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Activity Through Membrane Receptor Mediated Signaling Pathways

PRINCIPAL INVESTIGATOR: Ping Yi, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, TX 77030

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INTRODUCTION:

SRC-3/AIB1 (Steroid Receptor Coactivator-3, also named Amplified in Breast Cancer 1) interacts with steroid receptors in a ligand-dependent manner to activate receptor mediated transcription. Overexpression or gene amplification of SRC-3 has been found in more than 60% of breast tumors. Overexpression of SRC-3 in transgenic mice leads to the development of malignant mammary tumors(4), while SRC-3 knockout mice were found to have dramatically reduced breast tumor incidence(2). Phosphorylation of SRC-3 by kinases from growth factor signaling pathways is shown to regulate ER transcriptional activity (1, 5, 6) as well as its oncogenic potential. Furthermore, breast cancers overexpressing both SRC-3 and growth factor receptor HER2/neu are poorly responsive to the tamoxifen treatment (3). All these studies suggest that this post-translational modification of SRC-3 could be an important factor in normal mammary gland development and breast tumorigenesis as well as the observed endocrine resistance in breast cancer patients. However, it is still largely unknown as to how phosphorylation exactly affects coactivator function. Therefore, we investigate the mechanisms through which regulation of SRC-3 activity is influenced by extracellular signals induced phosphorylation.

BODY:

Determine the phosphorylation sites of SRC-3 in response to various extracellular signal stimulations

We have recently identified six phosphorylation sites in baculovirus expressed SRC-3(6). We found that these phosphorylation sites are induced upon estrogen stimulation and are required for coactivation of estrogen receptor. Furthermore, these phosphorylation sites were found to be important for the oncogenic potential of SRC-3. Since these phosphorylation sites were identified from baculovirus expressed SRC-3, it is most likely that there are additional phosphorylation sites present in mammalian expressed SRC-3. Moreover, there are possible phosphorylation sites that are only present when cells are stimulated with different extracellular signals. To identify these potential sites, I decided to generate an inducible stably expressed SRC-3 mammalian cell line, and then purify phosphorylated SRC-3 protein for identification of phosphorylation sites using mass spectrometry. I generated a HEK 293 cell line with tetracycline inducible expression of flag-SRC-3. As shown in Fig. 1, the expression of SRC-3 is dramatically induced with tetracycline induction for 24 hours.

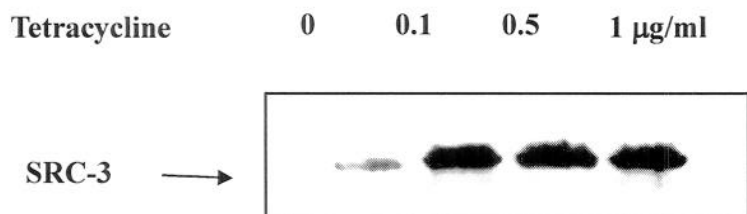


Fig.1. Tetracycline induced expressions of SRC-3 in Flp-In T-Rex HEK293 cells. Western blot analysis was done using anti-SRC-3 antibody.

After establishing the inducible SRC-3 stable cell line, I then adapted the adherent HEK293 cells into suspension culture in order to grow large amount of cells. 10 liters of cells were treated with estrogen for one hour and then harvested. SRC-3 was immunoprecipitated using anti-SRC-3 antibody. The precipitated SRC-3 was resolved in SDS-PAGE and subjected to mass spectrometry. The amount of SRC-3 precipitated is shown in Fig. 2 by coomassie staining.

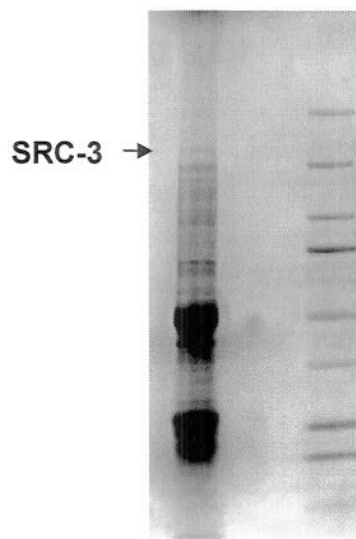


Fig. 2. Coomassie staining on SRC-3 purified from inducible stable HEK 293 cells.

However, the mass spectrometry result shows that the amount of SRC-3 protein purified was not sufficient for identification of phosphorylation sites. I tried several times but was not able to obtain enough amount of protein. Therefore, I was not successful on identification of SRC-3 phosphorylation sites in mammalian cells.

Regulation of phosphorylated SRC-3 function by peptidyl-prolyl isomerase 1 (Pin1)

See attached manuscript.

KEY RESEARCH ACCOMPLISHMENTS:

Demonstrate that the peptidyl-prolyl isomerase 1 (Pin1), which catalyzes the isomerization of phosphorylated Ser/Thr-Pro peptide bonds to induce conformational changes of its target proteins, interacts selectively and specifically with phosphorylated SRC-3.

Pin1 and SRC-3 activate nuclear receptor regulated transcription synergistically.

Depletion of Pin1 by siRNA reduces hormone-dependent transcription from both transfected reporters and an endogenous steroid receptor target gene.

Pin1 modulates interactions between SRC-3 and CBP/p300. The interaction is enhanced *in vitro* and *in vivo* by Pin1 and diminished when cellular Pin1 is reduced by siRNA.

Depletion of Pin1 in MCF-7 human breast cancer cells reduces endogenous estrogen-dependent recruitment of p300 to the promoters of estrogen receptor-dependent genes.

Pin1 over-expression enhanced SRC-3 cellular turnover and depletion of Pin1 stabilized SRC-3.

REPORTABLE OUTCOMES:

Manuscript:

Yi, P., Wu, R.C., Sandquist, J., Wong, J., Tsai, S.Y., Tsai, M.J., Means, A.R., O'Malley, B.W. Peptidyl-prolyl Isomerase 1 (Pin1) Serves as a Coactivator of Steroid Receptor by Regulating the Activity of Phosphorylated Steroid Receptor Coactivator-3 (SRC-3/AIB1)

CONCLUSIONS:

We found that the peptidyl-prolyl isomerase 1 interacts specifically with phosphorylated SRC-3 and it activates estrogen and progesterone receptor mediated transcription synergistically when co-expressed with SRC-3. We demonstrated that depletion of Pin1 reduces estrogen receptor target gene transcriptiton in breast cancer cell line. Pin1 functions in the stabilizing SRC-3 and CBP/p300 complex formation. Furthermore, it also plays a role in regulating SRC-3 protein turnover. Our results suggest that Pin1 functions as a transcriptional coactivator of nuclear receptors by modulating SRC-3 coactivator protein-protein complex formation, and ultimately, by regulating the turnover of the activated SRC-3 oncoprotein.

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Peptidyl-prolyl Isomerase 1 (Pin1) Serves as a Coactivator of Steroid Receptor by Regulating the Activity of Phosphorylated Steroid Receptor Coactivator-3 (SRC-3/AIB1)

Ping Yi¹, Ray-Chang Wu¹, Joshua Sandquist², Jiemin Wong¹, Sophia Y. Tsai¹, Ming-Jer Tsai¹, Anthony R. Means² and Bert W. O'Malley^{1*}

¹Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas 77030. ²Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710

* Corresponding author. Mailing address: Department of Molecular and Cellular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Phone: (713) 798-6205. Fax: (713) 798-5599. E-mail: berto@bcm.tmc.edu.

Abstract:

Steroid receptor coactivator-3 (SRC-3/AIB1) interacts with steroid receptors in a ligand-dependent manner to activate receptor mediated transcription. A number of intracellular signaling pathways initiated by growth factors and hormones induce phosphorylation of SRC-3, regulating its function and contributing to its oncogenic potential. However, the range of mechanisms by which phosphorylation affects coactivator function remains largely undefined. We demonstrate here that the peptidyl-prolyl isomerase 1 (Pin1), which catalyzes the isomerization of phosphorylated Ser/Thr-Pro peptide bonds to induce conformational changes of its target proteins, interacts selectively and specifically with phosphorylated SRC-3. In addition, Pin1 and SRC-3 activate nuclear receptor regulated transcription synergistically. Depletion of Pin1 by siRNA reduces hormone-dependent transcription from both transfected reporters and an endogenous steroid receptor target gene. We present evidence that Pin1 modulates interactions between SRC-3 and CBP/p300. The interaction is enhanced *in vitro* and *in vivo* by Pin1 and diminished when cellular Pin1 is reduced by siRNA. Depletion of Pin1 in MCF-7 human breast cancer cells reduces endogenous estrogen-dependent recruitment of p300 to the promoters of estrogen receptor-dependent genes. Pin1 over-expression enhanced SRC-3 cellular turnover and depletion of Pin1 stabilized SRC-3. Our results suggest that Pin1 functions as a transcriptional coactivator of nuclear receptors by modulating SRC-3 coactivator protein-protein complex formation, and ultimately, by regulating the turnover of the activated SRC-3 oncoprotein.

Steroid receptors, in response to their cognate ligands, regulate a variety of physiological processes including reproduction, development and cellular homeostasis. They activate gene transcription by binding to hormone responsive elements at target genes and recruiting coactivators. SRC (p160) coactivators are among the first cloned steroid receptor coactivators. Members of p160 coactivator family, including SRC-1 (16, 30), SRC-2 (GRIP1/TIF2)(14, 43) and SRC-3 (AIB1/ACTR/pCIP/RAC3/TRAM-1) (2, 8, 21, 38-40), interact with ligand bound receptors through conserved LXXLL motifs in their nuclear receptor interaction domains (10). In addition, they also contain functional activation domains that recruit proteins to modify histones and remodel chromatin. The recruited proteins include CBP/p300 which has intrinsic histone acetyltransferase activity (8) and CARM1 that has histone methyltransferase activity (7).

