Global gene repression by the steroid receptor coactivator SRC-1 promotes oncogenesis

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Abstract

Transcriptional control is the major determinant of cell fate. The steroid receptor coactivator SRC-1 enhances the activity the estrogen receptor in breast cancer cells where it confers cell survival benefits. Here we report that a global analysis of SRC-1 target genes suggested that SRC-1 also mediates transcriptional repression in breast cancer cells. Combined SRC-1 and HOXC11 ChIPseq analysis identified the differentiation marker, CD24, and the apoptotic protein, PAWR, as direct SRC-1/HOXC11 suppression targets. Reduced expression of both CD24 and PAWR was associated with disease progression in breast cancer patients, and their expression was suppressed in metastatic tissues. Investigations in endocrine-resistant breast cancer cell lines and SRC-1-/PyMT mice confirmed a role for SRC-1 and HOXC11 in downregulation of CD24 and PAWR. Through bioinformatic analysis and LC mass spectrometry, we identified AP1 proteins and Jumonji 2C (JMD2C/KDM4C), respectively, as members of the SRC-1 interactome responsible for transcriptional repression. Our findings deepen understanding of how SRC-1 controls transcription in breast cancers.
Introduction

The steroid receptor coactivator protein SRC-1 (NCoA1) was first identified in a yeast two-hybrid system as an enhancer of the progesterone receptor (1), with a role as a global nuclear receptor coactivator quickly being established thereafter. More recently, a steroid-independent role for SRC-1 has been recognized and the coactivator has been shown to interact with other transcription factors including members of the Ets family, Ets2 and PEA3 (2,3) and the developmental protein HOXC11 (4). SRC-1, a member of the p160 family of steroid coactivator proteins, is a master transcriptional regulator. It was recently identified as the gene with the strongest selective pressure among ethnic populations analysed by the International HapMap Project (5), illustrating the importance of the coactivator protein in human evolutionary adaptation.

There is now substantial evidence that SRC-1 is central to the ability of endocrine tumors to adapt and overcome targeted therapy (4,6,7). Though other members of the p160 family including SRC-2 (GRIP1/TIF2/NCoA2) and SRC-3 (AIB1/ACTR/RAC-3/pCIP/TRAM-1/NCoA3) have been investigated in endocrine related cancer (8), a specific role for SRC-1 in the development of metastatic disease has been described (9,10). Global analysis of SRC-1 targets in breast cancer cells has identified pathways involved in growth factor signaling and cellular adaptability (11). Furthermore, molecular and in vivo studies have provided evidence that SRC-1 promotes disease progression and the development of metastasis, through the transcriptional activation of tumorigenic genes including integrin-alpha5, ADAM22 and Myc (3, 7, 12).

All of the p160 family members have been described as transcriptional coactivators. However, SRC-2 has a somewhat amphipathic role in transcriptional regulation, working as both a trans-activator and repressor of the glucocorticoid receptor (GR) depending on the transcriptional context (13,14). This dual regulatory role for SRC-2 opens up the possibility that SRC-1 could also suppress transcriptional activity to fulfill its oncogenic potential.

In this study we used global analysis to identify a set of SRC-1 suppression targets, a number of which are co-regulated by HOXC11. We investigated the mechanism of SRC-1 transcriptional repression of two of these targets, CD24 and PAWR. In a similar manner to SRC-2, SRC-1 utilized AP1 sites to identify suppression targets, which were then silenced through combined recruitment of HDAC proteins and histone methylation. Direct SRC-1 suppression targets included genes important in apoptosis and cellular differentiation and both CD24 and PAWR were found to be markers of prolonged disease free
survival in breast cancer patients. This study describes a novel mechanism of coregulatory gene repression and adds another layer of complexity to the known functions of SRC-1.

Materials and Methods

Cell culture, treatments and transfections

Endocrine-sensitive MCF7 cells were obtained from American Type Culture Collection and endocrine-resistant LY2 cells were a kind gift from R. Clarke, Georgetown University, Washington DC (15) and have been classified as luminal B (16). Cells were grown as previously described (17). Cells were maintained in steroid-depleted medium for 72 hours before treatment with hormones [estradiol (E2), 10-8 mol/L; 4-hydroxytamoxifen (4-OHT), 10-7 mol/L; Sigma-Aldrich] over varying time periods. All cell lines were tested (Source Biosciences, Life Sciences) for authenticity in accordance with American Type Culture Collection guidelines.

siRNA directed against SRC-1 (Ambion, AM16706), HOXC11 (Qiagen, Hs_HOXC11_2) and JMJD2C (Santa Cruz, sc-92765) were used to transiently knock down gene expression. The pcDNA3.1 and pcDest47 (both Invitrogen) plasmids containing full-length SRC-1 and HOXC11, respectively, were used for over-expression studies. Empty plasmids were used as a negative control. Transfections were carried out using Lipofectamine 2000 (Invitrogen) as per manufacturer’s instructions. Experiments were carried out 72 hours after transfection.

