphorylate SRC-3Δ4 at this tyrosine residue (Y1159, equivalent to Y1357 of SRC-3), and the phosphorylation of SRC-3Δ4 at Y1159 may be important for the function of SRC-3Δ4 in coactivating AR and promoting prostate cancer cell growth and invasion in response to EGF stimulation.

Task 3a To determine whether SRC-3Δ4 is tyrosine-phosphorylated by EGFR. First, we tested whether SRC-3Δ4 is tyrosine-phosphorylated by EGF/EGFR signaling in cultured cells. As shown in Fig. 8, EGF stimulated the tyrosine phosphorylation of SRC-3Δ4, which was inhibited by the treatment with EGFR inhibitor AG1478. Mutation of Y1159 to phenylalanine (Y1159F) abolished EGF-induced tyrosine phosphorylation of SRC-3Δ4. These results suggest that EGF/EGFR signaling stimulates tyrosine phosphorylation of SRC-3Δ4 at Y1159. We then tested whether EGFR phosphorylates SRC-3Δ4 by performing *in vitro* kinase assay and found that EGFR did not phosphorylate SRC-3Δ4 in vitro (data not shown), which is actually consistent with the previous finding that c-Ab1, a tyrosine kinase downstream of EGF/EGFR signaling, phosphorylates SRC-3 (14).

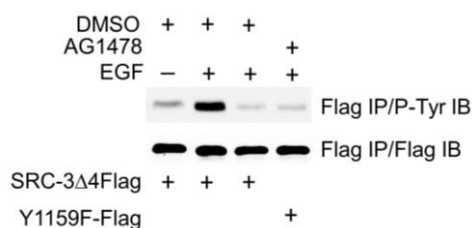


Figure 8 EGF/EGFR signaling stimulates tyrosine phosphorylation of SRC-3Δ4 at Y1159. LNCaP stable cell pools expressing either SRC-3Δ4Flag or SRC-3Δ4Y1159F-Flag were serum-starved for 24 hours, and then were treated with 1 μM AG1478 or vehicle (DMSO) for 30 min, followed by the stimulation with or without 25 ng/ml EGF for 30 min. Flag-tagged SRC-3Δ4 or SRC-3Δ4Y1159F proteins were immunoprecipitated from cell lysates using a Flag antibody (Flag IP), resolved by SDS-PAGE and immunoblotted for tyrosine phosphorylation using a phospho-tyrosine (P-Tyr) specific antibody. Immunoprecipitated proteins were probed with a Flag antibody.

Task 3b To determine the effect of SRC-3Δ4 tyrosine phosphorylation at Y1159 on EGF-induced interaction between SRC-3Δ4 and AR. As EGF stimulates tyrosine phosphorylation of SRC-3Δ4 at Y1159 (Fig. 8) and promotes the interaction of SRC-3Δ4 with AR (Fig. 2), we then tested whether tyrosine phosphorylation at Y1159 is involved in EGF-induced SRC-3Δ4/AR interaction. As shown in Fig. 9, mutation of Y1159 to phenylalanine (Y1159F) greatly reduced SRC-3Δ4/AR interaction that is stimulated by EGF.

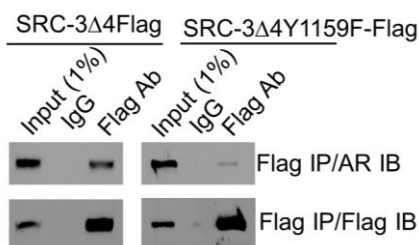


Figure 9 Phosphorylation site Y1159 is important for EGF-stimulated interaction of SRC-3Δ4 with AR. LNCaP stable cell pools expressing either SRC-3Δ4Flag or SRC-3Δ4Y1159F-Flag were serum-starved for 24 hours, and then were stimulated with 25 ng/ml EGF for 30 minutes, followed by cell lysis. The interaction between AR and SRC-3Δ4 (or SRC-3Δ4Y1159F) was analyzed by immunoprecipitation (IP) using a Flag antibody or a control IgG, followed by Western blotting. Ab: antibody. IB: immunoblots.