The SRC-3 coactivator is involved in important physiological processes, including reproductive function, cytokine signaling, cell proliferation, and somatic growth (24, 45, 51, 57). It is believed to be an oncogene (41, 57). It is amplified and over-expressed in breast and ovarian cancers (2). SRC-3 knockout mice display delayed mammary gland development, growth retardation and impaired vasoprotection (24, 45, 51, 54). A number of extracellular signals including steroid hormones, growth factors and cytokines can induce SRC-3 phosphorylation (47, 48). Phosphorylation of SRC-3 has been shown to be important for its interaction with CBP/p300 and nuclear receptors (12, 48) as well as for its oncogenic potential (48).

Peptidyl-prolyl isomerases are an evolutionarily conserved group of proteins that promote the *cis/trans* isomerization of the peptide bond preceding Pro residues (11, 35). Pin1 is a unique member of one of three such proteins families, the parvulins, as it specifically interacts with and isomerizes phospho-Ser/Thr-Pro motifs. The study of Pin1 has merged the prolyl isomerase modification with the more extensively studied protein modification of phosphorylation, and implicated a role for Pin1 in cell signaling. Pin1 was originally identified as a protein that interacted with the important fungal cell cycle regulatory protein kinase NIMA in both human and *Aspergillus nidulans* cDNA library screens (9, 28). It is comprised of an N-terminal WW domain that is involved in protein interaction and a C-terminal prolyl isomerase domain. Interestingly, both domains recognize phospho-Ser/Thr-Pro motifs (31, 42). Thus, multiple such motifs within a target protein sequence, such as the 7 amino acid repeat that comprises the CTD of RNA polymerase II, markedly increase the affinity and efficacy of Pin1 (15, 29). The

isomerization activity of Pin1 frequently results in conformational changes that can alter the function, localization or stability of the target protein (59). In addition, Pin1-induced isomerization can result in PP2A-mediated dephosphorylation of the phospho-Ser/Thr-Pro motif as PP2A is a *trans*-specific phosphatase (58). A number of Pin1-interacting proteins including c-Jun, NF- κ B, p53, β -catenin and c-Myc are transcription factors and for all of these proteins the primary role of Pin1 seems to be regulation of stability (33, 34, 50, 53, 55). At least in the case of c-Myc, Pin1 binding facilitates PP2A-mediated dephosphorylation, which is required for ubiquitination and degradation (53).

As Pin1 is important for the function of reproductive tissues in mice (4, 5, 23) we reasoned that Pin1 could play a role in the function of sex steroid receptors such as ER and PR by influencing the activities of one or more of their coactivators. Our attention was directed first to SRC-3/AIB1 because it is a dominant coactivator of PR and ER in certain reproductive tissues (19, 41, 51). Recently Wu et al (48) identified several phosphorylation sites in SRC-3 using mass spectrometry and found that different combinations of phosphorylation sites modulate SRC-3 transactivation function in response to different signaling pathways. Five out of the six phosphorylation sites identified in SRC-3 contain Ser/Thr-Pro motifs, raising the possibility that Pin1 could be involved in the post-phosphorylation regulation of SRC-3. In addition, activation of SRC-3 by phosphorylation appears to be coupled to its degradation. However, how transactivation by SRC-3 was coupled to its degradation has remained an enigma. Here, we demonstrate that Pin1 can function as a novel coactivator by interacting with phosphorylated SRC-3 and modulating its protein-protein interactions with other coregulators in a manner that enhances PR and ER function as well as directs the cellular turnover of SRC-3.

Materials and Methods

Plasmids and reagents

The Pin1 cDNA was synthesized by RT-PCR from MCF-7 cell mRNA and was inserted into a mammalian expression vector pCM5, pGEX (Amersham Biosciences) for GST fusion protein, or pMGal4 (BD Biosciences) for Gal4DBD fusion protein. The Pin1 mutant C113A or W34A was generated using a Quickchange Mutagenesis kit (Stratagene). The SRC-3 fragments

were inserted into pACT (Promega) for VP16-AD fusion proteins. VP16-CBP was kindly provided by Dr. Carolyn Smith. The PKA catalytic subunit, c-Jun, c-Fos and CREB expression vectors were kindly provided by Drs. Barbara Sanborn, Tse Hua Tan and Michael Greenberg. Different transcription elements driven luciferase vectors were from Mercury Pathway profiling luciferase systems (BD Biosciences).

The anti-SRC-3 antibody was generated as described previously (47). The anti-Pin1 antibody was provided by Dr. Anthony Means. Antibodies used in chromatin immunoprecipitation assay, anti-ER α (H-184), anti-ACTR (C-20) and anti-p300 (N-15), were from Santa-Cruz Biotechnology. Other antibodies were from various sources: anti-flag (Sigma, Affinity Bioreagents), anti-hemagglutini (HA, Roche Molecular Biochemicals), anti-p300 (Upstate).

Cell lines and Transfections

HeLa (ATCC), MCF-7 (from Dr. Richard Santen) and other cell lines were maintained in DMEM supplemented with 5% FBS unless otherwise noted. T47D (CAT0) cells were maintained in 0.2 mg/ml Geneticin (Invitrogen) containing medium and the Flp-In T-Rex 293 cells (Invitrogen) were maintained in 15 μ g/ml blasticidin, 100 μ g/ml zeocin containing medium. The tetracycline inducible stable SRC-3 Flp-In T-Rex 293 cell line was generated according to the manufacturer's manual (Invitrogen) and was maintained in blasticidin and hygromycin (150 μ g/ml) containing medium; 0.5 μ g/ml of tetracycline was used to induce the expression of flag-SRC-3. For assays of steroid hormone dependent transcription, cells were maintained in charcoal-stripped FBS (Gemini) containing phenol red free DMEM medium for at least three days before the addition of steroid hormones.

All plasmid DNAs were transfected using TransIT-LT1 transfection reagent (Mirus) according to the manufacturer's protocol. For measuring luciferase, steroid hormones were added to the medium 16 hours after transfection. After an additional 24-hour incubation, cells were lysed and cell lysates were used for luciferase assay. The smartpool siRNA or scrambled siRNA was obtained from Dharmacon. 4 nM siRNA was transfected into cells by TransIT-TKO transfection reagent (Mirus). Cells were harvested four days after transfection. In the case of co-transfection of siRNA and plasmid DNA experiments, cells were transfected with siRNA first; two days after transfection, cells were co-transfected with siRNA and plasmid DNA according to

the manufacturer's protocol (Mirus) followed by the same procedure as plasmid DNA transfection alone.

GST pull down and *in vitro* protein-protein interaction

E.coli expressed GST-Pin1 was bound by 10 μ l glutathione sepharose 4B (Amersham Biosciences) followed by the addition of 300 μ g HeLa cell extracts or *in vitro* transcribed or translated SRC-3. After four hours incubation at 4°C, the beads were washed five times with wash buffer (50 mM Tris.Cl (pH 7.5), 200 mM NaCl, 50 mM NaF, 1mM Na₃VO₄, 10% glycerol, 1% NP-40, 1mM DTT). The bound proteins were resolved in 7% SDS-PAGE and detected by Western blot analysis.

50 ng purified baculovirus recombinant SRC-3 was immunoprecipitated by anti-SRC-3 antibody in 150 μ l buffer (20 mM Hepes (pH7.6), 150 mM KCl, 0.1% NP-40, 8% glycerol, 1 mM DTT, 0.5 mM PMSF and protease inhibitor cocktail) and then incubated with purified GST or different concentrations of GST-Pin1 (2 and 10 ng) at R.T. for 5 min. The samples were incubated at 4°C for an additional 10 min and the excess Pin1 was removed by wash buffer. Purified baculovirus recombinant p300 was then incubated with SRC-3 at 4°C overnight and the beads were washed extensively with wash buffer. The bound p300 were resolved in 4-15% SDS-PAGE and detected by Western blot analysis.

Immunoprecipitation and Western blot analysis

Cells were washed with PBS and lysed in the lysis buffer (20 mM Tris.Cl (pH8.0), 125 mM NaCl, 0.5% NP-40, 20 mM NaF, 0.2 mM Na₃VO₄, 2 mM EDTA and protease inhibitor cocktail) for 30 min. After centrifugation at 13,400xg for 15 min, the supernatant was pre-cleared by normal IgG for 1 hour, and then incubated with desired antibody for 2 hours followed by the addition of 20 μ l protein A and G agarose beads overnight. The antibody bound complex was washed extensively with the lysis buffer. The proteins were then resolved in 4-15% gradient SDS gel and transferred to nitrocellulose membranes (Bio-Rad). The primary antibody was diluted in TBST buffer (50 mM Tris.Cl (pH 7.5), 150 mM NaCl, 0.1% Tween 20, 5% dried nonfat milk) with overnight incubation at 4°C followed by the appropriate secondary antibody with 1 hour incubation at RT. The Western blot was visualized by chemiluminescence (Pierce).

Real time quantitative PCR and RT-PCR

MCF-7 cell total RNA was extracted using Tri-reagent (Molecular Research Center, Inc.). The pS2 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 pS2 mRNA were as follows: forward, 5'-GGTCGCCTTGGAGCAGA, reverse, 5'-GGGCGAAGATCACCTTGTT, probe, 5'-FAM-TCCATGGTGGCCATTGCCTCCT-TAMRA. The primers for the cyclophilin mRNA were as follows: forward, 5'-GACAAGGTCCCAAAGACAGCAG, reverse, 5'-FCAGGAACCCTTATAACCAAATCC, probe, 5'-FAM-AAATTTTCGTGCTCTGAGC-TAMRA. The primers for the pS2 promoter in the ChIP assay were as follows: forward, 5'-CGTGAGCCACTGTTGTCAGG, reverse, 5'-TGGTGAGGTCATCTTGGCTG, probe, 5'-FAM-CAAGCCTTTTTCCGGCCAT-TAMRA. The primers for the cyclophilin intron in the ChIP assay were as follows: forward, 5'-TGTTTAATGACATTTAGTACAAAAGGCTTC, reverse, 5'-GAACAACATTATGACTGGCAACC, probe, 5'-FAM-AGCTACCTTTCTCGTCTTG-TAMRA.