Endocrine resistant LY2 SRC-1 stable knockdown cells were made using MISSION® shRNA lentiviral pLKO.1-puro-CMV-tGFP particles (Sigma). LY2 cells were transduced (multiplicity of infection of 5) with either SRC-1 shRNA or shRNA control particles and cultured in 1ng/ml puromycin to select transduced cells. Stable knock down of SRC-1 was confirmed by qRT-PCR and cells were continuously cultured in 0.5 ng/ml Puromycin.

ChIP sequencing

To identify SRC-1 target genes, chromatin immunoprecipitation (ChIP)-seq was conducted in endocrine-resistant LY2 cells that were treated with vehicle or tamoxifen (4-OHT, 10⁻⁷M) for 45 minutes and immunoprecipitated with anti-SRC-1 (sc-8995; Santa Cruz) and anti-HOXC11 (Ab-37844; AbCam) antibodies as previously described (11). To identify functionally relevant SRC-1 target genes, DNA was hybridized to whole genome expression arrays as previously described (11). Overlapping peaks between the SRC-1 ChIP-seq data set and the HOXC11 ChIP-seq data set were identified from ChIP sequencing of
SRC-1 immunoprecipitated DNA with a false discovery rate (FDR) of <1 and the identified peaks from the HOXC11 immunoprecipitated DNA with a false discovery rate (FDR) of <10. Genes downregulated by SRC-1 were submitted to DAVID bioinformatic database and arranged by functional annotation (11). Hypergeometric testing as implemented in R through the “phyper” function was used to calculate the p-value significance between the SRC-1 ChIP data and SRC-1 down regulated gene from the expression array data.

Accession codes. All ChIP-seq and expression array data are available from the Gene Expression Omnibus (GEO) database under series entry codes GSE28987 (SRC-1 ChIP-seq), GSE54027 (HOXC11 ChIP-seq) and GSE28645 (SRC-1 expression array) respectively.

LC-Mass spectrometry assay

MCF7 and LY2 cells were treated with 4-OHT (10^{-7}M) for 45 minutes. Cells were harvested and lysed. Nuclear protein extracts were combined and resolved by SDS–PAGE. Peptides were analyzed by LC–MS/MS on a Bruker HCT Ultra ion-trap system. The.mgf files were then searched using the X!Tandem search algorithm against the human SWISS-PROT protein database. Proteins identified via this search were checked for peptides which had E-values that did not exceed -3. MS spectra were then examined to identify the corresponding peak doublets.

Co-immunoprecipitation and Western blotting

Western blotting was carried out as previously described (3). Primary antibodies used were rabbit anti-human PAWR (1:1000, 2328 Cell Signaling), rabbit anti-human SRC-1 (1:100, sc-8995, Santa Cruz), rabbit anti-human HOXC11 (1:200, sc-788, Santa Cruz), or b-actin (1:7500; Sigma-Aldrich). Protein was immunoprecipitated with rabbit anti–SRC-1. Briefly, protein AG beads were blocked overnight in BSA at 4°C and beads were subsequently washed with protease inhibitors. IgG or SRC-1 antibodies along with protein lysate was added to the beads. Following incubation, protein bound beads were isolated and prepared for immunoblot for SRC-1, c-Jun, JMJD2C and HDAC1. Primary antibodies used were rabbit anti-human SRC-1 (1:100, sc-8995, Santa Cruz), rabbit anti-human c-Jun (1:2000, sc-44, Santa Cruz), rabbit anti-human JMJD2C (1:2000, NB110-38884, Novus Biologicals), rabbit anti-human HDAC1 (1:2000, ab7028, Abcam).
Knockout mouse

Knockout (KO) and wild-type (WT) mammary tumor cell lines were developed from primary tumors in SRC-1-/-/PyMT and WT/PyMT mice as described in the work of Xu and colleagues (9).

