SRC3 phosphorylation at Serine 543 is a positive independent
prognostic factor in ER positive breast cancer

Running Title: SRC3-pS543 in ER positive breast cancer

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Abstract

Purpose:
The steroid receptor coactivator SRC3 is essential for the transcriptional activity of oestrogen receptor α. SRC3 is sufficient to cause mammary tumourigenesis, and has also been implicated in endocrine resistance. SRC3 is posttranslationally modified by phosphorylation, but these events have not been investigated with regard to functionality or disease association. Here we investigate the spatial selectivity of SRC3-pS543/DNA binding over the human genome and its expression in primary human BC in relation with outcome.

Experimental Design
Chromatin Immunoprecipitation, coupled with sequencing (ChIP-seq) was used to determine the chromatin binding patterns of SRC3-pS543 in the BC cell line MCF-7 and two untreated primary breast cancers. Immunohistochemistry was used to assess the expression of SRC3 and SRC3-pS543 in 1650 primary breast cancers. The relationship between the expression of SRC3 and SRC3-pS543, disease free survival (DFS) and breast cancer specific survival (BCSS) was assessed.

Results
While total SRC3 is selectively found at enhancer regions, SRC3-pS543 is recruited to promoters of ERα responsive genes, both in the MCF-7 cell line and primary breast tumour specimens. SRC3-pS543 was associated with
both improved DFS (p=0.003) and BCSS (p=0.001) in tamoxifen untreated high-risk patients, such a correlation was not seen in tamoxifen-treated cases, the interaction was statistically significant (p=0.001). Multivariate analysis showed SRC3-pS543 to be an independent prognostic factor.

Conclusion

Phosphorylation of SRC3 at S543 affects its genomic interactions on a genome-wide level, where SRC3-pS543 is selectively recruited to promoters of ERα-responsive genes. SRC3-pS543 is a prognostic marker, and a predictive marker of response to endocrine therapy.
Translational Relevance
SRC3 is an essential co-activator for the transcriptional activity of ERα and is required for estrogen-dependent cell proliferation. Analogous to ERα, SRC3 is typically found at enhancer regions involved in long-range regulation of hormonal-responsive genes. In this study we establish the genome-wide chromatin binding preferences for activated SRC3 (SRC3-pS543) in ERα positive breast cancer (BC), as well as the association between SRC3-pS543 expression and clinico-pathological features and clinical outcomes in 1650 primary BCs. Our findings reveal that while ERα and total SRC3 are predominately found in distal enhancers and introns, SRC3-pS543 is predominately located at promoters of genes. SRC3-pS543 was associated with favourable clinic-pathological features, and was found to be an independent prognostic factor as well as a predictive marker with regard to tamoxifen treatment. These observations illustrate that high expression of SRC3-pS543 can potentially identify a population of early ER positive breast cancer with a good clinical outcome without receiving adjuvant therapy.
Introduction

Oestrogen receptor alpha (ERα) is a key transcription factor in normal breast development and plays a central role in the pathogenesis of ~70% of all breast cancers. Consequently, ERα is recognised as the key therapeutic target in this group of patients (1). The p160 steroid receptor co-activator (SRC) family member, SRC3 (also known as amplified in breast cancer 1; AIB1) is an essential co-activator for the transcriptional activity of ERα, which recruits SRC3 to chromatin (2). SRC3 is required for estrogen-driven proliferation of MCF-7 breast cancer cells (3) and is critical for normal mammary development (3). Overexpression of SRC3 can induce mammary tumorigenesis in mice (4), while its absence protects against breast cancer development (5,6). SRC3 was originally identified as a gene mapping to a region of chromosome 20 (20q12-13), which is frequently amplified in breast cancer and was shown to be amplified in 10% of breast cancers (7). SRC3 mRNA levels are significantly higher in breast cancer as compared to normal mammary tissue (8,9) and SRC3 protein levels are elevated in 16–83% (depending on the study) of human breast tumours (10).

