Steroid Receptor Coactivator-3/AIB1 Promotes Cell Migration and Invasiveness through Focal Adhesion Turnover and Matrix Metalloproteinase Expression

Jun Yan, Halime Erdem, Rile Li, Yi Cai, Gustavo Ayala, Michael Ittmann, Li-yuan Yu-Lee, Sophia Y. Tsai, and Ming-Jer Tsai

1Department of Molecular and Cellular Biology, 2Department of Pathology, 3Department of Medicine, 4Program of Development, and 5Baylor Prostate Cancer Specialized Programs of Research Excellence, Baylor College of Medicine, Houston, Texas

Abstract

Steroid receptor coactivator-3 (SRC-3)/AIB1 is a member of the p160 nuclear receptor coactivator family involved in development and cell cycle progression. We previously showed that SRC-3/AIB1 is required for prostate cancer cell proliferation and survival. Here, we reported that the elevated SRC-3/AIB1 expression is significantly correlated with human prostate cancer seminal vesicle invasion and lymph node metastasis. Furthermore, SRC-3/AIB1 is associated with increased prostate cancer cell migration and invasion. SRC-3/AIB1 is required for focal adhesion turnover and focal adhesion kinase activation. In addition, SRC-3/AIB1 directly regulates transcription of matrix metalloproteinase (MMP)-2 and MMP-13 through its coactivation of AP-1 and PEA3. Taken together, these data suggest that SRC-3/AIB1 plays an essential role in prostate cancer cell invasion and metastasis.

Introduction

Prostate cancer is the most common cancer and the second leading cause of cancer death in men in Western countries. About 10% of the initial cases of prostate cancer present with metastatic disease. However, in 90% of prostate cancer, metastases will develop despite treatment with surgery, radiation, or medical therapy, and those metastases will eventually become refractory to hormonal treatment. To date, invasion and metastasis, the primary causes of death, still represent a major clinical challenge. Therefore, the identification of target proteins responsible for the control of metastasis in prostate cancer is important for treating this disease.

The steroid receptor coactivator-3 (SRC-3)/AIB1 is a member of the p160 family of coactivators that associate with hormone-bound nuclear receptors to mediate the transcriptional function of these receptors (2–4). SRC-3/AIB1 has been found to be amplified and/or overexpressed in many human carcinomas that are not limited to steroid targeted tissues (5). Consistently, SRC-3/AIB1 is required for prostate cancer cell motility and invasiveness through enhanced focal adhesion turnover and induction of matrix metalloproteinase (MMP)-2 and MMP-13 expressions. Our studies supported that SRC-3/AIB1 plays a role in prostate cancer cell migration and invasiveness.

Materials and Methods

Materials and antibodies. Puromycin, nocodazole, gelatin, and Brij35 were obtained from Sigma-Aldrich. The mifepristone came from Merck. The antibodies used in this study are SRC-3/AIB1, tyrosine phosphorylation antibody (BD Biosciences), IRS-2, Rac1 (Upstate Biotechnology), NCoA3, phosphatidylinositol-3-kinase (PI3K) p85, focal adhesion kinase (FAK), c-Jun, junB, junD, c-Fos, Fra1, PEA3, ETS1 (Santa Cruz Biotechnology), FAK pY397 (Biosource International), MMP-2 and MMP-13 (Chemicon), and actin (Sigma-Aldrich).

Analysis of prostate cancer tissues. A quantitative PCR was performed on RNAs from 58 prostate cancers from men undergoing radical prostatectomy for clinically localized prostate cancer (with clinical characteristics previously described; ref. 14). The tissues were 70% to 90% cancerous based on histopathologic analysis. A keratin-18 quantitative reverse transcription-PCR (RT-PCR) was performed as described previously (14). Primers for SRC-3/AIB1 were 5'-TCTCAACCCACTTCTTCG-3' (forward) and 5'-GGTCTGCCAAGCCATAGG-3' (reverse).

Cell culture and transfections. The inducible SRC-3/AIB1 LNCaP cells (15) and PC3 cells were maintained in RPMI 1640, whereas DU145 and HeLa were maintained in DMEM, all containing 10% fetal bovine serum (FBS). For transient transfection of small interfering RNA (siRNA; Dharmacon Research, Inc.), 40 nmol/L siRNA was transfected using Lipofectamine 2000 (Invitrogen) as transfection agent according to the manufacturer’s protocol.