Chromatin immunoprecipitation assay and PCR

LY2 cells were steroid depleted for 72 hours then treated with vehicle or 4OHT for 45 minutes, and chromatin immunoprecipitation (ChIP) analysis was carried out as previously described (4). Cell lysates were quantified after shearing using a Nanodrop (Thermo Scientific) to ensure equal starting material in each sample. The following antibodies were incubated overnight at 4°C with rotation: 3μg rabbit anti-human SRC-1 (sc-8995, Santa Cruz), rabbit anti-human c-Jun (sc-44, Santa Cruz), 3μg rabbit anti-human JMJD2C (NB110-38884, Novus Biologicals), 3μg rabbit anti-human HDAC1 (ab7028, Abcam), rabbit anti-human H3K4 trimethylation (ab8580, Abcam), 3μg mouse anti-human H3K27 (ab6002, Abcam), 3μg rabbit anti-human Anti-Histone H3K36 (ab9050, Abcam) 3μg rabbit immunoglobulin G (IgG) or 3μg mouse IgG (sc-2343, Santa Cruz) as a negative control and 3.5μL antiacetylated H4 (Millipore) as a positive control. Reverse cross-linking and DNA recovery was carried out with Chelex 100 (Bio-Rad). Real time PCR was performed in duplicate by SYBR Green PCR (Qiagen) using the 7500 Real Time PCR system (Applied Biosystems). The primers for qRT-PCR were as follows: CD24 promoter: forward TCAGGATAAGTGGTAAAGAG, reverse GAGATCGGAGACCATCTCTGG and the PAWR promoter: forward CTAGCAGCTTCGGGCGGCTCC, reverse GAAGGGCGTGGAGGTGCGTT. For ChiP of gene body methylation the following primer sequences were used: CD24 gene body: forward TAGTAGATTCCGATGAAC, reverse TATGAGATCGTGGAGGT and the PAWR gene body: forward CAGCCAGATGGAAGTGAAT and reverse CTCACCCTAAGCCTCGT. For measuring transcript levels the primers used were CD24 forward primer: AACTAATGCCACCACCAAGG, reverse: CCTTGTTTTTCCTTGCCACAT. PAWR forward primer: ACATCCCTGCCGCCAGACTG, reverse primer: TCTCGTTTTCCGCTTTCTG.
equally among the experimental samples. The cells were then stained with FITC conjugated-CD44 and PE conjugated-CD24 (555478, 555428 BD Biosciences) for 1 hour in the dark at 4°C. Unstained sample and PE and FITC isotype controls were used as experimental controls.

**Immunohistochemistry**

Patient breast tumor samples were collected the tissue microarray (TMA) constructed and data recorded as previously described (18). Primary tumors from the knockout mouse were formalin fixed and paraffin embedded. The TMA and full faces of knockout mouse tumors were immunostained using rabbit anti-PAWR (2 μg/mL, 2328 Cell signaling) and mouse anti-CD24. Immunostained slides were called using the Allred scoring system (18).

**Statistics**

Associations of CD24 and PAWR with clinicopathologic variables and SRC-1 were examined using Fisher exact test. Kaplan–Meier graphs were used as an estimate of disease-free survival. Statistical analyses were conducted using STATA 10 data analysis software (Stata Corp. LP) and GraphPad Prism 6 (GraphPad software Inc, CA) and values of p < 0.05 were considered significant. Statistical significance for molecular experiments was performed using a paired Student’s t-test, with significance accepted at p<0.05.

**Results**

**SRC-1 directly down regulates genes in endocrine resistant breast cancer.**

Increased intensity of SRC-1 binding events was observed in endocrine resistant breast cancer cells in comparison to sensitive cells, in particular in the presence of the SERM tamoxifen (Fig.1A). Combining expression array and ChIP-seq analysis we identified 1,061 genes directly repressed by SRC-1. The number of genes down regulated by SRC1 were found to have more SRC1 peaks in the promoter/first exon than expected by chance (p-value = 3.111317e-06 ) (Fig. 1B). Pathway analysis of SRC-1 repressed genes revealed an over-representation of pathways important in tumor suppression, including apoptosis and cell differentiation (Fig. 1C). Grouping direct SRC-1 suppression targets by function identified CD24 as a gene important in both cell surface signal transduction and Wnt signaling processes (Fig. 1D).
**SRC-1 and HOXC11 are recruited to the promoters of luminal markers CD24 and PAWR.**

Enhanced recruitment of SRC-1 to the CD24 promoter was observed in the presence of tamoxifen (Fig. 2A). Furthermore multiple HOXC binding sites, capable of binding the defined SRC-1 transcription partner, HOXC11 were identified up-stream of the CD24 start-site (Fig 2B). In order to identify further common SRC-1/HOXC11 targets we performed HOXC11 ChIP-seq in luminal B LY2 cells. In a similar manner to SRC-1, enhanced HOXC11 genome binding was observed in the presence of tamoxifen (Supplementary Table S1). Recruitment of HOXC11 was found to be mainly to introns and distal intergenic regions, with relatively little binding to promoter regions (Fig. 2C). 54 genes were identified as common SRC-1/HOXC11 targets, 32 of which were protein coding genes (Fig. 2D and Supplementary Table S2). The pro-apoptotic protein PAWR was identified amongst the top 5 common targets (Supplementary Table S2). Consistent with the ChIP-seq studies, multiple HOXC family motifs were located in the PAWR promoter (Fig. 2E). Treatment induced recruitment of both SRC-1 and HOXC11 to the CD24 and PAWR promoters was confirmed in ChIP studies (Fig. 2F, G).