The RT-PCR analysis was performed using the Access RT-PCR kit (Promega). The primers for the flag-SRC-3 were as follows: forward, ATTACAAGGATGACGACGATAAG, reverse, CGTATCTGTCTTACTGTTTCCTTTAAAATC.

Chromatin immunoprecipitation (ChIP)

MCF-7 cells were transfected with Pin1 siRNA or scrambled siRNA. Four days after transfection, cells were treated with 10^{-8} M estradiol for 1 hour and were harvested for the ChIP analysis. The procedure is essentially the same as described previously (22). The protein bound DNA precipitated from the ChIP assay was purified with the QIA quick PCR purification kit (Qiagen) and eluted in 50 μ l of elution buffer. The DNA was quantitated using the pS2 promoter specific primers by real time quantitative PCR. The cyclophilin intron 3 specific primers were used as a control.

Statistical analysis and quantitative analysis

Statistical analysis was performed by t-test using SISA (Simple Interactive Statistical Analysis). Data quantitation was carried out with NIH Image software version 1.62.

Results:

Pin1 interacts specifically with phosphorylated SRC-3

To test the potential role of Pin1 in SRC-3 function, we first tested whether Pin1 interacts with SRC-3 using a GST-pull down assay of a HeLa cell extract. As shown in Fig. 1A, SRC-3 was detected in the Western blot following pull-down by GST-Pin1, but not by GST alone or a GST-Pin1W34A mutant which has a mutation in the WW domain and is defective in the target protein binding function (46). Similar results also were obtained when *in vitro* transcribed and translated SRC-3 was incubated with GST-Pin1 (data not shown). Given that all known Pin1 substrates are phosphoproteins and that five of the six identified phosphorylation sites of SRC-3 are Ser/Thr-Pro, we tested whether the interaction between SRC-3 and Pin1 is dependent on the phosphorylation status of SRC-3. Flag-SRC-3 was immunoprecipitated from extracts of transfected HeLa cells by anti-flag antibody and dephosphorylated by treatment with λ -phosphatase. After elution from antibody precipitates with flag peptides, the dephosphorylated SRC-3 was incubated with GST-Pin1. As shown in Fig. 1B, the interaction between SRC-3 and Pin1 was lost when SRC-3 first was treated with the active phosphatase; heat inactivated λ -phosphatase treatment had no effect on this interaction. We conclude that Pin1 only interacts with phosphorylated SRC-3.

We next determined whether the SRC-3/Pin1 interaction also occurs in cells using a mammalian two hybrid assay. Pin1 or Pin1W34A was fused to a Gal4 DNA binding domain and SRC-3 was fused to a VP16 activation domain. The co-transfection of Gal4-Pin1, but not Gal4-Pin1W34A, with VP16-SRC-3 markedly increased reporter activity (Fig. 1C), indicating that Pin1 also interacts with SRC-3 in cells. To confirm this interaction, co-immunoprecipitation was carried out using extracts derived from cells stably expressing flag-SRC-3 (Fig. 1D). Flag-SRC-3 was detected in an anti-Pin1 antibody precipitation while it could not be detected in control IgG precipitations. Together, these results indicate that SRC-3 interacts with Pin1 both *in vitro* and *in vivo*.

Pin1 interacts with multiple regions of SRC-3

To determine potential Pin1 interaction domains in SRC-3, we used a series of *in vitro* transcribed and translated SRC-3 fragments in GST pull-down assays. SRC-3, like other members of the p160 coactivator family, contains several functional domains as shown in Fig. 2A. It interacts with nuclear receptors through the conserved LXXLL motifs in the Receptor Interaction Domain (RID, aa581-840). The N-terminal region contains a highly conserved bHLH/PAS domain (aa1-320) and a Ser/Thr rich region (aa321-581). The C-terminal regions are the CBP/p300 interaction domain (CID, aa841-1080) and the domain that contains HAT activity (HAT, aa1081-1417). Three fragments of SRC-3 (aa321-581, 582-840 and 1081-1417) were found to interact with GST-Pin1 (Fig. 2B). Since the interaction with Pin1 is dependent on the phosphorylation of SRC-3 (Fig. 1B) and the *in vitro* translated products may not have exactly the same phosphorylation pattern as *in vivo*, we next analyzed the interaction of different SRC-3 fragments with Pin1 *in vivo* using the mammalian two hybrid assay.

Consistent with the *in vitro* interaction results, the co-transfection of VP16-AD fused to the SRC-3 fragments and Gal4-Pin1 enhanced transcription activity although at a lower level than full length SRC-3; the fragment (aa841-1080) that did not interact with Pin1 in the GST pull-down assay also did not interact with Pin1 in cells (Fig. 1C). Our results indicate that Pin1 can interact with SRC-3 at multiple sites and raise the question of whether Pin1 plays a role in regulating SRC-3 activity.

Two of the interacting fragments (aa321-581 and aa582-840) contain previously identified phosphorylation sites. However, the third fragment (aa1081-1417), which does not contain a previously identified phosphorylation site, still interacts with Pin1, implying that additional phosphorylation sites may be present in that fragment. Indeed GST-Pin1 pull-down experiments comparing wild type SRC-3 with the mutant in which all six Ser/Thr are changed to Ala show that GST-Pin1 still interacts with the mutant (data not shown). These results suggest that additional phosphorylation sites in SRC-3 may influence the interaction with Pin1.

Pin1 synergizes with SRC-3 to coactivate steroid receptor transcriptional activity

To determine if the interaction between Pin1 and SRC-3 plays a functional role, we examined the effects of Pin1 on SRC-3 activated progesterone receptor (PR) activity. A PR expression vector and progesterone response element (PRE) driven luciferase reporter were co-

transfected into HeLa cells. Transfection of SRC-3 enhanced luciferase activity in the presence of 10 nM progesterone (Fig. 3A). Transfection of Pin1 also stimulated PR target gene transcription. However, co-transfection of both SRC-3 and Pin1 synergistically increased PR-dependent luciferase activity (Figs. 3A and 3B). In contrast, the Pin1C113A mutant, which is impaired in PPIase activity (46, 58), did not produce any activation of PR-mediated transcription. Similarly, neither of the two separate domains of Pin1 (WW or PPIase domain) could activate transcription synergistically with SRC-3. These experiments show that a fully intact and enzymatically active Pin1 is required for SRC-3 dependent synergistic activation of the PR target gene reporter.

Although originally identified as a nuclear receptor coactivator, SRC-3 was reported to coactivate several other transcription factors including CREB, AP1, E2F, serum response factor, p53 and STAT (3, 13, 17, 18, 20, 27). To test whether Pin1 and SRC-3 can synergize to activate other transcription factors, we examined estrogen, AP1 and CRE responses. As shown in Fig. 3B-E, Pin1 or SRC-3 alone moderately enhanced transcription of each of the four reporter genes tested. When Pin1 and SRC-3 were co-transfected, only the estrogen response element (ERE) and PRE reporter genes were synergistically activated. Additive or weak synergy of the AP1 response was observed for SRC-3 and AP-1. Finally whereas either Pin1 or SRC-3 caused small increases in CRE-mediated transcription, no additivity of these responses was observed upon co-transfection of SRC-3 and Pin1. On the other hand, transcription of SV40 promoter-driven renilla luciferase was not altered when cells were co-transfected with Pin1 and SRC-3 (data not shown), thus serving as a negative internal control.

Down regulation of cellular Pin1 impairs steroid receptor target gene transcription

Since over-expression of Pin1 significantly enhanced SRC-3 activated steroid receptor-mediated transcription, we next wished to examine whether a decrease in endogenous Pin1 decreased such transcription reactions. To examine this question, we first transfected HeLa cells with a siRNA against Pin1 or a scrambled control siRNA. Two days after transfection, PR, SRC-3 and PRE driven luciferase reporter were co-transfected and progesterone simulated luciferase activity was measured. As shown in Fig. 4A (upper panel), the Pin1 siRNA reduced the Pin1 protein level in HeLa cells and decreased the SRC-3 activated PR activity by 50% compared to control siRNA (Fig. 4A lower panel).

We next tested whether Pin1 depletion affects expression of an endogenous steroid receptor target gene. For this purpose we used an MCF-7 cell line to examine estrogen receptor regulated pS2 transcription. As shown in Fig. 4B (upper panel), Pin1 siRNA reduced Pin1 expression in MCF-7 cells. Consistent with the result observed in transfected HeLa cells, the endogenous estrogen-dependent pS2 mRNA level decreased 30% when Pin1 was knocked down, as revealed by real-time quantitative PCR (Fig. 4B lower panel, $p < 0.05$).

Thus, in both HeLa and MCF-7 cells, a decrease in Pin1 results in a reduction in PR and ER-dependent transcription.