DNA constructs and stable cell lines. The short hairpin RNA (shRNA) for human SRC-3/AIB1 (5'-ATCGAGACGGAAACATTGTATTTGTAT-3') was cloned into pSuper (OligoEngine) according to the manufacturer’s instructions, and stable clones were obtained after selection with 1 μg/mL puromycin for 3 wk.

Wound-healing assays. A wound was created by using a 10 μl pipette tip on confluent cell monolayers. Photographs were taken at 0 and 18 h. A quantitative analysis of the wound closure was measured by NIH Image program and determined by the percentage of the covered wound area at 18 h.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Ming-Jer Tsai, Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX 77030. Phone: 713-798-6253; Fax: 713-798-8227; E-mail: mtai@bcm.tmc.edu.

Research Article
Two-chamber migration and invasion assay. Cell migration and invasion assays were performed as described previously (16). For both assays, 4 × 10^5 cells were seeded in serum-free medium in the upper chamber, and the lower chamber was filled with 10% FBS/RPMI 1640. A quantification was performed by percentage of migratory cells on the bottom out of total seeding cells.

Fluorescent phagokinetic migration assay. The assay was performed as described previously (17). Briefly, cells were fixed with 4% paraformaldehyde 24 h after seeding. Cells were stained with Texas red–coupled phalloidin. The ability of the cells to create nonfluorescent tracks was then assessed by fluorescent microscopy and 50 cell tracks were quantified using NIH Image program.

Analysis of focal adhesion regeneration and Rac1 activity assay. Methods for disassembly and regeneration of focal adhesions after nocardazole treatment have been previously described (18). Briefly, serum-starved cells were plated onto coverslips and treated with 10 µmol/L nocardazole for 4 h to depolymerize microtubules. Nocardazole was replaced with fresh serum-free medium, and at the indicated times, cells were fixed and vinculin stained for immunofluorescence. The Rac1 activity assays were performed using a Rac1 activity kit (Upstate Biotechnology) according to the manual.

Western blotting and immunoprecipitation. Cleared protein lysates (30 µg) were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with the appropriate primary antibodies. For immunoprecipitation, cell lysates containing 500 µg protein were preclarified by protein G agarose beads and then incubated with anti-FAK antibody overnight at 4°C. The beads were washed and resuspended in SDS protein sample buffer before the immunoprecipitated protein was subjected to Western blotting.

Immunocytochemistry. Cells grown on a coverslip were fixed in 4% paraformaldehyde for 15 min, incubated with 0.1% Triton X-100 for 5 min, washed in PBS, blocked with 5% bovine serum albumin for 30 min, and stained with anti-vinculin antibody (1:1200; Chemicon) and Texas red–coupled phallolidin (1:100; Molecular Probes). Images were acquired and analyzed with a laser scanning confocal microscope (Nikon TE2000 microscope system, Nikon Instrument, Inc.).

Semi quantitative RT-PCR. Total RNA was isolated using the RNequeous-4PCR kit (Ambion) and treated with RNase-free DNase I. Total RNA (2 µg) was used to produce cDNA with SuperScript II reverse transcriptase (Invitrogen). The primers are the following: SRC-3, 5'-CGTCC-TCCATAAACCAGGAC-3' and 5'-TCTATGTTTTTGGCGTCTTCC-3'; JunB, 5'-ATACAGAGACGCCTACCC-3' and 5'-GCCGCTGGGCTCCTACCTT-3'; PEA3, 5'-TCCCAAGGCCCCCTACCAACA-3' and 5'-CGGCTCCAGGCAATGA-Ac-3'; c-Fos, 5'-CACCCTGCTCGTACTAAT-3' and 5'-GCCGCTGGCCT-CCCCCTC-3'; MPP-2, 5'-TGAGCTCCGAAAAATGTTG-3' and 5'-TCAG-CAGCCTAGCAGCT-3'; MPP-3, 5'-GCAGTTCTTCTGCGCTAG-3' and 5'-CAGGCTCTGGAGTTGCA-3'; and -actin, 5'-ATCATGTTGGA-GACCTTCA-3' and 5'-CATCTTTGCTGAGCTC-3'.

Gelatin zymography. Unconcentrated conditioned media from the cell cultures were analyzed for MMP gelatinolytic activities by gelatin zymography as described previously (19). The volume of supernatant collected was normalized to the protein concentration to ensure equal loading.

Luciferase reporter assays. The luciferase assay was performed as previously described (13). pGL2-MMP-2 is a kind gift from Dr. Yi Sun (Warner-Lambert Co., Ann Arbor, MI), pGL3-MMP-13 and its mutants are kind gifts from Dr. Carlos Lopez-Otin (Universidad de Oviedo, Oviedo, Spain).