SRC3 expression has been associated with decreased risk of relapse in breast cancer patients who did not receive adjuvant endocrine therapy (11). Conversely, in tamoxifen-treated patients SRC3 expression was associated with poor outcome and increased risk of relapse (11). Others have found no such association with tamoxifen treatment, although an increased risk was seen with high SRC3 when expressed alongside HER1, HER2 or HER3 (12). In group of women of whom 82% were postmenopausal, SRC3 expression
has been associated with early recurrence on tamoxifen treatment (13), while high SRC3 in pre-menopausal women was an independent predictive factor of improved response to tamoxifen (14). ERα-associated SRC3 is typically found at enhancer regions, where expression of genes proximal to shared ERα/SRC3 chromatin binding events correlates with poor outcome of ERα positive breast cancer patients treated with tamoxifen (15). This feature was only found for genes where SRC3 was selectively enriched as compared to the other two p160 family members, SRC1 and SRC2.

Phosphorylation of SRC3 is required for it to function as a potent transcriptional activator, and SRC3 possesses multiple phosphorylation sites that may be functionally involved in this process. (16,17). Oestradiol treatment increases SRC3 phosphorylation, but the kinase responsible for this remains elusive. However, a number of kinases are known that induce phosphorylation at these residues of SRC3 (16,17). In addition, dephosphorylation of serine-101 and -102 regulates SRC3 protein stability by preventing proteasome-dependent turnover (18). Of note, the C-terminal domain of SRC3 has weak histone acetyltransferase activity raising the possibility that it may have a role in chromatin remodelling (10).

Previous studies have shown a requirement of SRC3 phosphorylation for SRC3 activity, including phosphorylation of SRC3 at serine residue 543 (SRC3-pS543) (16). Furthermore, the region around S543 is evolutionarily conserved in mammals, bird and reptiles, while no sequence homology around S543 is found in SRC1 or SRC2 (19). In addition, thus far no studies explored the genomic consequences and clinical significance of SRC3 phosphorylation. Given all the above, we decided to investigate the chromatin
associations of SRC3 in vitro and in vivo when phosphorylated at serine 543 (SRC3-pS543) together with its clinical significance in ERα positive breast cancer. To separate the prognostic from the predictive potential of SRC3 phosphorylation, tamoxifen-treated patients were compared with patients who did not receive any adjuvant endocrine therapy.

**Material and Methods**

*Cell lines, plasmids and antibodies.*

MCF-7 and CHO cells were from the American Type Culture Collection (ATCC), which utilises short tandem repeat profiling with no authentication being performed by authors. All experiments were performed within 10 passages from the original stock. Cell lines were grown in DMEM with 10% FCS and standard antibiotics. Wild type SRC3 and mutant S543A plasmids kindly provided by BW O’Malley (Baylor College of Medicine). The antibodies used were SRC3 (Immunohistochemistry, BD Transduction Laboratories; 611105), SRC3 (ChIP-seq experiments, SC-9119; Santa Cruz), ERα (SC-543; Santa Cruz). A rabbit polyclonal antiserum for SRC3-pS543 was generated by immunising animals with the peptide 537-LLSTLSPGPKLDN-550, where the residue in bold is phospho-serine (Moravian Biotechnologies, Czech Republic).