**CD24 and PAWR in endocrine treated breast cancer patients.**

In a cohort of breast cancer patients, expression of CD24 and PAWR was found to inversely associate with SRC-1 and positively associate with luminal A status (Supplementary Table S3). In line with the defined role of SRC-1 in promoting increased tumor aggression, the luminal marker CD24 was found to associate with increased time to disease progression in the total patient population, in luminal patients and in luminal A patients (Fig. 3A and Supplementary Fig. S1). In primary cell cultures derived from ER positive tumors, elevated levels of CD24 expression also associated with luminal A status (Fig. 3B). In a similar manner, the pro-apoptotic marker PAWR also associated with prolonged disease-free survival (Fig 3C). Furthermore, in endocrine resistant luminal A patients who did express CD24 and PAWR in their primary tumor, expression of both the luminal marker and the apoptotic protein were lost in the metastatic setting (Fig 3D and Supplementary Table S4). Conversely, expression of SRC-1 was gained in the metastatic tumors. These clinical studies support a role for CD24 and PAWR in advancing a good prognosis and suggest that loss of these proteins may have a role in promoting a more aggressive phenotype.
SRC-1 and HOXC11 regulate CD24 and PAWR.

Over-expression and knockdown studies in luminal B breast cancer (LY2) cells with subsequent transcript analysis confirmed a role for SRC-1 and its transcriptional partner HOXC11 in the transcriptional suppression of CD24 (Fig. 4A) and PAWR (Fig 4B). Knockdown of SRC-1 enhanced protein expression of CD24 (Fig. 4C), whereas knockdown of either SRC-1 or HOXC11 restored PAWR expression in luminal B breast cancer cells (Fig. 4D). Furthermore, in the PyMT SRC-1 knockout mouse model (SRC-1⁻/⁻PyMT), elevated levels of CD24 and PAWR transcript levels (Fig. 4E) and protein expression (Fig. 4F and Supplementary Fig. S2) were observed in tumor tissue from the SRC-1⁻/⁻ in comparison to the wild type animals. These data confirm a role for SRC-1 in the transcriptional repression of CD24 and PAWR.

SRC-1 uses AP-1 binding sites to identify candidates for transcriptional silencing.

As SRC-1 is a known transcriptional activator we wanted to investigate how the coregulatory protein marked target genes for transcriptional repression. Using bioinformatic studies we undertook motif analysis of the SRC-1 cistrome. Similar to SRC-2 down-regulated genes, AP1 sites were observed to be over-represented in the promoters of SRC-1 repressed genes in comparison to SRC-1 activated genes (Supplementary Table S5). 4-OHT treatment-induced interactions between SRC-1 and the AP-1 protein Jun were observed in LY2 breast cancer cells (Fig. 5A and Supplementary Fig. S3), this is due at least in part to increased levels of SRC-1 in the presence of 4-OHT. Recruitment of Jun to both the CD24 and PAWR promoters was confirmed by ChIP analysis (Fig. 5B).

SRC-1 utilizes JMJD2C to silence CD24 and PAWR.

To explore potential mechanisms of SRC-1 transcriptional repression we undertook LC-mass spectrometry of SRC-1 interacting proteins in 4-OHT treated (45 mins) luminal B (LY2) and luminal A (MCF7) breast cancer cells. 126 proteins were identified as specific SRC-1 interacting proteins in the luminal B cells (Supplementary Table S6). Arranging proteins by functional annotation we identified jumonji domain containing 2C (JMJD2C/KDM4AC) as a potential transcriptional regulator (Fig. 6A). JMJD2 proteins are demethylases that target histone H3 on lysines 9 and 36 (Reviewed extensively in 19). Immunoprecipitation confirmed treatment regulated SRC-1/JMJD2C interactions in LY2 breast cancer cells (Supplementary Fig S4). 4-OHT treatment also induced recruitment of JMJD2C to the CD24
and PAWR promoters (Fig. 6B). Furthermore, knockdown of JMJD2C in LY2 cells induced a concomitant increase in expression of both CD24 and PAWR (Fig. 6C).

We looked at epigenetic marks in the promoter and gene body of CD24 and PAWR (Fig. 6D). Previous studies have reported that both AP1 proteins and JMJD2C can induce transcriptional silencing by recruiting the histone deacetylase protein, HDAC1 (20,21). In this study treatment with tamoxifen was observed to induce SRC-1/HDAC1 interactions in LY2 cells (Fig. 6E) and recruitment of HDAC1 to the PAWR promoter was found to be SRC-1 dependant (Fig. 6F and Supplementary Fig. S5).