Chromatin immunoprecipitation assay. The chromatin immunoprecipitation (ChiP) assay was performed as described previously (13). The primers used for detection of the MMP-2 and MMP-13 promoters were the following: MPP-2 promoter, 5'-TCCCCACCCAGCCTCC-3' and 5'-TGCGCACCAAGAATCC-3'; MPP-13 promoter, 5'-GCCAGATGGGTTTTGGAG-3' and 5'-GTGATGCCCTGGGACTT-3'. Primers, located 2 kb downstream of transcription start sites, were 5'-CAGGCGTGCTGGGGAG-TGTC-3' and 5'-CAGGCGTATCCTTTAGTCA-3' for MPP-2 and 5'-TTTGG-CATGCTTGGGAGATAA-3' and 5'-TCTAGGTGTTGGAGTAAAGGA-3' for MMP-13. As for checking the cis-element ChiP assay for MMP-13 promoter, forward primer is the same as described above (5'-GCGGATGTTTGGAGAC-3') and downstream primer located in plasmid is the GLprimer2 (5'-GTTTATTTTTGGGCTTCC-3').

**Results**

**Overexpression of SRC-3/AIB1 is significantly correlated with invasion and metastasis in human prostate cancers.** To determine if expression of SRC-3/AIB1 is correlated with prostate cancer invasion and metastasis, we analyzed the SRC-3/AIB1 mRNA level in 58 prostate cancer tissues from radical prostatectomy specimens by quantitative RT-PCR. To correct for variability in the tissue content of cancer, expression levels were normalized using keratin-18, an epithelial-specific marker uniformly and strongly expressed in both androgen-dependent and androgen-independent prostate cancer of all grades (20). The normalized SRC-3/AIB1 expression was then correlated with clinical and pathologic variables of invasion, metastasis, and aggressive disease. As shown in Fig. 1A, the increased SRC-3/AIB1 mRNA level was associated with seminal vesicle invasion (P = 0.014, Mann-Whitney rank sum test) and pelvic lymph node metastasis (P = 0.046, Mann-Whitney), both of which are indicative of local invasion and metastatic potential and are associated with adverse outcome. The increased SRC-3/AIB1 mRNA levels correlate well with biochemical [prostate-specific antigen (PSA)] recurrence (P = 0.04, Mann-Whitney), which is significantly associated with aggressive disease. Higher SRC-3/AIB1 mRNA levels were also present in cancer with Gleason score >7 (versus 5/6) and extracapsular extension (versus no extracapsular invasion) but the differences were not statistically significant (P = 0.146 and 0.096, respectively). To confirm these results at the protein level, we analyzed the expression of SRC-3/AIB1 by immunohistochemistry using an anti-SRC-3/AIB1 antibody in a tissue microarray containing 480 cancers. The slides were digitized and a nuclear SRC-3/AIB1 protein expression was scored in each core using a staining index that ranged from 0 (no staining) to 9 (extensive strong staining) as previously described (Supplementary Fig. S1; ref. 12). SRC-3/AIB1 staining intensity in the nuclei of cancer cells was significantly higher in cases with seminal vesicle invasion (P = 0.0001, Mann-Whitney; data not shown) and pelvic lymph node metastasis (P = 0.0001, Mann-Whitney; Fig. 1B). There was no statistically significant correlation for extracapsular extension or Gleason score. As described previously (12), higher SRC-3/AIB1 protein expression was associated with increased risk of PSA recurrence. Thus, higher SRC-3/AIB1 expression in prostate cancer is significantly associated with invasion into adjacent structures, metastasis, and disease recurrence in men undergoing radical prostatectomy.