*(Chromatin) Immunoprecipitations*

Chromatin immunoprecipitations were performed according to standard protocols (20). Primary breast cancer specimens and proliferation MCF-7 breast cancer cells were used. Tissue was cryosectioned in 30 slices of 30
µm, and subsequently defrosted in solution A (50 mM Hepes, 100 mM NaCl, 1mM EDTA, 0.5 mM EGTA) supplemented with 1% formaldehyde. For MCF-7 cells, formaldehyde was added to the culture medium at a final concentration of 1%. After 10 minutes of fixation, 100 mM Glycine was added, after which cells were washed in PBS in presence of Protease Inhibitor (Roche Diagnostics 11836145001) and Phosphatase Inhibitors (Roche Diagnostics: 04906837001) at concentrations recommended by the supplier. Tissue was homogenized, and nuclei were isolated, washed and sonicated using a Diagenode Bioruptor as described before (21,22). After sonication, the lysate was cleared by centrifugation. For each ChIP 100µl of Dynabeads (Invitrogen) was used, precoupled overnight with 10µg of SRC3 or SRC-pS543 antibody. Immunoprecipitation was performed overnight while rotating at 4 degrees. Subsequently, beads were washed 10 times using RIPA buffer and reverse crosslinked at 65 degrees for 6 hours. Supernatant was removed, cleaned using a phenol/chloroform column and precipitated as described before (20). For standard Immunoprecipitations, beads were resuspended in sample buffer, boiled at 95°C for 5mins and the samples run onto 8% Polyacrylamide gel for protein analysis for S543P, Input SRC3 and Immunoprecipitated (IP) SRC3. Immunoprecipitations were performed in presence of Protease Inhibitor (Roche Diagnostics 11836145001) and Phosphatase Inhibitors (Roche Diagnostics: 04906837001) using standard concentrations recommended by the supplier. When indicated, samples were incubated with Lambda Protein Phosphatase (Millipore; cat # 14-405) for 30 min prior to ChIP (2000 units). Primer sequences for QPCR analysis are in Supplementary Table S1.
For MCF-7 experiments, ~6x10^7 asynchronous proliferating cells were used, and two independent replicates were performed for each ChIP-seq, where only consensus peaks that were found in both experiments were considered. For tumour specimen ChIP-seq, fresh snap-frozen primary ERα-positive, HER2-negative untreated breast cancers were obtained from the Imperial College Healthcare NHS Trust Tissue Bank which has the requisite ethical approval.

**Solexa sequencing and enrichment analysis**

ChIP DNA was amplified as described (20). Sequences were generated by the Illumina Hiseq 2000 genome analyser (using 50 bp reads), and aligned to the Human Reference Genome (assembly hg19, February 2009). Enriched regions of the genome were identified by comparing the ChIP samples to mixed input using the MACS peak caller (23) version 1.3.7.1. Total read count, percentage of aligned reads and number of peaks for each ChIP-seq is shown in Supplementary Table S2. For histone modification data, publically available MCF-7 ChIP-seq data for H3K4me1 (GEO: GSM588568), H3K4me2 (GEO: GSM822392), H3K4me3 (GEO: GSM945269) and H3K27Ac (GEO: GSM945854) was used. For ERα ChIP-seq data, peaks from (24) were used. ChIP-seq data for SRC1, SRC2 and total SRC3 were from (15). For heatmap visualizations, Seqminer software was used.

**Motif analysis, heatmaps and genomic distributions of binding events**

ChIP-seq data snapshots were generated using the Integrative Genome Viewer IGV 2.1 (www.broadinstitute.org/igv/). Motif analyses were performed
through the Cistrome (cistrome.org), applying the SeqPos motif tool (25). For visualization purposes, data were represented in Motif Radar Plots (MRPs). Motifs datafiles were first generated using Galaxy Cistrome with equal numbers of peaks for each subset, randomly downsamples towards the lowest number. All motifs with $p<0.001$ were included in the analysis, corresponding to a lower bound absolute z-score of 3.09. Subsequently, motifs for all different genomic datasets were combined in a single radarplot visualization. The genomic distributions of binding sites were analysed using the cis-regulatory element annotation system (CEAS) (26). The genes closest to the binding site on both strands were analysed. If the binding region is within a gene, CEAS software indicates whether it is in a 5′UTR, 3′UTR, coding exon, or an intron. Promoter is defined as 3 kb upstream from RefSeq 5′ start. If a binding site is $>3$ kb away from the RefSeq transcription start site, it is considered distal intergenic.

**Nottingham Tenovus Primary breast cancer Series**

Primary operable breast cancer cases ($n = 1,650$) from the Nottingham Tenovus Primary Breast Carcinoma Series was utilised as described before (27,28), supplementary table S3. Patients were under the age of 71 years (median, 55 years), diagnosed between 1986 and 1999, and treated uniformly in a single institution. Patients within the good prognosis group (Nottingham Prognostic Index (NPI) $\leq 3.4$) did not receive adjuvant therapy. Premenopausal patients within the moderate (NPI $>3.4$ to $\leq 5.4$) and poor (NPI $>5.4$) prognosis groups were candidates for chemotherapy. Conversely, postmenopausal patients with ERα positive breast cancer with moderate or
poor NPI were offered hormonal therapy, while ERα-negative patients received chemotherapy. Clinical data were maintained on a prospective basis with a median follow-up of 130 months (Range 2-311 months).