To further elucidate the mechanism of SRC-1 mediated transcriptional repression we examined the role of SRC-1 and JMJD2C in promoter histone methylation. Histone 3 lysine 27 (H3K27) trimethylation, which is a known mark of transcriptional silencing, was diminished with SRC-1 knockdown in LY2 breast cancer cells at both the CD24 and PAWR promoters (Fig. 6G). There was no significant alteration in H3K27 trimethylation seen with JMJD2C knockdown (Supplementary Fig. S6A). Conversely, no alteration was observed in SRC-1 dependant H3K4 trimethylation, a known activation mark, at either of the promoters (Supplementary Fig. S6B).

In the gene body, H3K36 trimethylation facilitates transcription through elongation (22,23). JMJD2C has previously been shown to demethylate H3K36, leading to transcriptional activation (24). In this study, knockdown of JMJD2C was found to activate H3K36 gene methylation in the gene body of CD24, and to a lesser extent PAWR (Fig 6H). These data implicate JMJD2C in SRC-1 mediated transcriptional repression in breast cancer cells.

Taken together these functional studies suggest that SRC-1 can select genes for repression by utilizing AP1 transcription factors and can induce transcriptional repression through both histone deacetylation and JMJD2C mediated histone methylation mechanisms (Fig. 7).

Discussion

SRC-1 functions at several levels to regulate gene expression including transcription initiation, elongation, RNA splicing, receptor turn over and translation. Its predominant role however is as a transcriptional coactivator. In breast cancer, over-expression of SRC-1 confers on the cell the ability to adapt and overcome targeted therapy by up-regulating developmental and de-differentiation genes (7,11). Challenging the classic role of SRC-1 as a transcriptional activator, global analysis of direct SRC-1 targets described here, uncovered a significant number of repressed genes. Pathway analysis revealed
these genes to be important in cellular differentiation and apoptosis, which is consistent with SRC-1’s role in promoting an aggressive phenotype.

In previous studies we have identified HOXC11 as a transcription factor partner for SRC-1 which can activate genes to mediate increased tumor plasticity and adaptation to the therapeutic environment (4). Here we identified the differentiation marker CD24 and the apoptotic protein PAWR as direct SRC1/HOXC11 suppression targets. CD24 has been identified as a transcriptional target gene for HIF1α and has been linked to HIF1α mediated tumor growth in both primary and metastatic tumors (25). Functions of CD24 however may depend on the cellular context, in particular with regard to endocrine cancer. Both CD24 and PAWR have documented roles as endocrine tumor suppressors (26-29). Moreover, loss of CD24 and PAWR are associated with poor differentiation and tumor progression (29,30). Establishing the functional consequences of SRC-1 target gene expression, CD24 and PAWR were found to inversely associate with SRC-1 in primary and metastatic tumors from breast cancer patients. Furthermore, where SRC-1 associates with poor disease free survival in patients (2,7), CD24 and PAWR were found to be markers of good prognosis. The fact that SRC-1 silences these functionally relevant targets is of prime importance when considering the role of SRC-1 as a transcriptional co-regulator. Not only does SRC-1 promote expression of oncogenes such as Myc (7), it can simultaneously work to suppress expression of genes which are striving to maintain a well differentiated phenotype.

As there is now evidence that SRC-1/HOXC11 can both promote and repress gene expression, the question therefore arises as to how SRC-1 can mark some genes for activation and others for repression. Clues may be resident in the SRC-2-GR pairing. SRC-2 serves as a GR coactivator at palindromic GREs, however when SRC-2 is tethered to GR-AP1 sites and GR-NF-kappaB sites it mediates transcriptional repression (31, 32). From motif analysis of the SRC-1 cistrome we found AP-1 sites to be over-represented in the promoters of SRC-1 repressed genes in comparison to activated genes. Furthermore, treatment induced SRC-1-jun interactions and recruitment of jun to the promoter of both CD24 and PAWR were confirmed, suggesting that SRC-1 can regulate gene expression in a promoter specific manner similar to that of SRC-2 (14).

To elucidate potential mechanisms of SRC-1 mediated repression we investigated putative proteins of the SRC-1 interactome. A number of SRC-1 interacting proteins specific to the luminal B breast cancer sub-type were defined. Several transcriptional regulators were identified including the histone demethylase, jumonji domain containing 2C (JMJD2C). Both AP1 proteins and JMJD2C have been shown previously to induce transcriptional repression through recruitment of the histone
deacetylating protein HDAC1 (20, 21). Molecular studies confirmed that SRC-1 can mediate transcriptional repression, at least in part, by facilitating histone deacetylation.