Task 3c To determine the role of SRC-3Δ4 tyrosine phosphorylation at Y1159 in AR transcriptional activity by PSA-luciferase assay and real-time PCR analysis of AR target genes' expression. Our finding that tyrosine phosphorylation of SRC-3Δ4 at Y1159 is important for the interaction of SRC-3Δ4 with AR implies that this phosphorylation regulates the function of SRC-3Δ4 in coactivating AR-mediated gene transcription. Indeed, in comparison with SRC-3Δ4, SRC-3Δ4Y1159F has significantly decreased ability in promoting EGF-stimulated PSA-luciferase reporter activity (Fig. 10A) and PSA gene expression (Fig. 10B) in LNCaP cells.

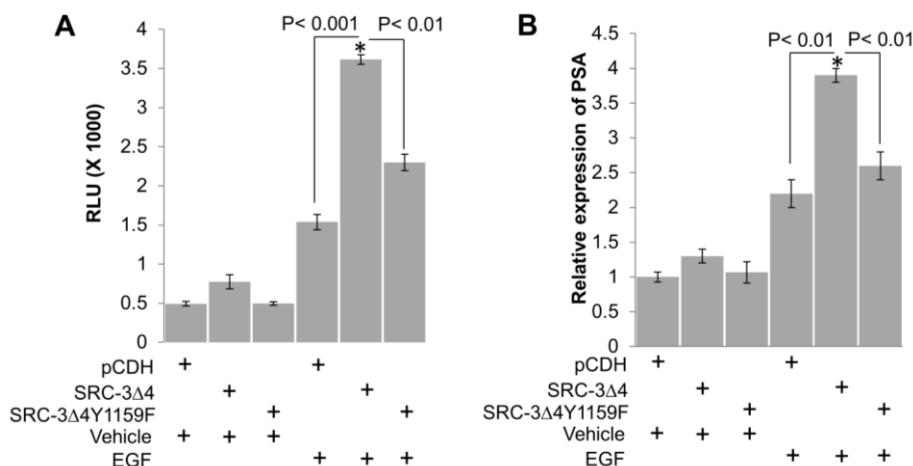


Figure 10 Phosphorylation site Y1159 is important for SRC-3Δ4 in co-activating AR-mediated PSA luciferase reporter activity and PSA gene expression in response to EGF stimulation. LNCaP stable cell pools expressing either SRC-3Δ4Flag or SRC-3Δ4Y1159F-Flag were cultured in phenol-red free DMEM media containing 5% charcoal-stripped FBS. Cells were transiently transfected with a plasmid expressing luciferase driven by PSA-promoter (PSA-Luc). 24 hours post-transfection, cells were starved overnight in serum-free medium. Cells were then treated with EGF (50 ng/ml) or vehicle for 24 hours in serum-free medium and harvested for either measuring luciferase activity by luciferase assay (A) or measuring PSA gene expression by RT-qPCR. Values of the relative luciferase units (RLU) or PSA gene expression are means \pm s.e of three separate experiments. “*” indicates significant difference upon Student’s *t* test.

Task 3d To determine the role of SRC-3Δ4 tyrosine phosphorylation at Y1159 in androgen-independent prostate cancer cell growth and invasion by MTS assay and Matrigel-transwell cell invasion assay, respectively. Lastly, we investigated the importance of tyrosine phosphorylation at Y1159 in SRC-3Δ4-mediated androgen-independent prostate cancer cell growth and invasion. While SRC-3Δ4 significantly increased EGF-induced prostate cancer cell growth and invasion under hormone-free culture condition (Fig. 7), SRC-3Δ4Y1159F was incapable of promoting either growth or invasion of prostate cancer cells (Fig. 11), suggesting that in line with its role in mediating the interaction of SRC-3Δ4 with AR and AR-regulated gene transcription, Y1159

phosphorylation site is important for SRC-3 Δ 4 in promoting EGF-stimulated prostate cancer cell growth and invasion.