**Immunohistochemistry**

Breast cancer tissue microarrays (TMAs) from 1,650 cases were immunostained with SRC3 (Dilution 1:20) and SRC3-pS543 (Dilution: 1:2000) antibodies overnight at room temperature, after antigen retrieval (citrate buffer (pH 6.0) and microwave (20 minutes at 750 Watts)). Negative controls were performed by omission of the primary antibody. Nuclear staining was scored based on H-score. Determination of the optimal cut-offs was performed using X-tile bioinformatics software (Yale University, USA) and according to histogram distribution of H-score. For immunohistochemistry blocking experiments, peptides were used which contained the phospho-moiety (CLLSTLS-phosphoS-PGPKLDN) or the non-phospho peptide (CLLSTLSSPGPKLDN).

The peptides were pre-incubated overnight at 100 fold excess over antibody prior to staining as described previously.

**Statistical analysis**

Data analysis was performed using SPSS (SPSS, version 17 Chicago, IL). Where appropriate, Pearson’s Chi-square, Fisher’s exact, Student’s t and ANOVAs one-way tests were used. Cumulative survival probabilities were estimated using the Kaplan–Meier method and differences between survival rates were tested using the log-rank test. Multivariate analysis for survival was
performed using the Cox hazard model. The proportional hazards assumption was tested using standard log-log plots. Hazard ratios (HR) and 95% confidence intervals (95% CI) were estimated for each variable, with $p < 0.05$ as significant. For multiple comparisons, a stringent $p$ value ($<0.001$) was considered significant.
Results

Specificity of SRC3-pS543 Antibody

An antibody for SRC3-pS543 was generated which recognised wild-type phosphorylated SRC3, but not S543-mutated SRC3 (S543A) (Figure 1A). The SRC3-pS543 antibody detected SRC3 in immunoprecipitates from MCF-7 cell lysates (Figure 1A, B; full size blot supplementary figure 1 and 2), where signal was lost after phosphatase treatment (Figure 1B). Consistent with a previous report (17), we find estrogen treatment to increase S543 phosphorylation in a dose-dependent manner, further validating our SRC3-pS543 antibody (Figure 1C). To ensure the specificity of the SRC3-pS543 antibody by immunohistochemistry, the antibody was incubated with either peptide containing the phospho-moiety or the non-phospho peptide. Subsequently, the pre-incubated antibody (bound to non-phospho-peptide and phospho-peptide) was tested on four tumours with different levels of signal for SRC3-pS543 (negative, low/medium, high), Figure 1D. In addition, phosphatase treatment was applied. While the phospho-peptide strongly decreased pS543-SRC3 signal, this was not observed with the non-phospho peptide. In addition, phosphatase treatment strongly diminished pS543-SRC3 signal in all tested specimens (Figure 1D).

ChIP-seq analysis shows a genomic preference of SRC3-pS543 to bind promoter regions

Next, we determined the genome-wide distributions of this SRC3-pS543 phosphorylation as compared to total SRC3 and ERα in asynchronously proliferating luminal breast cancer cells MCF-7 as exemplified for well-known ERα binding sites proximal to the XBP-1 locus (Figure 2A). For
ERα genomic locations, data from (24) were used, while total SRC3 data was from (15). In total 47,214 ERα binding sites were detected, of which 15,329 were also bound by SRC3 (15) (Figure 2B). Between ERα, SRC3 and SRC3-pS543, 2365 peaks were shared (for raw data visualizations, see Figure 2C). Not all SRC3 binding events were phosphorylated and 2795 peaks for SRC3-pS543 were apparently not shared with total SRC3. However, there was weak total SRC3 signal observed at these positions, which did not reach the detection threshold for peak calling (Supplementary Figure S3A and B).