Pin1 activity enhances the interaction between SRC-3 and CBP/p300

SRC-3 exerts its function as a nuclear receptor coactivator primarily through recruiting additional coregulator proteins such as CBP/p300 to the promoter-bound receptor-coactivator complex. We previously showed that mutations of the phosphor Ser/Thr-Pro SRC-3 phosphorylation sites impair the ability of SRC-3 to form complexes with coactivators such as CBP (48). Since Pin1 binds specifically to phosphorylated SRC-3, we wished to determine whether Pin1 affects the interaction between SRC-3 and CBP/p300. Mammalian two hybrid assays were carried out using Gal4-SRC-3 and VP16-CBP (Fig. 5A). The co-transfection of Gal4-SRC-3 and VP16-CBP significantly increased reporter transcription, indicating there is an interaction between SRC-3 and CBP. Transcription was further augmented by co-transfection of Pin1 but not the catalytically inactive Pin1 C113A mutant. Although there are several possible explanations for this result, one likely possibility is that Pin1 functions to facilitate the cellular interaction of SRC-3 and CBP/p300 to enhance the transcriptional activity. To confirm this idea, SRC-3 and p300 interactions were assessed directly using recombinant proteins purified from baculovirus-infected insect cells. SRC-3 was first immunoprecipitated with a specific antibody. Different concentrations of Pin1 were then incubated with bead-bound SRC-3 and the excess Pin1 was then washed away before addition of p300. After a further incubation period, the SRC-3/p300 complex was co-immunoprecipitated with SRC-3 antibody and detected by Western blot analysis (Fig. 5B). The amount of precipitated p300 associated with SRC-3 was increased as a function of Pin1 concentration. However, mutant Pin1 proteins with altered WW or PPIase domains (Fig. 5C) did not promote the interaction between SRC-3 and p300. These data confirm that Pin1 facilitates the interaction between SRC-3 and CBP/p300.

To further substantiate this finding, we next examined whether Pin1 affects the recruitment of p300 to an endogenous pS2 promoter in MCF-7 cells. Chromatin immunoprecipitation assays were carried out in MCF-7 cells transfected with control siRNA or Pin1 siRNA. The recruitments of ER, SRC-3 and p300 to the pS2 promoter upon E2 stimulation were detected by real time quantitative PCR (Fig. 5D). The results in Fig. 5D show that knocking down of Pin1 did not affect significantly the recruitment of ER α and SRC-3 to the pS2 promoter. However, the recruitment of p300 to the promoter was dramatically reduced ($p < 0.001$) when Pin1 was knocked-down. These results provide further evidence that Pin1 facilitates the recruitment of CBP/p300 to a steroid receptor targeted promoter.

Pin1 modulates the cellular levels of SRC-3 protein

Prolyl isomerization catalyzed by Pin1 has been shown to alter the stability of target proteins such as NF κ B (34), p53 (49, 55, 56) and c-Myc (53). We next addressed whether the concentration of Pin1 can also modulate the stability of SRC-3 in HeLa cells. A Pin1 expression vector (or an empty expression vector) was co-transfected with flag-SRC-3 into HeLa cells. The level of the flag-SRC-3 protein was detected by Western blot analysis using a flag specific antibody. As shown in Fig. 6A the amount of flag-SRC-3 was reduced when cells were co-transfected with Pin1. Conversely, treatment of HeLa cells with the proteasome inhibitor MG132, resulted in a substantial increase in flag-SRC-3, and this level was unaffected by over-expression of Pin1 (as shown by shorter exposure of the Western blot). As over-expression of Pin1 did not increase the amount of flag-SRC-3 mRNA (Fig. 6B) the data suggest that Pin1 affects SRC-3 protein stability rather than altering expression of the SRC-3 gene. To determine whether the effect of Pin1 on SRC-3 protein level is cell type-specific and to substantiate that Pin1 PPIase activity is involved in regulating SRC-3 level, we transfected different concentrations of Pin1 or Pin1C113A into a 293 cell line stably expressing an inducible flag-SRC-3 gene. As illustrated in Fig. 6C, increasing the concentration of Pin1 decreased the level of flag-SRC-3 whereas the Pin1C113A mutant had no effect on flag-SRC-3 protein levels. Thus, the effect of Pin1 on SRC-3 level requires the PPIase activity and is not cell specific.

We also addressed the relationship between Pin1 and flag-SRC-3 levels by using siRNA to reduce the amount of Pin1 in HeLa cells (Fig. 6D). In contrast to the effect caused by the over-expression of Pin1, administration of Pin1 siRNA increased the level of flag-SRC-3

compared to the addition of a scrambled control siRNA (Fig. 6D, lane 3 vs. lane 1). The increase in SRC-3 mediated by Pin1 siRNA was due specifically to the decrease of Pin1 protein level since co-transfection of Pin1 reverted the Pin1 siRNA effect and resulted in a decreased level of flag-SRC-3 (Fig. 6D, lane 4 and 2 vs. 1).

To determine if the regulation of SRC-3 protein level by Pin1 was due to a change in SRC-3 stability, we examined the half-life of endogenous SRC-3 in MCF-7 cells treated with either scrambled control siRNA or Pin1 siRNA. The cells were treated with estradiol overnight prior to addition of cycloheximide to inhibit cellular protein synthesis. Fig. 6E shows that the SRC-3 protein levels gradually decreased during the time course of cycloheximide treatment when control siRNA was transfected into cells and this rate of SRC-3 turnover is consistent with that previously reported (26). In cells transfected with Pin1 siRNA, however, the SRC-3 protein was more stable (Fig. 6E). To test whether the ability of Pin1 to alter SRC-3 turnover requires phosphorylation of SRC-3, we examined the effect of the over-expression of Pin1 on an SRC-3 mutant in which all six previously identified phosphorylation sites are mutated to Ala (SRC-3A1-6) (48). Flag-SRC-3A1-6 was co-transfected with increasing concentrations of Pin1 into HeLa cells. Although this mutant can still interact with Pin1 its level is unaffected by increasing levels of Pin1 (Fig. 6F). These data support our contention that Pin1 modulates SRC-3 turnover in a manner that requires phosphorylation of SRC-3, and in fact, may be required for its proteasome-mediated degradation.

Discussion:

Protein phosphorylation and dephosphorylation are important mechanisms regulating the activities of nuclear receptors and cofactors in response to extracellular signals. While phosphorylation of CBP by ERK1 increases its HAT activity (1), phosphorylation of ARA55 by Pyk2 decreases its activity by reducing its interaction with AR (44). Although, it is not entirely clear as to how phosphorylation regulates coactivator activities, it is suspected that phosphorylation induces conformational changes that provide surface binding sites for other proteins. Pin1 has been found to regulate post-phosphorylation events of a number of its target proteins by catalyzing the *cis* and *trans* isomerization of pSer/Thr-Pro bonds. Given that SRC-3 contains several phosphorylated Ser/Thr-Pro motifs (48) and SRC family coactivators are central to the recruitment of multiprotein coactivator complexes to promoters, we tested whether Pin1 is involved in the regulation of SRC-3 function.

We provide evidence here that Pin1 is a novel 'secondary' coactivator for steroid receptors. It synergizes with SRC-3 in the coactivation of steroid receptors (Fig 3) and depletion of Pin1 reduces hormone-dependent steroid receptor activity (Fig. 4). Pin1 binds SRC-3 in a phosphorylation-dependent manner and we found that phosphorylation of SRC-3 along with the cellular concentration of Pin1 is important for SRC-3 turnover. Indeed, the half-life of SRC-3 protein was significantly increased when Pin1 levels were reduced and the altered turnover is dependent on both SRC-3 phosphorylation and the prolyl isomerase activity of Pin1 (Fig. 6).

We also provide evidence that Pin1 promotes the formation of SRC-3 and CBP/p300 coactivator complexes as Pin1 enhances the functional interaction between SRC-3 and CBP/p300 in mammalian two hybrid assays (Fig. 5A). In addition, Pin1 also increases SRC-3 and CBP/p300 interaction in an *in vitro* purified system (Fig. 5B), supporting the idea that Pin1 may have a direct effect on SRC-3. This effect is reflected by decreased recruitment of p300 to the estrogen receptor targeted promoter in a ChIP assay (Fig. 5D), explaining in part the down-regulation of pS2 transcription by Pin1 depletion (Fig. 4).

We found that Pin1 interacts with several regions in SRC-3, using both *in vitro* GST pull-down and *in vivo* mammalian two hybrid analyses (Fig.2) and the interaction appears to be phospho-specific because phosphatase treatment of SRC-3 results in the loss of Pin1 binding

(Fig. 1B). We conclude from our studies that Pin1 may interact with multiple phosphor-Ser/Thr-Pro motifs in SRC-3. This idea has precedent as Pin1 interacts with the 7 amino acid repeats that constitute the CTD of RNA Pol II. In this case, phosphorylation of Ser 2 and Ser 5 of a single motif provides the highest Pin1 binding avidity and each repeat is capable of binding Pin1 (52). Although not formally proven, it is believed that Pin1 binding alters the conformation of the CTD. The mitotic phosphatase cdc25c also contains a SP rich region whose conformation is changed upon Pin1 binding (37) and, depending on the kinase that phosphorylates cdc25c, Pin1 can alter the activity of cdc25c in different ways (37). c-Myc is also a Pin1 binding protein and Pin1 binds to a form of c-Myc that is doubly phosphorylated on Thr 58 and Ser 62 (53). In this case, Pin1 binding increases the activity of c-Myc as a transcription factor but also facilitates the dephosphorylation of Ser 62 by PP2A, which is required for the ubiquitination and degradation of c-Myc. Thus, Pin1 apparently positively influences the activity and turnover of c-Myc.

Our data reveal that Pin1 also has multiple effects on SRC-3. On one hand, Pin1 activates SRC-3 mediated steroid receptor transactivation and promotes the interaction between SRC-3 and CBP/p300. On the other hand, it enhances the turnover of SRC-3. Given the multiple sites of interaction between Pin1 and SRC-3, we suggest that Pin1 could regulate different aspects of SRC-3 functions by interacting with different sites. However, based on our data, we can not exclude the possibility that Pin1 affects SRC-3 protein stability indirectly.