**SRC-3/AIB1 is required for migration and invasion.** To test the hypothesis that SRC-3/AIB1 contributes to prostate cancer invasion and metastasis, we established the stable SRC-3/AIB1 knockdown PC3 cells, which displayed a significant decrease in the SRC-3/AIB1 at protein level compared with vector control (Fig. 2A). We analyzed the effect of SRC-3/AIB1 on the migratory and invasive capability of the highly invasive PC3 cells by using several function assays. First, wound-healing assay showed that, 18 h after a wound was made on the monolayer of cells, the control cells extensively migrated into the denuded area, but this migratory capacity was significantly compromised by depletion of SRC-3/AIB1 in PC3 cells (Fig. 2B). To rule out bias due to differences in proliferation, we tracked single cells by fluorescent phagokinetic migration assay and video time-lapse microscopy. Fluorescent
phagokinetic migration assay examines the ability of individual cells to form migration tracks through a field of fluorescent microspheres (17). The average area migrated by an individual SRC-3/AIB1 knockdown cell was remarkably reduced compared with control cells (Fig. 2C). Time-lapse video also showed that random migration capacity was impaired by the depletion of SRC-3/AIB1 in PC3 cells (see Supplementary Videos). Compared with control cells, diminished SRC-3/AIB1 expression also reduced PC3 cell migration through a membrane (Supplementary Fig. S2A) and invasion ability (>2-fold) through a membrane with a Matrigel barrier (Fig. 2D) by two-chamber assay. To exclude the possibility of off-target effect of shRNA, in vitro invasion assay was confirmed using two alternative siRNA targeting independent SRC-3/AIB1 sequence (Supplementary Fig. S2B and C).

To confirm that these observations are not specific for PC3 cell line, we depleted SRC-3/AIB1 in another prostate cancer cell line DU145 by siRNA method. Compared with control cells, SRC-3/AIB1-depleted DU145 cells exhibited remarkably impaired random motility in the fluorescent phagokinetic migration assay (Supplementary Fig. S2D and E) and reduced cell migration and invasion capacities in two-chamber assay (Supplementary Fig. S2F and G). In contrast, ectopic overexpression of SRC-3/AIB1 in mifepristone-inducible SRC-3/AIB1 LNCaP cells (15) increased random cellular motility (Supplementary Fig. S2H and I) and invasion through Matrigel (Supplementary Fig. S2J). The induction of invasion capacities by SRC-3/AIB1 is not dependent on androgen treatment (Supplementary Fig. S2K). Overall, these data suggest that SRC-3/AIB1 expression is essential for the migratory and invasive behavior of prostate cancer cells.

**SRC-3/AIB1 is required for the turnover of focal adhesions.**

Cell migration is a complex event that depends on the coordinated remodeling of the actin cytoskeleton as well as the regulated assembly and turnover of focal adhesions (21, 22). The focal adhesion turnover rate is higher in motile cells than in stationary

---

**Figure 1.** The correlations between SRC-3/AIB1 and clinical variables. A, SRC-3/AIB1 transcript level was measured by quantitative RT-PCR and normalized to keratin-18 (K18) mRNA levels to correct for differences in epithelial content. Comparison of the normalized SRC-3/AIB1 transcript levels with prostate cancer patients without (−) or with (+) the indicated clinicopathologic variables was shown. Columns, mean; bars, SE. *, statistically significant differences.

**Figure 2.** SRC-3/AIB1 is required for prostate cancer cell migration and invasion. A, Western blotting of SRC-3/AIB1 in PC3 control cells (C) and cells depleted of SRC-3/AIB1 by shRNA-mediated knockdown (sh3). Actin was used as a loading control. B, wound-healing assay with PC3 controls and stably knockdown SRC-3/AIB1 cells. Bar, 200 μm. Quantification of cells migrated into a defined wound area after 18 h. Cells in three defined areas per group per experiment were quantified. Columns, mean of three independent experiments; bars, SD. *, P < 0.05. C, quantification of fluorescent phagokinetic migration assay of SRC-3/AIB1 knockdown PC3 and control cells. Cells were stained with Texas red–conjugated phalloidin (red) after migration for 24 h on the blue fluorescent bead-covered coverslips. Bar, 50 μm. At least 50 cells per group per experiment were quantified. The average track area of control cells is arbitrarily set as 1.0. Columns, mean of three independent experiments; bars, SD. *, P < 0.05. D, quantification of the two-chamber invasion assay of SRC-3/AIB1 knockdown PC3 and control cells. Columns, mean of three independent experiments; bars, SD. *, P < 0.05.
cells. As shown in Fig. 3A, the control PC3 cells showed extensive phalloidin-stained lamellipodial extensions and small punctuate adhesions located at the lamellipodia with vinculin immunolabeling (CTRL, arrow). Vinculin is typically found at the leading edge of motile cells and is thought to represent rapidly cycling adhesions. In contrast, depletion of SRC-3/AIB1 in PC3 cells showed increased size and reduced number of vinculin-positive focal adhesions around the cell periphery (Fig. 3A, shSRC-3/AIB1, arrow). No obvious lamellipodia was detected in SRC-3/AIB1 knockdown cells as shown by the phalloidin staining (Fig. 3A, shSRC-3/AIB1).