JMJD2C has previously been associated with endocrine tumors, including breast and prostate (33-36). It has to date predominantly been described as a transcriptional activator, specifically through lysine demethylation (37,38). A role for JMJD2C in transcriptional repression however has also been elucidated, where activation of the nuclear receptor PPAR γ is suppressed by the tudor domain of JMJD2C (20). Due to the substantial number of studies describing a role for JMJD2C in modulating histone methylation, we examined the methylation status of both CD24 and PAWR. Histone H3 lysine 27 trimethylation, a mark of transcriptional repression, was found to be SRC-1 dependent at both promoters, where no alteration in the activation mark, histone H3 lysine 4 trimethylation was observed. JMJD2C has previously been shown to demethylate gene body H3K36 thereby inhibiting transcriptional elongation (19). Here, JMJD2C was found to demethylate H3K36 in the gene body of CD24. Taken together these mechanistic studies suggest that SRC-1 achieves transcriptional silencing through a combination of histone deacetylation and histone methylation, which are mediated at least in part through JMJD2C.

In summary, here we have described for the first time that the coactivator protein SRC-1 can act as a transcriptional repressor and have proposed a model whereby SRC-1 can select candidate genes for repression or activation in a promoter specific context. SRC-1’s ability to bi-directionally regulate key genes can drive functional change and enforce its end phenotype. This novel mechanism of coregulatory gene repression significantly alters our perception of SRC-1 in the molecular pathogenesis of cancer.

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Figure Legends

Figure 1. SRC-1 directly down regulates genes in endocrine resistant breast cancer

(A) Heat map showing increased intensity of SRC-1 binding events in treated endocrine resistant breast cancer (LY2) cells compared to untreated resistant cells and endocrine sensitive (MC7) cells. The window represents ±5kb regions from the centre of the binding events.

(B) Venn diagram of cross over between SRC-1 cistrome and SRC-1 dependant gene suppression defined 1,007 SRC-1-dependant repressed genes. The venn diagram shows the number of genes from the set of Affymetrix hgu133plus2 genes that were downregulated by SRC1 (2,047, p-value <0.05) and that had an SRC1 ChIP-seq peak in the promoter (5Kbp upstream) or first exon (8,867 with a MACS FDR <1%). 1007 genes overlapped these two conditions. The overlap was found to be significant (p =3.111317e-06).

(C) Pathway analysis of direct SRC-1 repressed genes revealed a number of pathways being suppressed by SRC-1. An over representation of apoptotic and cell differentiation pathways following treatment was seen.

(D) The top 50 SRC-1 down regulated genes were submitted to the DAVID bioinformatics database and arranged by functional annotation. The top 7 functional groupings are represented. CD24 is represented in ‘cell surface receptor linked signal transduction’ and the ‘Wnt receptor signaling pathway’.
Figure 2. SRC-1 and HOXC11 are recruited to the promoters of luminal markers CD24 and PAWR

(A) Representative image of SRC-1 recruitment to the CD24 promoter displayed in the UCSC genome browser. A comparison is made between peaks under control conditions and following 4-OHT treatment, with 4-OHT treatment inducing a larger SRC-1 binding peak.

(B) Illustration of HOXC family binding motifs in the CD24 promoter as identified using Genomatrix.

(C) Location of HOXC11 binding sites seen in LY2 cells on tamoxifen treatment.

(D) Venn diagram of cross over between the HOXC11 and the SRC-1 cistrome defined 54 genes as common targets. 32 of these were for known protein coding regions.

(E) Illustration of HOXC family binding motifs in the PAWR promoter as identified using Genomatrix.

(F) Representative image of SRC-1 and HOXC11 recruitment to the PAWR promoter displayed in the UCSC genome browser under control conditions and following 4-OHT treatment.

(G) ChIP analysis of SRC-1 and HOXC11 recruitment to the CD24 and PAWR promoters in LY2 cells following treatment with either vehicle or 4-OHT. Treatment with 4-OHT lead to increased recruitment of SRC-1 and HOXC11 to the CD24 (*p=0.04 and *p=0.03) and PAWR (**p=0.007 and (*p=0.05) promoters respectively. Recruitment calculated relative to untreated samples (mean± SEM, n=3).

Figure 3. CD24 and PAWR in endocrine treated breast cancer patients

(A) Immunolocalisation of CD24 in breast cancer patient TMA. Kaplan Meier estimates of disease-free survival (DFS) according to CD24 in luminal breast cancer patients (n=243). CD24 positive patients have significantly improved 5 year survival compared to CD24 negative patients (p<0.0039).