The question arises as to how the effects of Pin1 on both SRC-3 turnover and its interaction with p300 contribute to overall transcriptional activation? While promotion of the SRC-3 and CBP/p300 interaction no doubt will enhance transcription, ample evidence indicates protein turnover may also directly contribute to transcriptional activation. Protein turnover has been shown to be tightly coupled to its transcriptional potential (25, 32). Cyclic turnover of ER was shown to be inherently linked to transcription (25, 32, 36). During the course of transcriptional regulation, coactivators are ubiquitinated and subjected to turnover (25, 32, 36). Proteasome subunits have been found to be associated with actively transcribed chromatin. Similarly, retinoic acid induces increased p300 HAT activity while it destabilizes the protein (6). It is likely that the Pin1 mediated enhancement of SRC-3 and p300 interaction and activity is sufficient to overcome any short-term decrease in SRC-3 protein level.

It is clear that phosphorylation of SRC-3 regulates its transcriptional coactivation function (12, 48). We showed recently that different cellular signals can induce different

combinations of phosphorylation sites in SRC-3, which in turn differentially regulate the function of SRC-3 in coactivating transcription factors downstream of diverse signaling pathways (48). Evidence suggests that phosphorylated SRC-3 needs to be either dephosphorylated or degraded so that SRC-3 can properly function when new signals arise. We have observed that SRC-3 phosphomutants can be neither activated nor degraded and that activation of this oncogene is closely coupled with its degradation. Importantly, we observed herein that Pin1 is ineffective in regulating the turnover of phosphomutant SRC-3. Thus, it is entirely logical that Pin1 binds to the phosphorylated form of SRC-3, enhancing its function initially, and then promoting its subsequent degradation. Based on this, we proposed a model on how Pin1 regulates SRC-3 function (Fig. 7). By this mechanism, potent coactivators such as SRC-3 can quickly respond to changes in environmental signals but activated SRC-3/AIB1 does not survive sufficiently long to promote unwanted enhancement of oncogenic functions. The role of Pin 1 in this series of reactions emphasizes the critical requirements for precise conformational interactions among coactivators in NR-mediated transcriptional regulation. Pin 1 serves as an important 'secondary' coactivator for steroid receptors that functions in the post-phosphorylation regulation of primary coactivators to achieve these requisite functional conformations.

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Figure legends:

Fig.1. Pin1 interacts with phosphorylated SRC-3. (A) *in vitro* interaction of SRC-3 and Pin1. HeLa cell extracts were subjected to GST pull-down assay and immunoblotted for SRC-3 (upper panel). The lower panel shows the coomassie staining of GST-Pin1, GST-Pin1W34A and GST. (B) Phosphorylation is required for SRC-3 to interact with Pin1. Flag-SRC-3 was immunoprecipitated from transfected HeLa cell extracts, treated with λ -phosphatase or heat inactivated λ -phosphatase, and then eluted from the antibody. The eluted SRC-3 was used for GST-Pin1 pull-down assay. (C, D) *in vivo* interaction of SRC-3 and Pin1. (C) Mammalian two hybrid assay. HeLa cells were co-transfected with 100 ng of plasmids expressing Gal4DBD-Pin1, VP16AD-SRC-3 and the Gal4 binding reporter luciferase. (D) Co-immunoprecipitation. The cell lysates from SRC-3 inducible expressed Flp-In T-Rex 293 cells were immunoprecipitated with anti-Pin1 antibody or normal IgG and western blotted for anti-flag.

Fig.2 The Pin1 interacting regions in SRC-3. (A) The schematic representation of SRC-3 domains. (B) *in vitro* interaction of Pin1 and SRC-3 fragments. A series of ^{35}S -labeled *in vitro* transcribed and translated SRC-3 fragments were used in GST-Pin1 pull-down assay and were detected by autoradiograph. (C) Mammalian two hybrid assay. The same procedure as described in Fig.1.

Fig.3 Pin1 and SRC-3 activate steroid receptor transactivation synergistically. (A) HeLa cells were co-transfected with vectors expressing PRB, SRC-3, Pin1 or Pin1 mutants and PRE-luciferase reporter in the absence and presence of 10^{-8} M progesterone. (B-E) HeLa cells were transfected with expressing vectors containing SRC-3, Pin1, various transcription factors and the corresponding reporters. (B) PRE-luciferase, and the co-expression of PRB in the presence of 10^{-8} M progesterone. (C) ERE-luciferase, and the co-expression of ER α in the presence of 10^{-7} M estradiol. (D) AP1-luciferase and the co-expression of c-jun, c-fos. (E) cAMP-response element-luciferase, stimulated by the co-transfection of the vector expressing PKA catalytic subunit.

Fig. 4 Depletion of Pin1 impairs the steroid receptor targeted transcription (A) Knock-down of Pin1 reduces transient transfected PR target gene transcription. HeLa cells were transfected with Pin1 siRNA or scrambled control siRNA and PRB, SRC-3 expressing vectors, PRE-Luciferase in the absence or presence of 10^{-8} M progesterone. Cell lysates were subjected to Western blot analysis for Pin1 and β -actin antibodies (upper panel) and the reporter luciferase activities were measured (lower panel). (B) Knock-down of Pin1 decreases endogenous ER target gene transcription. MCF-7 cells were transfected with Pin1 siRNA and stimulated with 10^{-8} M estradiol. Cell lysates were used in Western blot (upper panel). Total RNA extracted from cells was subjected to real time quantitative RT-PCR using pS2 mRNA specific primers (lower panel). The pS2 mRNA level was normalized against the cyclophilin mRNA level (n=4, *p<0.05).

Fig. 5 Pin1 enhances the interaction of SRC-3 and CBP/p300. (A) Pin1 increases the functional interaction between SRC-3 and CBP in a mammalian two hybrid assay. (B, C) Pin1 increases *in vitro* interaction between SRC-3 and p300. Recombinant SRC-3 purified from baculovirus was immunoprecipitated by SRC-3 antibody, incubated with increasing concentrations of Pin1 (B) or Pin1 mutants (C), followed by the addition of purified recombinant p300 as described in Materials and Methods. The SRC-3 associated p300 was detected by Western blot analysis. (D) Depletion of Pin1 reduces the recruitment of p300 to pS2 promoter in response to estrogen stimulation. MCF-7 cells treated with ethanol or 10^{-8} M estradiol were subjected to ChIP analysis using ER α (*p<0.05), SRC-3 (**p=0.436) and p300 (**p<0.001) antibodies (n=5). The protein bound pS2 promoter DNA was quantitated using real time quantitative PCR (lower panel). The arrows (upper panel) indicate the pS2 promoter specific primers used in the PCR.

Fig. 6 Pin1 alters the steady state SRC-3 protein level. (A) Western blot analysis of transfected flag-SRC-3 in HeLa cells using flag antibody with the co-transfection of Pin1 or empty vector in the absence or presence of 10 μ M MG132 for 24 h. The middle panel shows the shorter exposure of the chemiluminescence for the upper panel. (B) RT-PCR analysis on flag-SRC-3 and GAPDH mRNA from transfected HeLa cells with the co-transfection of empty vector, Pin1 or Pin1 C113A. (C) PPIase activity of Pin1 is required for the destabilization of SRC-3. Western blot analysis of flag-SRC-3 from inducible flag-SRC-3 stable Flp-In T-Rex 293 cells with the co-transfection of increasing concentrations of HA-Pin1 or HA-Pin1C113A expression plasmids

was performed using flag, β -actin and HA antibodies. (D) Over-expression of Pin1 rescues the effect of Pin1 siRNA on SRC-3 protein level. HeLa cells were transfected with scrambled control and Pin1 siRNA followed by the transfection of Pin1 (lane 2 and 4) or empty vector (lane 1 and 3). Cell lysates were subjected to Western blot analysis using flag, HA and Pin1 antibodies. (E) Depletion of Pin1 increases the half-life of SRC-3. MCF-7 cells were transfected with Pin1 siRNA, stimulated with estradiol and treated with 200 μ g/ml cycloheximide for 0, 2 and 4 h. Cell lysates were subjected to Western blot analysis using SRC-3 antibody (left panel). The quantitation of SRC-3 protein levels normalized against β -actin during the course of cycloheximide treatment was shown in the right panel (n=4). (F) Pin1 does not affect SRC-3A1-6 phosphomutant protein level. HeLa cells were transfected with flag-SRC-3A1-6 and increasing concentrations of Pin1. Cell lysates were subjected to Western blot analysis.

Fig. 7 Model for Pin1 regulation on SRC-3 function. Extracellular signals induce the phosphorylation of SRC-3. Pin1 serves as a “secondary” coactivator by binding to the phosphorylated SRC-3 and inducing its conformational change. This conformational change increases the interaction between SRC-3 and CBP/p300, thereby enhancing steroid receptor (SR) mediated transcription. Ultimately, Pin1 promotes the degradation of the phosphorylated SRC-3.