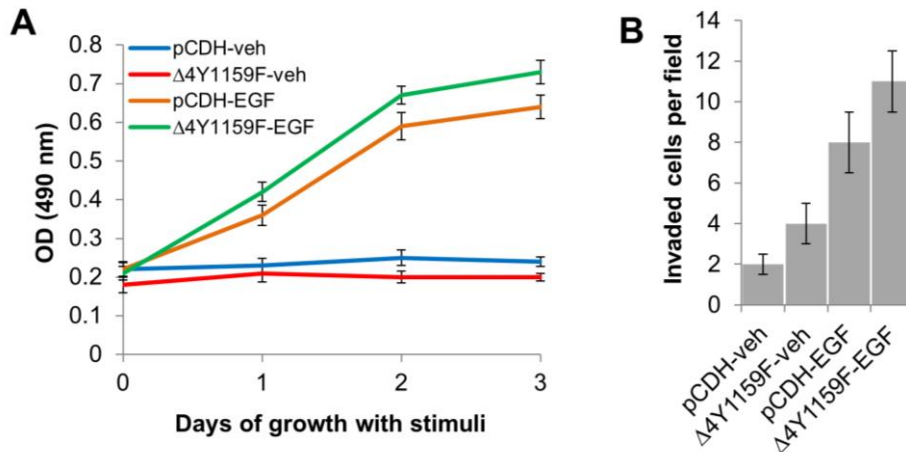


Figure 11 SRC-3 Δ 4Y1159F does not promote androgen-independent growth and invasion of LNCaP cells with the stimulation of EGF. (A). MTS growth assays of LNCaP stable cell pools expressing either SRC-3 Δ 4Y1159F-Flag or an empty control vector pCDH. Cells were plated into 96-well plate with serum-free RPMI media. 24 hours later (Day 0 with stimulation), cell numbers in one set of cells were measured by MTS assay and presented as OD (490 nm); other sets of cells were stimulated with either 25 ng/ml EGF or vehicle (veh, 1 x PBS) for different time periods. (B). Transwell-matrigel invasion assay of LNCaP stable cell pools with the stimulation of EGF (25 ng/ml) or 1 x PBS as vehicle (veh) control. Values represent the means \pm SE of three separate experiments.

Task 3e To determine whether SRC-3 Δ 4 regulates EGF-induced AR tyrosine phosphorylation. EGF induces AR phosphorylation on tyrosine Y534, and phosphorylation on Y534 is essential for AR nuclear localization, AR transcriptional activity, and AR-mediated androgen-independent prostate cancer cell growth (8). We previously demonstrated that SRC-3 Δ 4 interacts with EGFR and is important for EGF-induced cell signaling (7). These findings suggest that SRC-3 Δ 4 may regulate EGF-induced AR tyrosine phosphorylation, which may partly account for its regulatory role in AR transcriptional activity. As shown in Figure 12, EGF stimulated AR tyrosine phosphorylation at equivalent levels (AR IP/P-Tyr IB, Figure 12) in CWR22RV1-shCtrl and CWR22RV1-shSRC-3 Δ 4 stable cell pools, indicating that depletion of SRC-3 Δ 4 does not affect EGF-induced AR tyrosine phosphorylation.