(B) Significantly lower levels of CD24 protein were detected in patient luminal B cells in comparison to luminal A cells (p=0.03; n=3 patient tumors/group) by FACS analysis.
(C) Immunolocalisation of PAWR in total breast cancer patient population (n=560). PAWR positive patients have significantly improved 5 year survival compared to PAWR negative patients (p=0.01).

(D) CD24 and PAWR protein levels are reduced in metastatic tissue in comparison to matched breast cancer primary tissue from three SRC-1 negative luminal A patients. Tissue from three individual patients shown.

Figure 4. SRC-1 and HOXC11 regulate CD24 and PAWR

(A) Transcript levels of CD24 and PAWR as analysed by qRT-PCR following over-expression of SRC-1 using the pc3.1 vector in MCF7 cells and knock down of SRC-1 with siRNA in LY2 cells. There is a significant decrease in both CD24 and PAWR expression following over-expression of SRC-1 (*p=0.04 and *p=0.05, respectively). When SRC-1 is knocked down, there is a significant corresponding increase in CD24 and PAWR mRNA expression (*p=0.02 and *p=0.01 respectively). Results are mean ± SD, n=3.

(B) Transcript levels of CD24 and PAWR as analysed by qRT-PCR following over-expression of HOXC11 using the Dest47 vector in MCF7 cells and knock down of HOXC11 with siRNA in LY2 cells. There is a significant decrease in both CD24 and PAWR expression following over-expression of HOXC11 (*p=0.005 and *p=0.01 respectively). When HOXC11 is knocked down, there is a significant corresponding increase in CD24 and PAWR mRNA expression (*p=0.03 and *p=0.001 respectively). Results are mean ± SEM, n=3.

(C) Cell surface protein expression of CD24 in LY2 cells as measured by flow cytometry. Following SRC-1 knockdown there is a restoration of CD24 cell surface expression in LY2 cells.

(D) PAWR protein levels following knockdown of SRC-1 and HOXC11 with siRNA in LY2 cells. Knock down of both SRC-1 and HOXC11 leads to re-expression of PAWR protein (n=3).

(E) Elevated levels of CD24 and PAWR transcript, as determined by qRT-PCR was detected in mammary tumors from SRC-1−/PyMTmice in comparison to wild-type mice (WT/PyMTmice), (*p=0.019 and *p=0.02; n=4 mice tumors/group, results for qRT-PCR expressed as mean ± SEM).

(F) Immunohistochemistry was used to compare CD24 and PAWR expression in tumors from SRC-1−/PyMTmice and WT/PyMT mice. Elevated levels of CD24 and PAWR were found in the SRC-1−/− mouse (n=4, representative images shown).
Figure 5. SRC-1 uses AP-1 binding sites to identify candidates for transcriptional silencing

(A) SRC-1 was immunoprecipitated from whole cell extract from LY2 cells treated with vehicle or 4-OHT for 45 minutes and subsequently immunoblotted for Jun. Treatment with 4-OHT drives SRC-1-Jun interactions, representative blot of three independent experiments.

(B) Confirmation of the recruitment of the AP-1 protein Jun to the CD24 and PAWR promoters using ChIP analysis. LY2 were treated with 4-OHT (45 mins, $10^{-7}$M). Jun ChIP was performed to the CD24 and PAWR promoters. Mean recruitment calculated relative to input controls (p=0.001 and p=0.009 respectively; mean± SEM, n=3).

Figure 6. SRC-1 utilises JMJD2C to silence CD24 and PAWR

(A) LC-mass spectrometry was used to identify endocrine resistant specific SRC-1 interacting proteins. Endocrine sensitive (MCF7) and endocrine resistant (LY2) breast cancer cells were treated with 4-OHT (45 mins, $10^{-7}$M) and were immunoprecipitated with anti-SRC-1. SRC-1 interacting proteins were separated on a one dimensional gel and the resultant lanes were analysed by LC mass spectrometry. 126 proteins were identified as unique to the endocrine resistant phenotype. Proteins were arranged by functional annotation and Jumonji domain containing 2C (KDM4AC/JMJD2C) was identified as a potential transcriptional regulator.

(B) Confirmation of JMJD2C recruitment to the CD24 and PAWR promoters with ChIP analysis. Elevated recruitment of JMJD2C to both the CD24 and PAWR promoters was observed following treatment with 4-OHT (45 mins, $10^{-7}$M) (p=0.005 and 0.009). Mean recruitment calculated relative to untreated controls (mean± SEM, n=3).

(C) Elevated transcript levels of CD24 and PAWR as determined by q-RT-PCR following knockdown of JMJD2C using siRNA (*p=0.01 and *p=0.001). mRNA levels expressed as mean ± SEM, n=3.