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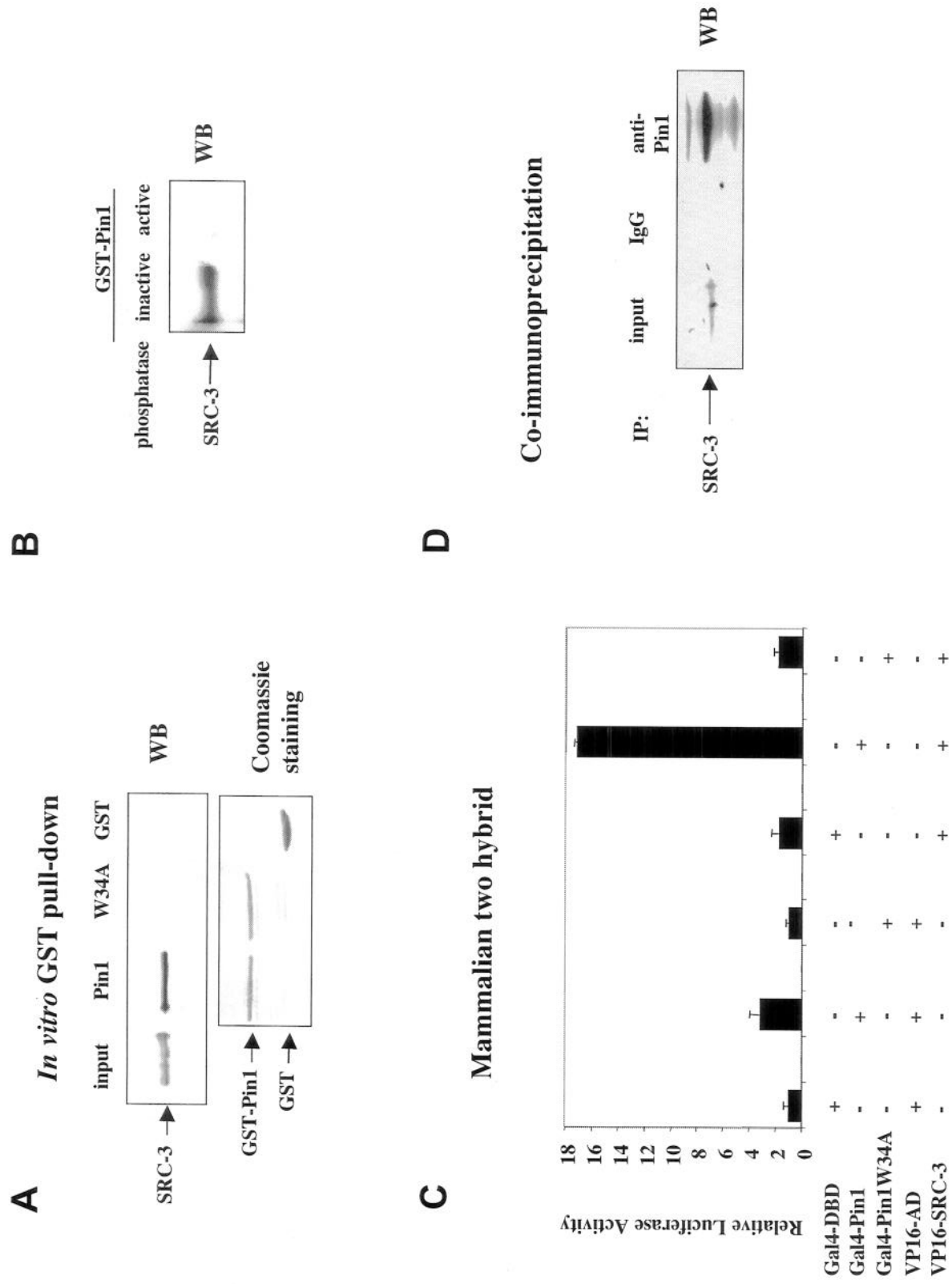


Figure 1

	1	320	580	840	1080	1417
SRC-3	bHLH/PAS	S/T	RID	CID	HAT	

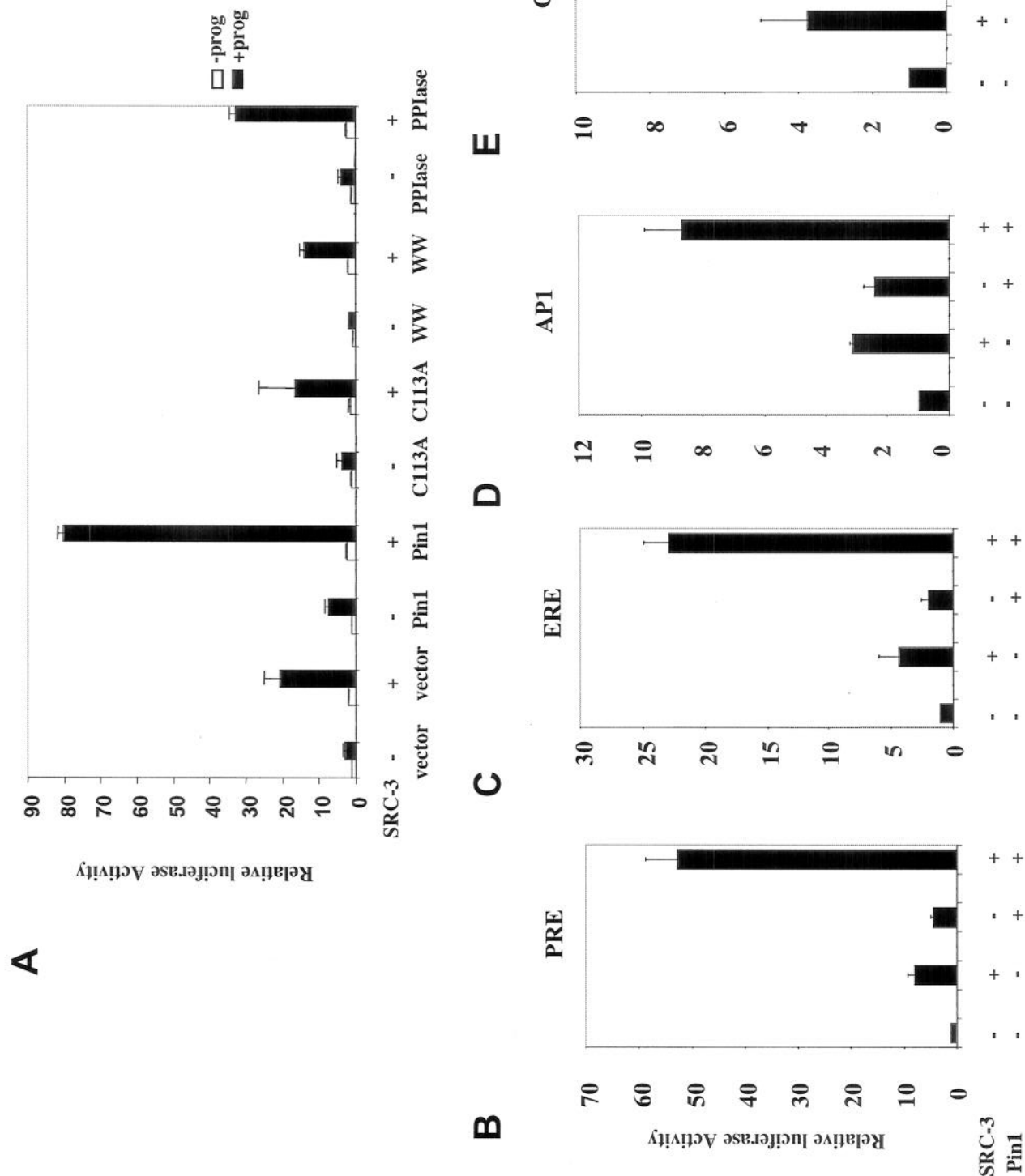
Protein Fragment	input	GST-Pin1
1-321	+	-
321-581	+	-
582-840	+	+
841-1080	+	-
1081-1417	+	-

	WT	1-580	581-830	831-1080	1081-1417
VP16-SRC-3	-	-	-	-	-
VP16-AD	+	+	+	+	+
Gal4-Pin1	-	-	-	-	-
Gal4-DBD	+	+	+	+	+

	WT	1-580	581-830	831-1080	1081-1417
Relative Luciferase Activity	~15	~15	~15	~15	~15
VP16-AD	~15	~15	~15	~15	~15
Gal4-Pin1	~15	~15	~15	~15	~15
Gal4-DBD	~15	~15	~15	~15	~15