Task 3f To determine whether SRC-3 Δ 4 acetylates AR and whether AR acetylation by SRC-3 Δ 4 is important for AR transcriptional activity. AR transcriptional activity is also regulated by protein acetylation (15). Interestingly, SRC-3 was reported to have histone acetyltransferase (HAT) activity (16). As SRC-3 Δ 4 protein remains the HAT domain in its c-terminus, it would be interesting to investigate whether SRC-3 Δ 4 has acetyltransferase activity toward AR. To test this, we analyzed AR acetylation in both CWR22RV1-shCtrl and CWR22RV1-shSRC-3 Δ 4 stable cell pools with or without EGF stimulation. As shown in Figure 12, equivalent levels of AR acetylation (AR IP/Ac IB) was observed in CWR22RV1-shCtrl and CWR22RV1-shSRC-3 Δ 4 stable cell pools in the absence or presence of EGF stimulation, indicating SRC-3 Δ 4 does not regulate AR acetylation.

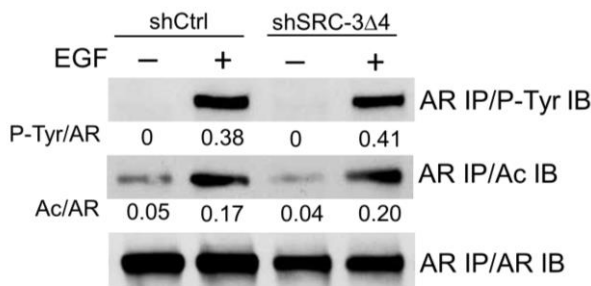


Figure 12 SRC-3Δ4 regulates neither tyrosine phosphorylation nor acetylation of AR. CWR22RV1-shCtrl and CWR22RV1-shSRC-3Δ4 stable cell pools (Fig. 6) were serum-starved for 24 hours, then stimulated with or without 25 ng/ml EGF for 30 minutes, followed by cell lysis. AR protein was immunoprecipitated (IP) using an AR antibody and was resolved by SDS-PAGE. Tyrosine phosphorylation (P-Tyr) and acetylation (Ac) of AR were analyzed by Western blotting using a phospho-Tyr-specific Ab and an acetylation specific Ab, respectively. IB: immunoblots.

Task 4 (Aim 4) was to determine the role of SRC-3Δ4 in prostate tumor progression and metastasis in castrated BALB/c mice.

Task 4a: To determine whether depletion of endogenous SRC-3Δ4 inhibits LNCaP-C4-2-induced prostate tumor growth and metastasis using a xenograft tumor model by orthotopic injection of the cells into prostates. LNCaP-C4-2 cell pools with stable depletion of SRC-3Δ4 (LNCaP-C4-2-sh SRC-3Δ4) and the control cell line LNCaP-C4-2-shCtrl were generated as described in Figure 6. 20 μl of each cell pool (2×10^6 cells) was injected (orthotopic injection) into prostates of castrated BALB/c male mice (6-8 weeks, 20 mice per group, Frederick National Laboratory/Charles River). Animal body weight was measured twice a week. Mice were sacrificed 60 days post tumor cell inoculation. Primary tumor growth and tumor metastasis to the lymph node and/or bone were analyzed by histological analysis. Overall, the tumor take rate was relatively low (13 out of 20 for LNCaP-C4-2-shCtrl and 12 out of 20 for LNCaP-C4-2-sh SRC-3Δ4). In addition, there was no tumor metastasis observed. Depletion of SRC-3Δ4 had little effect on animal body weight (Figure 13A) and prostate tumor growth derived by LNCaP-C4-2 cells (Figure 13B).