To test the focal adhesion turnover rate in SRC-3/AIB1 knockdown and control cells, we used nocodazole block and release assay to analyze the synchronous reassembly of focal adhesions by all of the cells (18). Treatment with nocodazole, a microtubule-depolymerizing agent, can stabilize focal adhesions of cells at periphery. After nocodazole removal, cells disassemble their focal adhesions, associated with a decline in the level of phosphorylated FAK (pFAK Y397), which were recovered 2 h after the removal of nocodazole. As shown in Fig. 3B, 4-h nocodazole treatment induced strong focal adhesion accumulation around the cell periphery in both the SRC-3/AIB1 knockdown PC3 and control cells (left). At 30 min after nocodazole washout, focal adhesions were disassembled in both control and SRC-3/AIB1 knockdown PC3 cells (Fig. 3B, 30 min). The loss of focal adhesions corresponded with a decline in pFAK levels in these cells (Fig. 3C). At 120 min after nocodazole washout, focal adhesion was regenerared at the cell periphery in control cells (Fig. 3B, 120 min, CTRL), which corresponded with a return in pFAK levels (Fig. 3C, CTRL). However, in SRC-3/AIB1 knockdown PC3 cells, focal adhesion assembly did not recover at 120 min (Fig. 3B, shSRC-3/AIB1) and pFAK levels did not return (Fig. 3C, shSRC-3/AIB1). These studies indicate that the turnover rate of focal adhesion in SRC-3/AIB1-depleted cells is slower than that of control cells.

**SRC-3/AIB1 activates FAK signaling.** The FAK acts as a protein-protein adapter critical to the formation of focal adhesions and mediates adhesion and growth factor–dependent signals into the cells (23, 24). Tyrosine phosphorylation of FAK is one of the key signaling events during cell motility. Consistent with our}

---

**Figure 3.** SRC-3/AIB1 expression is required for focal adhesion turnover. A, SRC-3/AIB1 knockdown and control PC3 cells were visualized by immunolabeling with anti-vinculin antibody (green) or staining with Texas red–coupled phalloidin (red) for F-actin. Arrows, vinculin-positive focal adhesions. Bars, 10 μm. B, SRC-3/AIB1 knockdown and control PC3 cells were visualized by staining with anti-vinculin antibody at the indicated time points after removal of the microtubule-depolymerizing agent nocodazole. Bar, 10 μm. C, Western blot with anti-FAK pY397 showing recovery of phosphorylation of FAK at Y397 120 min after removal of nocodazole in the control group but not in the SRC-3/AIB1 knockdown group.
PI3K p85. As shown in Fig. 4 for increased motility, we examined the association of FAK with and phosphorylation of FAK may increase its interaction with PI3K p85. Consistently, the activation of Rac1, a downstream target of FAK signaling, was reduced in PC3 with the SRC-3/AIB1 knockdown and elevated in LNCaP cells with the induced SRC-3/AIB1 expression in comparison with control cells (Fig. 4C; Supplementary Fig. S3B).

**SRC-3/AIB1 induces cell invasiveness through MMP-2 and MMP-13.** MMPs are a family of key zinc-dependent enzymes involved in the degradation of extracellular matrix in cancer metastasis (16). Because the depletion of SRC-3/AIB1 impaired the invasive potential of prostate cancer cells, we examined the expression of several important MMPs in prostate cancer. Semiquantitative RT-PCR and Western blotting revealed that the expression of MMP-2 and MMP-13 was reduced in SRC-3/AIB1 knockdown PC3 cells compared with control cells (Fig. 5A), whereas MMP-1 and MMP-9 did not change much (data not shown). Forced expression of SRC-3/AIB1 in LNCaP induces MMP-2 and MMP-13 at both mRNA and protein levels in a time-dependent manner (Fig. 5A). Next, gelatin zymograph assay revealed a marked reduction of MMP-2 activity in the conditioned medium of PC3 cells depleted of SRC-3/AIB1 (Fig. 5B). In contrast, overexpression of SRC-3/AIB1 increased the MMP-2 activity in LNCaP cells (Fig. 5B). No obvious change of MMP-9 activity was detected in these cells.

**Direct induction of MMP-2 and MMP-13 by SRC-3/AIB1 requires AP-1 and PEA3.** We then evaluated the contribution of SRC-3/AIB1 to MMP-2 and MMP-13 promoter activities. As shown in Fig. 5C, overexpression of SRC-3/AIB1 induced a 2-fold MMP-2 and MMP-13 promoter activities in HeLa cells, respectively. ChIP assays showed that SRC-3/AIB1 is directly recruited onto the promoters of MMP-2 and MMP-13 (Fig. 5D), whereas no signals were detected in the negative groups (P2), indicating that such recruitment is specific for each MMP promoter.