Based on these results, we conclude that differential binding patterns between SRC3-pS543 and total SRC3 should be considered as ‘enriched’ rather than ‘unique’. P160 family members share substantial sequence homology. Even though the sequence surrounding S543 is not conserved in SRC1 and SRC2, the SRC3-pS543 antibody might still detect the other two p160 members. Therefore, we determined potential overlap of SRC3-pS543 enriched sites with SRC1 and SRC2 in MCF-7 cells (15). The overlap of SRC3-pS543 enriched sites with SRC1 and SRC2 was limited, rendering it unlikely that the SRC3-pS543 enriched sites can be explained merely by cross-reactivity with one of the other p160s (Supplementary Figure S4). Differential chromatin binding between SRC3-pS543 and total SRC3 could be validated by QPCR analyses on asynchronous MCF-7 cells (Supplementary Figure S5A), using four primer sets that were designed for each subset of binding sites, i.e. SRC3-pS543-enriched, total SRC3-enriched and shared sites (Supplementary Table S1 for primer sequences and Supplementary Figure S5B for genome browser snapshots of SRC3 and SRC3-pS543 ChIP-seq signal at these sites). To assess hormone dependency of SRC3-pS543 chromatin
interactions, SRC3-pS543 ChIP-QPCR was performed on cells that were hormone deprived for 3 days, and subsequently treated for 3 hours with vehicle, E2 or tamoxifen (Supplementary Figure S6). SRC3-pS543/chromatin interactions at SRC3-pS543-enriched sites (that were not bound by ERα) were hormone independent and not affected by ligand. For SRC3-pS543 sites shared with total SRC3, no chromatin binding was observed in the absence of hormone and in tamoxifen-treated cells, but this interaction was actively induced by E2 treatment. Phosphatase treatment abrogated SRC3-pS543 enrichment at these sites (Supplementary Figure S6). Genomic distributions of ERα and SRC3 were comparable as reported by others(29) (Supplementary Figure S7). To further illustrate functional interplay between ERα and SRC3-pS543, ERα was depleted from MCF-7 cells using Fulvestrant (Figure S6). SRC3-pS543/ SRC3 shared, but not SRC3-pS543 enriched chromatin interactions were dependent on ERα levels, as shown by Fulvestrant-mediated ERα degradation (Supplementary Figure S8).

In order to identify potential selectivity of transcription factor usage, motif analyses were performed on the sites that were either or not shared between SRC3-pS543 and ERα, and visualized in a Motif Radar Plot (MRP) (Figure 2D). For the shared events, motif enrichment was found for classical luminal breast cancer-selective transcription factors including ESR1 and forkhead motifs. For SRC3-pS543 enriched binding events, No motifs for ESR1 or FOXA1 were enriched, but a clear enrichment was observed for ELK, ELF and ETV motifs (see also Supplementary Table S4 for the total list).

Differential motif enrichment could form the basis of differential gene expression programs. Therefore, GO pathway enrichment was assessed for
genes with an SRC3/chromatin binding event proximal (<20kb) from their transcription start sites. GO terms highly varied for the differential SRC3 subclasses (Supplementary Figure S9), with 1 out of 80 GO terms for SRC3 enriched sites (“biological process”) shared with the other two subsets. Interestingly, the majority of SRC3-pS543/SRC3 shared peak GO terms, 28 out of 37, were overlapping with those found for the SRC3-pS543 enriched sites, including “metabolic process”, “gene expression” and “translation”. For a complete list of all GO terms, see Supplementary Table S5.