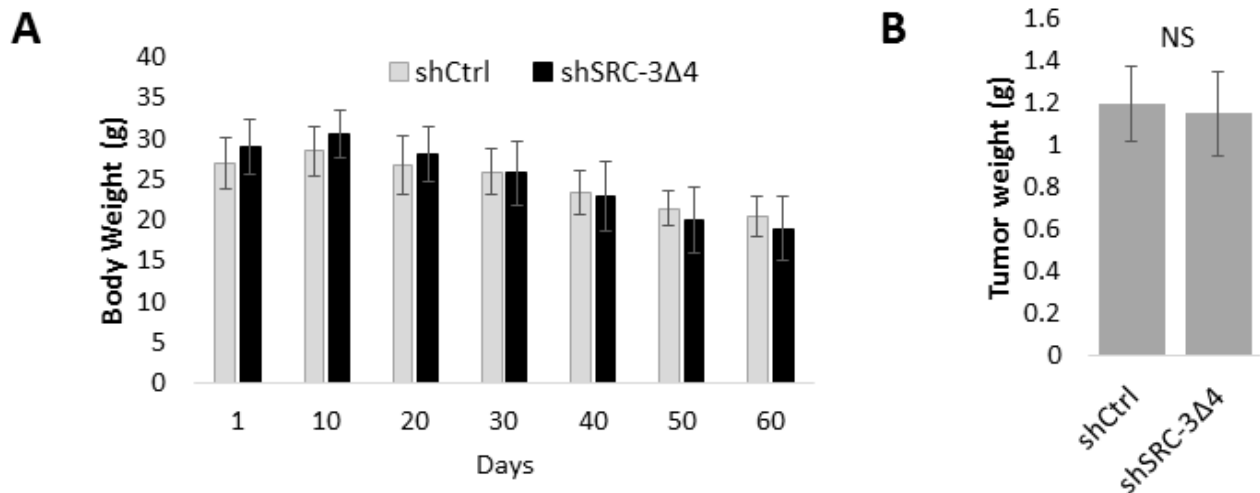


Figure 13 SRC-3Δ4 depletion had no significant effect on prostate tumor growth derived by LNCaP-C4-2 in castrated BALB/c mice. (A) mouse body weight (gram) during 60 days post inoculation with either LNCaP-C4-2-shCtrl (n=20) or LNCaP-C4-2-sh SRC-3Δ4 (n=20). There was no significant difference between two groups (Students' *t* test). (B) Average prostate tumor weight (gram) of LNCaP-C4-2-shCtrl group (n=13) and LNCaP-C4-2-sh SRC-3Δ4 group (n=12). NS: no significant difference by Students' *t* test.

Task 4b: To determine whether overexpression of exogenous SRC-3Δ4 in LNCaP cells promotes prostate xenograft tumor growth and/or metastasis in castrated mice. LNCaP cells was incapable of forming tumors in castrated nude mice (17). We aimed to test whether overexpression of exogenous SRC-3Δ4 in LNCaP cells induces prostate xenograft tumor growth and promotes metastasis in castrated mice. 2 x 10⁶ LNCaP cells with stable expression of SRC-3Δ4 or the lentiviral control vector (described in Figure 7) were resuspended in 100 μl PBS and 100 μl Matrigel (BD Biosciences) and s.c. injected into the left and right flanks of castrated BALB/c mice (20 mice each group). Consistent with the previous report (17), LNCaP-Ctrl cells did not induce tumors in castrated mice. In addition, no measurable tumor was observed in LNCaP-SRC-3Δ4 group either. These results suggest that although SRC-3Δ4 promoted prostate cancer cell growth and invasion under in vitro culture conditions, it did not affect prostate tumor growth and progression in vivo under the conditions in the present study.

Opportunities for training and professional development the project has provided

1. This DOD Idea award contributed to the professional development of Dr. Weiwen Long (the principle investigator) from an Instructor to an independent faculty.

2. It provided training opportunities to two postdoctoral fellows in the PI's lab. Dr. Wei Wang, a postdoctoral fellow from 2011-2013 at Baylor College of Medicine, participated in performing Aims1-3. He attended an AACR special conference "Tumor Invasion and Metastasis" in San Diego in January, 2013 and had a poster presentation entitled "SRC-3Δ4 coactivates AR target gene expression in an androgen-independent manner upon EGF stimulation in prostate cancer cells." Dr. Cheng Zhang, a postdoctoral fellow in the PI's lab at Wright State University, participated in performing Aim 4 and presented her work in the department's Brown bag seminars (a seminar program for trainees).