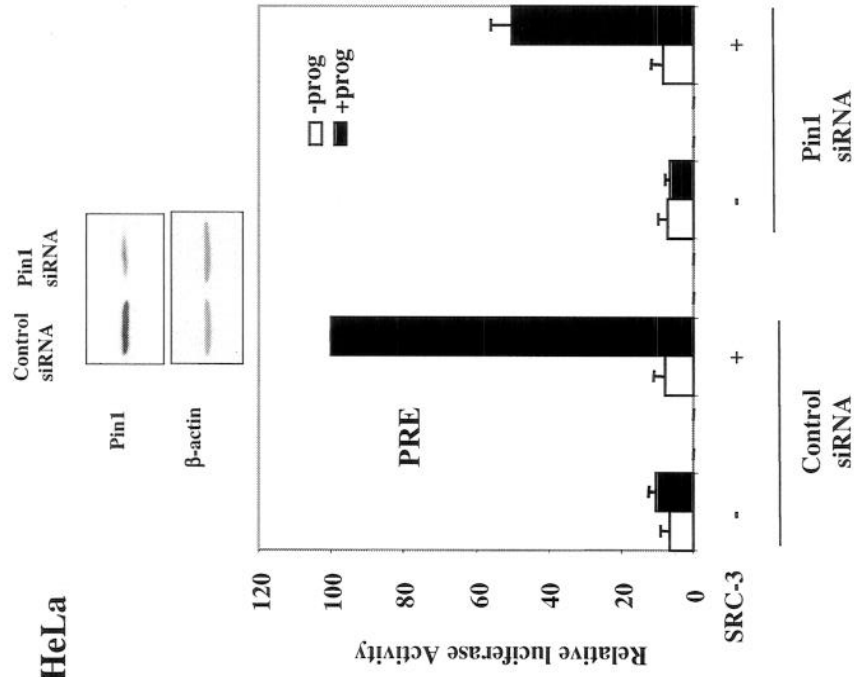
Figure 2

Figure 3



A

HeLa



B

MCF-7

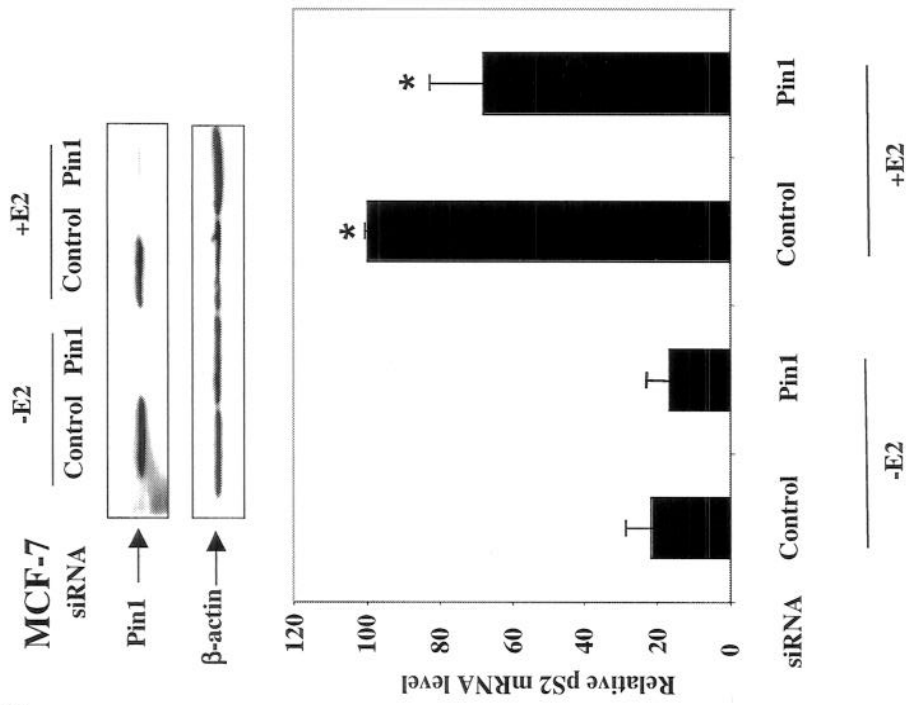


Figure 4

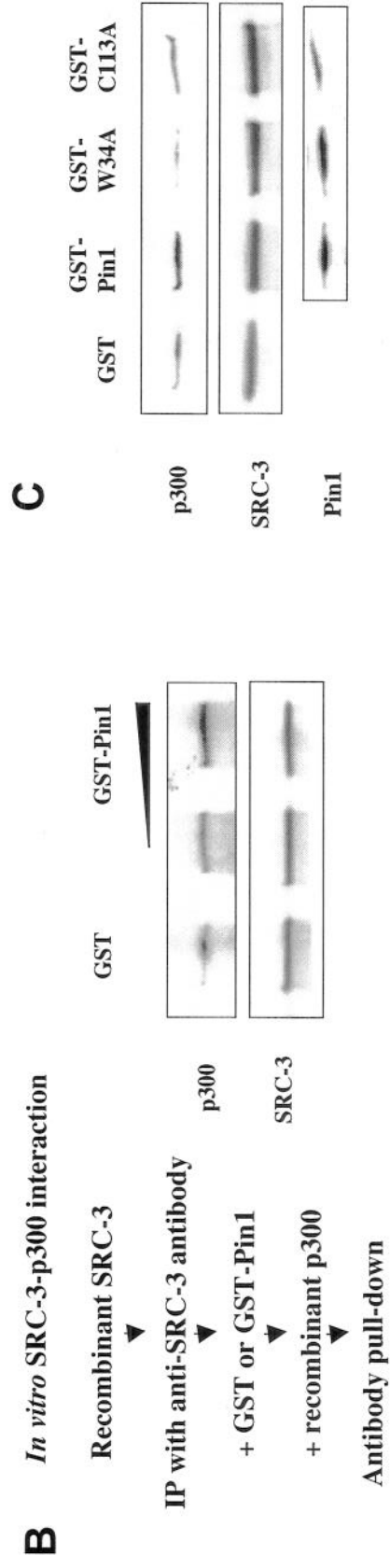
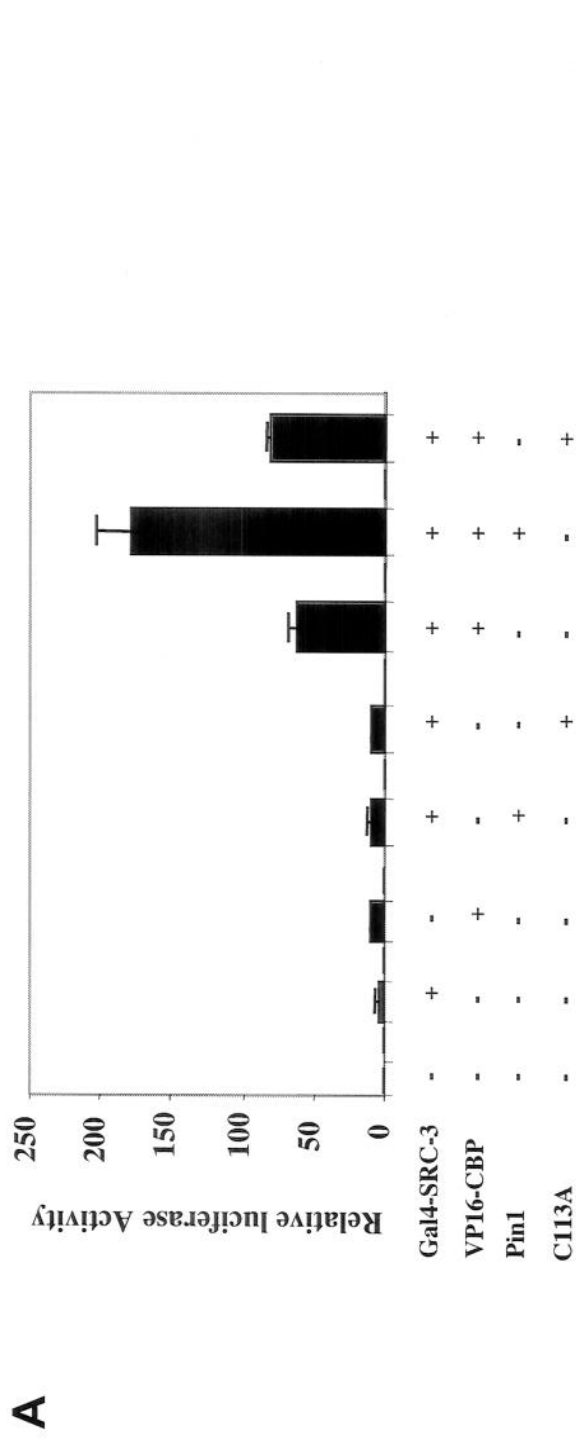


Figure 5

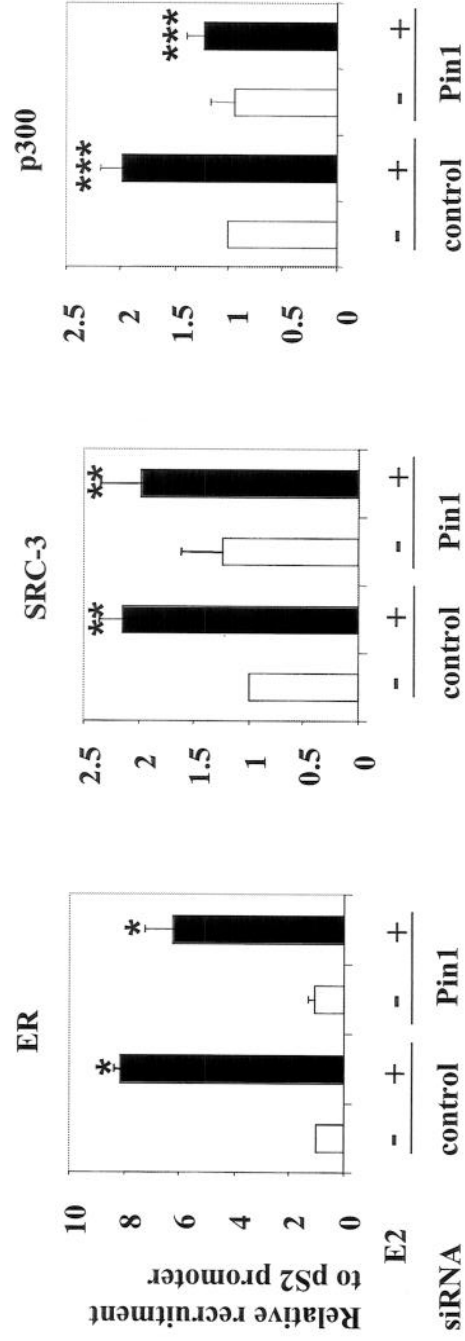
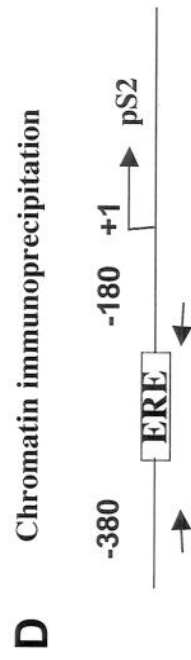