(D) Schematic representing location for ChIP qPCR of HDAC1 (repression mark), H3K27me3 (repression mark) and H3K4me3 (activation mark) in the promoters of CD24 and PAWR (P) and for H3K36me3 (elongation mark) in the gene body of CD24 and PAWR (GB).

(E) SRC-1 was immunoprecipitated from LY2 cells treated with vehicle or 4-OHT (45 mins, $10^{-7}$M) and subsequently immunoblotted for JMJD2C and HDAC1. Treatment with 4-OHT drives SRC-1 interactions with JMJD2C and HDAC1, representative blot of three independent experiments.
(F) Mechanism of suppression of PAWR: there is a significant reduction in HDAC1 recruitment to the PAWR promoter in LY2 cells stably transfected with shSRC-1. Mean recruitment calculated relative to non-targeting controls (*p=0.01; mean± SEM, n=3).

(G) There is a decrease in H3K27 trimethylation at the CD24 and PAWR promoters (*p=0.05 and *p=0.04, respectively) in LY2 cells stably transfected with shSRC-1. Mean recruitment calculated relative to the non-targeting control cells (mean± SEM, n=3).

(H) There is an increase in H3K36 trimethylation in the CD24 gene body in LY2 cells stably transfected with siJMJD2C (*p=0.045). Alterations in H3K36 trimethylation in the PAWR gene body were not found to be significant. Mean recruitment calculated relative to the scrambled siRNA control cells (mean± SEM, n=3).

**Figure 7. Putative SRC-1 interactome**

(A) Schematic illustrating the putative SRC-1 interactome regulating transcriptional repression of target genes.
Figure 1

**A**

Heatmap distribution of SRC-1-Chipseq data sets

**B**

Affymetrix DNA Microarray
- 2047
- Chip-sequencing peaks 8867

**C**

Pathways associated with SRC-1 downregulated genes

- Apoptosis
- Mitogenic Signalling
- Immune System Response
- Inflammatory response
- DNA damage response
- Cell differentiation
- Cystic fibrosis disease
- Vascular development
- Tissue remodelling and wound repair
- Cardiac hypertrophy
- Cell cycle and its regulation

**D**

Network diagram of SRC-1 down-regulated genes

- Transcription regulators
- DNA damage response pathways
- Mitogenic signalling pathways
Figure 2

A) Vehicle treated LY2 vs. Tamoxifen treated LY2

B) 5Kb Promoter
Forward Strand HOXC binding sites
Reverse Strand HOXC binding sites
CD24

C) SRC-1 recruitment HOXC11 recruitment

D) SRC-1 ChIPseq peaks
HOXC11 ChIPseq peaks
51,563 54 1,515

E) CHR12
5Kb Promoter
Forward Strand HOXC binding sites
Reverse Strand HOXC binding sites
PAWR

F) SRC-1 recruitment
HOXC11 recruitment

G) SRC-1 recruitment to the CD24 promoter
HOXC11 recruitment to the CD24 promoter
SRC-1 recruitment to the PAWR promoter
HOXC11 recruitment to the PAWR promoter

Relative recruitment

Figure 3

A. CD24 expression in patient primary cultures

Survival Analysis of CD24 in a Luminal population

B. CD24 expression in patient primary cultures

C. PAWR expression in clinical patient population

Survival analysis of PAWR in a clinical patient population

D. Recurrence of disease in clinical patient population

Recurrence
**Figure 4**

**A** SRC-1 inhibits CD24 mRNA expression

**B** HOXC11 inhibits CD24 mRNA expression

**C** HOXC11 inhibits PAWR mRNA expression

**D** HOXC11 inhibits PAWR mRNA expression

**E** SRC-1 inhibits CD24 mRNA expression

**F** CD24 and PAWR expression in wild-type and SRC-1 -/- tumors.
Figure 5

A

SRC-1 c-Jun co-immunoprecipitation

<table>
<thead>
<tr>
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<th>Vehicle</th>
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<tr>
<td>c-Jun</td>
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B

c-Jun recruitment to CD24 and PAWR promoters

![Graph showing relative recruitment to CD24 and PAWR promoters](image)
Figure 6

A

Recruitment of JMJD2C to CD24 and PAWR promoters

B

JMJD2C regulates CD24 and PAWR mRNA expression

C

Recruitment of HDAC1 to PAWR promoter

D

H3K27 trimethylation at CD24 promoter

E

H3K36 trimethylation in the CD24 gene body

F

H3K27 trimethylation at PAWR promoter

G

H3K36 trimethylation in the PAWR gene body
Figure 7
Global gene repression by the steroid receptor coactivator SRC-1 promotes oncogenesis

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