4. IMPACT

Our findings demonstrate that SRC-3Δ4 coactivates AR target gene expression in an androgen-independent manner and promotes prostate cancer cell growth and invasion upon EGF stimulation in vitro, although it did not affect prostate tumor growth and progression in vivo under the conditions in the present study. There is no technology transfer to report.

5. CHANGES/PROBLEMS

While the project was prolonged due to the delay of grant transfer, no significant changes were made to the project. No major technical problems were encountered either.

6. PRODUCTS

Reportable outcomes (please refer to 3. ACCOMPLISHMENTS)

1. Generated lentiviruses expressing SRC-3Δ4Flag or the pCDH empty vector following the manufacturer's instructions (System Biosciences).
2. Generated VCaP cell pools stably expressing SRC-3Δ4Flag or the pCDH empty vector by lentiviral transduction with lentiviruses expressing either SRC-3Δ4Flag or the empty vector pCDH, respectively.
3. Generated lentiviruses expressing a shRNA specifically targeting SRC-3Δ4 (shSRC-3Δ4) or a scrambled shRNA sequence (shCtrl) using the pSIH-H1 shRNA-expressing system (System Biosciences).
4. Generated LNCaP-C4-2 and CWR22Rv1 cell pools stably expressing either shSRC-3Δ4 or shCtrl.
5. Generated LNCaP and CWR22 cell pools with stable overexpression of SRC-3Δ4Flag or a control vector pCDH.
6. Generated LNCaP cell pools with stable overexpression of SRC-3Δ4Y1159F-Flag.
7. Attended an AACR special conference "Tumor Invasion and Metastasis" in January, 2013 in San Diego and had a poster presentation entitled "SRC-3Δ4 coactivates AR target gene expression in an androgen-independent manner upon EGF stimulation in prostate cancer cells."
8. We are about to write a manuscript.

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

Name	Role	Effort	Contribution to project	Active other support
Weiwen Long	PI	9 months (09/30/11-09/29/13)	Experimental design and conducting experiments and data analysis	None
		3.6 months (07/01/16-06/30/18)	Experimental design and participating in conducting experiments and data analysis	NCI1R01CA193264-01 (06/01/05-05/31/20)
Wei Wang	Postdoctoral Fellow	12 months (01/2012-09/2013)	conducting experiments and data analysis (Aims 1-3) and partially participating experimental design	None
Cheng Zhang	Postdoctoral Fellow	12 months	conducting experiments and data analysis (Aim 4) and partially participating experimental design	None

Other collaborating organizations: nothing to report.

8. Special reporting requirements: Not applicable.

9. APPENDICES: None

Abstract: submitted to AACR special conference “Tumor Invasion and Metastasis” in San Diego in January, 2013

SRC-3 Δ 4 coactivates AR target gene expression in an androgen-independent manner upon EGF stimulation in prostate cancer cells

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SRC-3 Δ 4, an N-terminus deletion isoform of steroid receptor coactivator (SRC-3), was shown to act as a signaling adaptor of EGF signaling in activating FAK. Its role in prostate cancer (PCa) progression is unclear. Interestingly, we found that SRC-3 Δ 4 is upregulated in castration resistant PCa cells as compared to androgen-dependent PCa cells. As such, we determined whether SRC-3 Δ 4 coactivates AR in an androgen-independent manner and promotes prostate cancer cell growth and invasiveness in response to EGF signaling. We have found that EGF stimulated the interaction of SRC-3 Δ 4 with AR, the recruitment of SRC-3 Δ 4 to the promoters of AR target genes, and AR target genes' transcription in androgen-depleted culture conditions. In addition, SRC-3 Δ 4 promotes androgen-independent prostate cancer cell growth and invasion, during which EGF-induced phosphorylation of SRC-3 Δ 4 plays a critical role. Taken together, these results demonstrate that SRC-3 Δ 4 acts as a coactivator of AR and regulates the transcription of AR target genes and prostate cancer cell growth and invasion in response to EGF signaling.

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