Figure 5

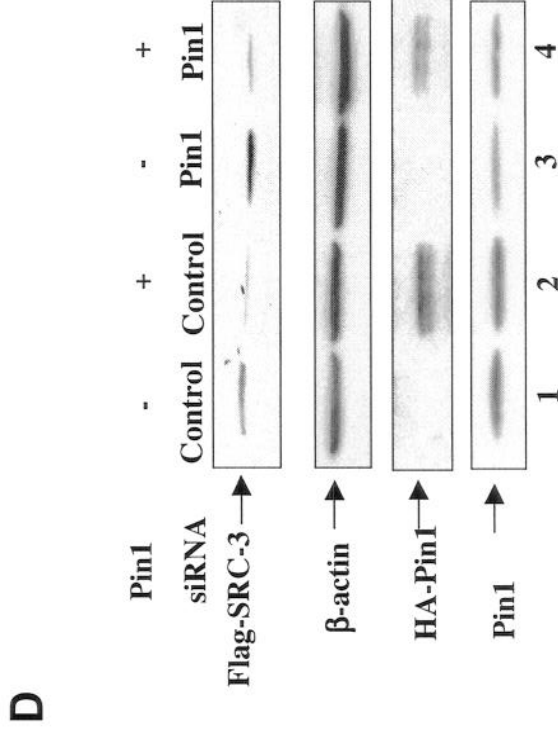
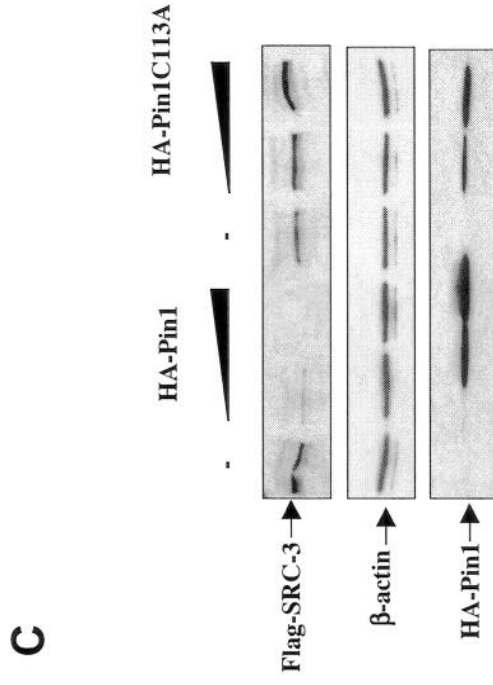
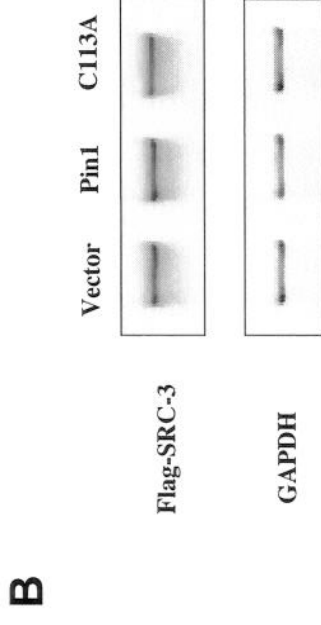
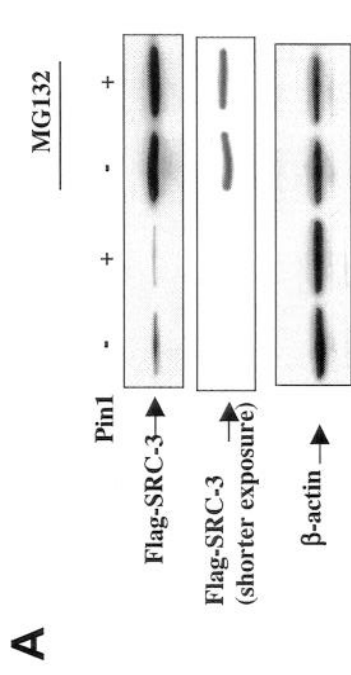
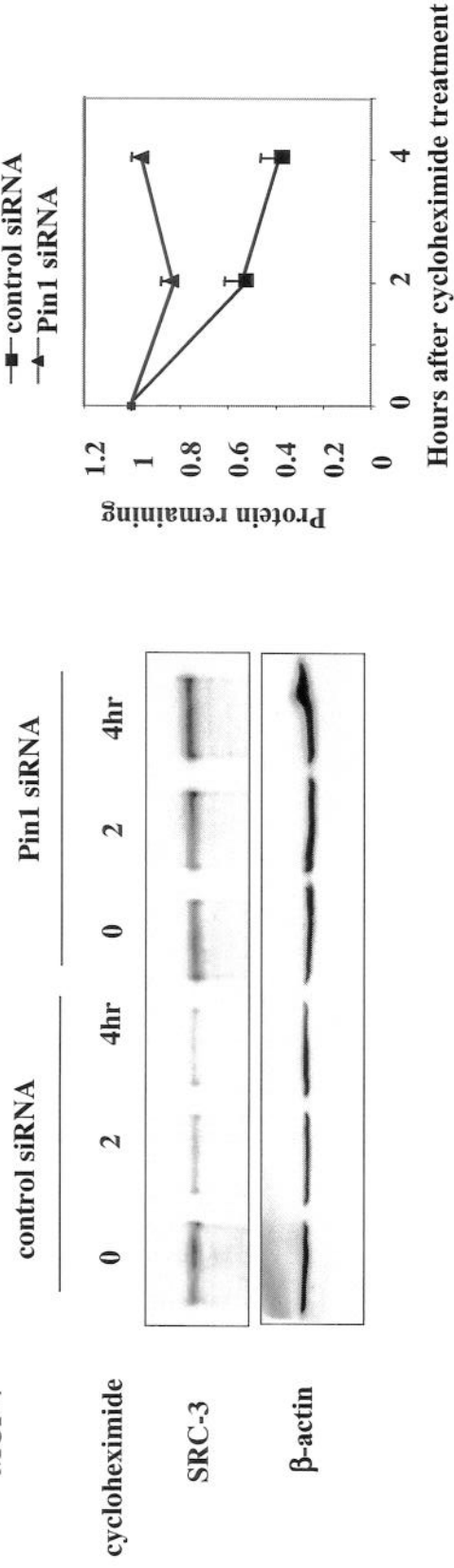


Figure 6

MCF-7

E



F

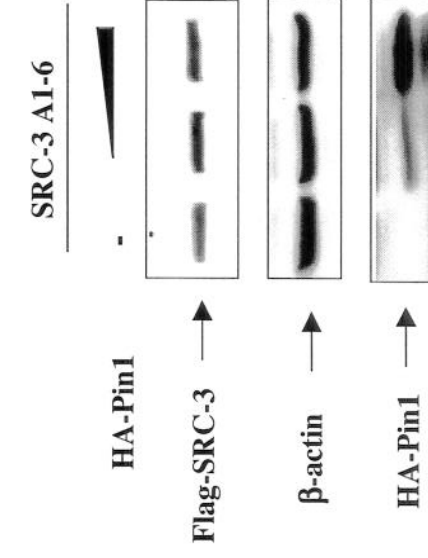


Figure 6

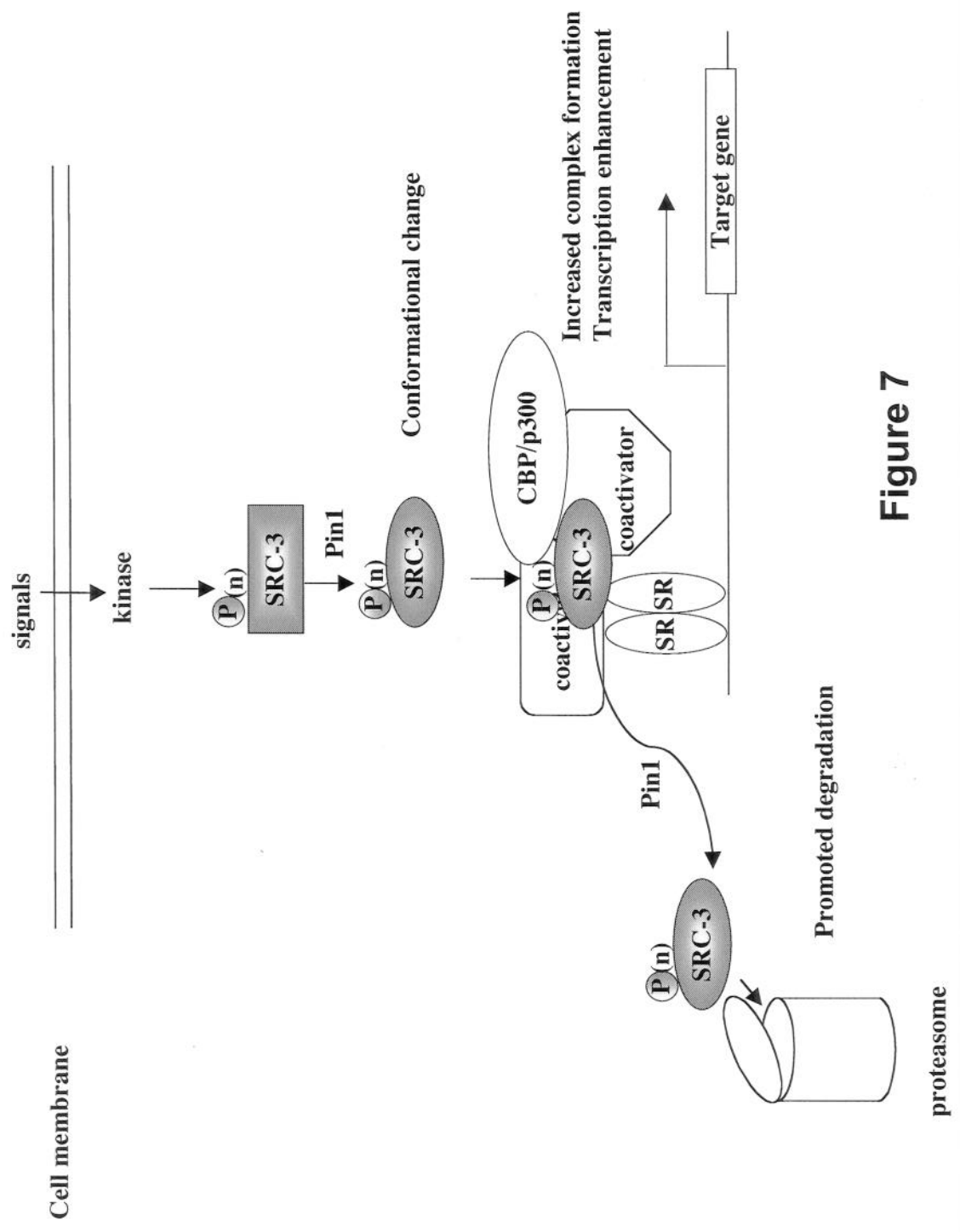


Figure 7