It is well known that MMPs are regulated by AP-1 and ETS family members (25), which can be coactivated by SRC-3/AIB1 in other promoters (26, 27). To define which factor is responsible for the recruitment of SRC-3/AIB1 onto the MMP promoters, we focused our analysis on the MMP-13 promoter. We analyzed the activities of four MMP-13 promoters: wild-type (WT), mutant AP-1 site (mAP-1), mutant PEA3 site (mPEA3), and double mutants (DM). Luciferase reporter assays showed that the AP-1 binding site on MMP-13 is the major element responsible for basal MMP-13 promoter activity (Fig. 6A). Mutation in the PEA3 binding site had little effect on basal MMP-13 promoter activity. The DM lost basal MMP-13 promoter activity. Cotransfection with SRC-3/AIB1 resulted in a 2.4-fold induction of MMP-13 promoter activity, which was blunted in response to mutations in the AP-1 and/or PEA3 site. These results suggest that the PEA3 as well as AP-1 binding sites are both important for SRC-3/AIB1–induced MMP-13 promoter activity.

To further dissect which cis-element is essential for the SRC-3/AIB1 recruitment onto MMP-13 promoter, we transfected the WT or mutant MMP-13 promoter constructs into HeLa cells, used antibody against SRC-3/AIB1 to precipitate endogenous SRC-3/AIB1, and analyzed the recruitment of SRC-3/AIB1 on these four MMP-13 promoter constructs. The primer pair encompasses the observation above, PC3 cells with SRC-3/AIB1 knockdown showed decreased FAK phosphorylation at Y397 site (Fig. 4A). Consequently, paxillin, one of the downstream FAK targets, also showed reduced phosphorylation in SRC-3/AIB1 knockdown cells. Consistently, overexpressed SRC-3/AIB1 in LNCaP cells showed increased phosphorylation of FAK at Y397 and paxillin at Y118 (Supplementary Fig. S3A).

Because our previous study showed that SRC-3/AIB1 coordinately regulates the IGF/AKT signaling pathway components (13), and phosphorylation of FAK may increase its interaction with PI3K for increased motility, we examined the association of FAK with PI3K p85. As shown in Fig. 4B, SRC-3/AIB1 knockdown PC3 cells contained less tyrosine-phosphorylated FAK and a reduced level of FAK association with PI3K p85 as determined by coimmunoprecipitation. We also detected a decrease in IRS-2, another FAK interacting partner. These data suggest that SRC-3/AIB1–induced migration activity is associated with the activation of FAK signaling through IRS-2 and PI3K p85. Consistently, the activation of Rac1, a downstream target of FAK signaling, was reduced in PC3 with the SRC-3/AIB1 knockdown and elevated in LNCaP cells with the induced SRC-3/AIB1 expression in comparison with control cells (Fig. 4C; Supplementary Fig. S3B).

![Figure 4](https://example.com/f4.png)

**Figure 4.** SRC-3/AIB1 increases phosphorylation of FAK, interaction of FAK with IRS-2 and PI3K, and an induction in Rac1 activity. *A*, protein lysates from control PC3 (C) or SRC-3/AIB1 knockdown PC3 cells (sh3) were analyzed by Western blot with the indicated antibodies. *B*, cell extracts of control (C) or SRC-3/AIB1 knockdown PC3 cells (sh3) were immunoprecipitated (IP) with anti-FAK or IgG control antibodies and immunoblotted (IB) with the indicated antibodies. C, pull-down assays were performed to measure active GTP-bound Rac1 in SRC-3/AIB1 knockdown PC3 cells (sh3) and control cells (C).
AP-1 and PEA3 binding sites of the MMP-13 promoter (Fig. 6A). As shown in Supplementary Fig. S4, recruitment of SRC-3/AIB1 was impaired at the mutant PEA3 binding site. AP-1 binding site mutation alone as well as the DM showed a striking reduction in SRC-3/AIB1 recruitment to the MMP-13 promoter. These results indicate that although both binding sites are important for SRC-3/AIB1 recruitment, the AP-1 binding site is absolutely necessary for SRC-3/AIB1 recruitment to the MMP-13 promoter.

The AP-1 and ETS family contain multiple members. Within each family, their members share similar in vitro binding activity to the same consensus binding sequence. To address the identity of the binding protein to AP-1 and PEA3 sites in PC3 cells, a supershift study was conducted using specific antibodies against AP-1 family members and against ETS family members. First, using an oligonucleotide probe containing AP-1 binding site, electrophoretic mobility shift assays (EMSA) showed DNA-protein complexes in the nuclear extract of the PC3 cells (Supplementary Fig. S5A, lane 2). The specificity of binding was confirmed by a marked depletion of the band with the addition of JunB or c-Fos antibodies and a supershift with the addition of Fra1 antibody. Furthermore, a marked depletion of these bands was detected by the addition of PEAS antibody, whereas less reduction of band intensity was observed with ETS1 antibody (Supplementary Fig. S5B). Together, these data suggest that in PC3 prostate cancer cells, JunB, c-Fos, and Fra1 bind to the AP-1 site, and PEA3 and ETS1 bind to the PEA3 site of MMP-13 promoter.

Because JunB can form homodimers or heterodimers with Fos family members, whereas Fos members can only form heterodimers with Jun family members to bind to the AP-1 site (29), we used JunB to investigate the importance of the AP-1 binding site for MMP-2 and MMP-13 transcription. ChIP assays revealed that JunB and PEA3 are recruited to MMP-2 and MMP-13 promoters in PC3 cells (Fig. 6B), which agreed with the EMSA results. Next, semiquantitative RT-PCR showed that depletion of JunB, PEA3, or c-Fos reduced MMP-2 and MMP-13 expression level (Fig. 6C). Finally, using the ChIP analysis, we found that knockdown of PEA3 in PC3 cells slightly affected the recruitment of SRC-3/AIB1, whereas knockdown of JunB or JunB + PEA3 at the same time drastically reduced the recruitment of SRC-3/AIB1 to both MMP promoters (Fig. 6D). Overall, these data indicated that AP-1 is essential for SRC-3/AIB1-mediated induction of MMP-2 and MMP-13 expressions in prostate cancer cells, whereas PEAS may play an additional role.

Positive correlations between SRC-3/AIB1 and pFAK in a cohort of primary prostate carcinomas. We revealed using cultured cells that overexpression of SRC-3/AIB1 increases FAK signaling. To confirm the relationship in the human prostate tumor specimens, we analyzed SRC-3/AIB1 and pFAK Y397 expression level in a cohort of 36 human prostate carcinomas. Supplementary Fig. S6A shows two representative tumors in which the upper case contained high SRC-3/AIB1 and pFAK Y397 and the lower case contains low SRC-3/AIB1 and pFAK Y397 staining. We found that...
levels of nuclear SRC-3/AIB1 correlate significantly with those of cytosolic pFAK Y397 (Rho = 0.491; P = 0.0024; Supplementary Fig. S6B). Taken together, the tissue microarray data further strengthened the notion that SRC-3/AIB1 overexpression is essential for the activation of FAK signaling.

Discussion

The ability to migrate and invade through the basement membrane into surrounding tissues and blood vessels is one of the hallmarks of cancer and a prerequisite for local tumor progression and metastatic spread (30). Our data indicate that SRC-3/AIB1 expression is associated with local invasion and metastasis in human prostate cancer. We found that SRC-3/AIB1 is involved in promoting prostate cancer cell motility and invasiveness through the increase of focal adhesion turnover and the induction of MMP-2 and MMP-13. In support of our findings, SRC-3/AIB1 in metastatic prostate cancer was independently confirmed in different cohort by RNA profiling study of prostate cancer patients (31). These data are available from the ONCOMINE database. SRC-3/AIB1 expression level was significantly higher in metastatic prostate cancer than in prostate carcinoma (P = 2.1E-5, t test).

FAK is a nonreceptor protein tyrosine kinase that localizes to focal contact sites. Activation of FAK by both integrin and growth factors can increase focal adhesion turnover and facilitate cell migration. Many IGF signaling components, such as IGF-I, IRS-1, IRS-2, and PI3K, have been implicated in cell migration and metastasis (32–34). When stimulated by integrins and growth factors (e.g., IGF-I), activated FAK can interact with IRS-1, IRS-2, and PI3K and enhance cell migration (34–36). SRC-3/AIB1 coordinately regulates and activates the IGF signaling, involved in a variety of systems (8, 13, 15). Activated IGF signaling by SRC-3/AIB1 can enhance the interaction of activated FAK with PI3K (p85) as well as IRS-2, a target regulated by SRC-3/AIB1. Activated FAK phosphorylates its downstream target paxillin and stimulates the focal adhesion turnover rate. The activation of PI3K through interaction with FAK contributes to cell migration by producing phosphatidylinositol-3,4,5-triphosphate, and subsequent activation of Rac1 that induces cytoskeleton changes (32). Overall, SRC-3/AIB1 plays an important role in cell motility by activation of FAK signaling.

We also found that SRC-3/AIB1 can directly regulate the expression of both MMP-2 and MMP-13, which are two important MMP proteins implicated in many cancer invasion and metastasis (37–39). SRC-3/AIB1 is recruited to the MMP-2 and MMP-13 promoters via two transcription factors, AP-1 and PEA3. PEA3 is an ETS superfamily member that prefers to form complexes with other factors, such as AP-1, to bind to DNA (40). Interestingly, AP-1 and PEA3 binding sites are found in close proximity to each other on these two MMP promoters. We showed that mutations of either or both binding sites can affect SRC-3/AIB1 recruitment and MMP-13 promoter activity, suggesting that both AP-1 and PEA3 sites are essential for SRC-3/AIB1-induced MMP-13 expression. This is consistent with previous findings that these two binding sites are important for MMP-13 expression (28). Furthermore, Fos can interact with the C terminus of SRC-3/AIB1 that contains the histone acetyltransferase domain (26), whereas PEA3 subfamily
members can interact with the N terminus of SRC-3/AIB1 (30). Therefore, SRC-3/AIB1 may interact with AP-1 (JunB:c-Fos) and PEA3 simultaneously via different domains. Both AP-1 and PEA3 subfamily members have been implicated as important players in tumor progression. JunB and PEA3 are amplified in hormone-resistant or androgen receptor (AR)-negative prostate cancer, respectively (41, 42). Recent studies also showed that most prostate cancer patients have ETS family members fused to 5'-untranslated region of androgen-regulated TMPRSS2, resulting in the overexpression of the various ETS family members (43). Our findings raise the interesting possibility that SRC-3/AIB1 contributes to AP-1-mediated and PEA3-mediated tumor metastasis by coregulating AP-1 and PEA3 simultaneously to up-regulate MMP-2 and MMP-13 expression levels in prostate cancer. Recently, it was reported that SRC-3/AIB1 can promote breast cancer cell metastasis by specifically inducing MMP-7 and MMP-10 expressions (44). It seems that SRC-3/AIB1 can induce different MMPs expression in a cell context–specific manner. It may be dependent on the limiting amount of SRC-3/AIB1 for each MMP promoter or the availability of different transcription factors for the recruitment of SRC-3/AIB1 onto the different MMP promoters.

The SRC-3/AIB1 overexpression in prostate cancer can coactivate AR and induce cyclin A expression in an androgen-independent manner (45). At the same time, SRC-3/AIB1 may also interact with transcription factors, such as AP-1 and ERBB1 (26, 27). These findings suggest that overexpression of SRC-3/AIB1 in the late stage of prostate cancer may function through AR as well as other transcription factors to contribute to metastasis in prostate cancer cells. In a subset of AR-negative prostate cancer, the coactivation of transcription factors by SRC-3/AIB1 is also sufficient for tumor metastasis. In support of this notion, our study showed that in AR-null PC3 and DU145 prostate cancer cells, depletion of SRC-3/AIB1 strongly reduces cell motility and invasiveness. These evidences indicate that SRC-3/AIB1 is essential for prostate cancer metastasis by regulation of multiple gene expression related to cell migration and invasiveness. Our data show that the nuclear receptor coactivator SRC-3/AIB1 plays a critical role to facilitate cancer cell migration and invasiveness in prostate cancer cells. This strongly suggests that suppression of SRC-3/AIB1 activity or its downstream targets, such as FAK, MMP-2, and MMP-13, may be a good therapeutic approach for prostate cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
Received 3/12/2007; revised 4/30/2008; accepted 4/30/2008.

Grant support: NIH grant DK 62821 (M.-J. Tsai and M. Ittmann) and NIH Prostate Specialized Program of Research Excellence grant U01CA105352 (M.-J. Tsai, M. Ittmann, and G. Ayala).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Drs. Carlos Lopez-Otin and Yi Sun for providing reporter plasmids, Drs. Charles Foulds and Larbi Amazit for technical assistance, and Deanna Killen and Anna F. Schroeder for their assistance in the tissue microarray analysis.

References
Steroid Receptor Coactivator-3/AIB1 Promotes Cell Migration and Invasiveness through Focal Adhesion Turnover and Matrix Metalloproteinase Expression

Jun Yan, Halime Erdem, Rile Li, et al.