Since the SRC3-pS543 chromatin binding events only recapitulated a proportion of the total SRC3 binding events, genomic distributions could deviate as well. As described before (15,30), the majority of ER$\alpha$ and SRC3 binding events are found within enhancers and introns (Figure 2E). In contrast, SRC3-pS543 binding was clearly enriched at promoter regions and 5'UTR, with promoter-bound SRC3-pS543 colocalizing with SRC3, ER$\alpha$ and histone modifications indicative for active gene transcription (H3K4me2, H3K4me3, H3K27Ac) (Supplementary Figure S10). As expected, SRC3 and ER$\alpha$ signal was found centered on the SRC3-pS543 signal. Since transcription factors bind accessible chromatin, histone marks indicative for active promoters (H3K4me3, H3K27Ac) are found around the SRC3-pS543 sites instead of directly centered on them. Interestingly, this was not the case for H3K4me2, which was clearly enriched on the SRC3-pS543 peak. The enhancer-selective histone mark H3K4me1 was not enriched at SRC3-pS543 promoters. Even though less pronounced than the total of SRC3-pS543 peaks, the sites shared between ER$\alpha$ and SRC3-pS543 were also more promoter enriched (13.9%) as compared to ER$\alpha$/SRC3 shared regions.
(4.9%). Furthermore, SRC3-pS543 signal was highly enriched at promoters of E2 regulated genes (31) as compared to all Refseq genes (p<0.0001) (Figure 2F).

**SRC3-pS543 and ERα chromatin binding events in primary tumour tissue**

Next, we determined phosphorylated SRC3/chromatin interactions in primary tumors (Figure 3). ChIP-seq was performed on two independent ER+/PR+/HER2- breast tumour specimens as described (21,24), and directly compared with the MCF-7 cell line data. Peak intensities between the two tumours and the MCF-7 sample were comparable, and peaks are well-conserved (Figure 3A). Between the two tumours and MCF-7 cells, 2670 binding events of SRC3-pS543 were shared (Figure 3B, visualized in a raw heatmap in Figure 3C). Furthermore, peaks were also found selectively enriched for only MCF-7 (2523 sites), tumour #1 (1816 sites) or tumour #2 (4631 sites). The SRC3-pS543 binding sites from all samples were annotated for their genomic distributions, and again enrichment for promoters and 5'UTR regions was found (Figure 3D). This was even more strikingly observed for binding events shared by all three samples, with 52% of all sites being localised to promoters and 20% at 5'UTR.

For tumor #1, the lysate was divided in two prior to immunoprecipitation, and the second half of the lysate was used for ERα ChIP-seq, enabling a direct comparison of SRC3-pS543 and ERα binding patterns within the same tumor (Figure 3E). Between ERα and SRC3-pS543, 1727 binding events were shared, while 15050 binding events were only found for ERα and 5537 binding sites were only found for SRC3-pS543.
(Figure 3F). The shared and enriched binding patterns were not dictated by different thresholds in peak calling algorithms, as shown by heatmap analyses (Figure 3G). The genomic distributions of ERα in the tumour sample closely resemble what was found in the cell line (Figure 2E) and previously reported in breast tumour tissue (21,24), with ∼5% of ERα binding events being found at promoters, with the vast majority of sites located at distal enhancers and introns (Figure 3H). The SRC3-pS543 sites (either or not shared with ERα), were highly enriched at promoters and 5′UTR regions.

Motif analyses for the ERα binding events in the tumour specimen revealed enrichment in ERα, FOXA1 and AP-2 motifs, as expected (30,32) (Figure 3I). Enrichment for these specific motifs was found both for ERα-enriched sites as well as for sites shared between ERα and SRC3-pS543. Furthermore, practically all motifs found at SRC3-pS543 enriched sites were also found at the regions shared by ERα and SRC3-pS543 bounds, including motifs for ELK, ETV and ETS factors as were observed in the MCF7 cells (see Supplementary Table S6 for the total list).

Association of total SRC3 and SRC3-pS543 with clinicopathological features

Total SRC3 and SRC3-pS543 expression was evaluated in 1,650 breast carcinomas, with protein expression predominately nuclear (Supplementary Figure S11). The total SRC3 mean histoscore was 157 (interquartile range, 0-300) and for SRC3-pS543 mean histoscore was 31 (interquartile range, 0-300). For subsequent analysis, total SRC3 expression was categorized as high (above mean, H-score>157) or low (below mean, H-score<157) and for SRC3-pS543 high (above mean, H-score >31) or low (below mean, H-score
A scatterplot analysis of total SRC3 versus SRC3-pS543 in these tumors shows that the relative levels between total SRC3 versus SRC3-pS543 were strongly variable between tumors. Most importantly, a large proportion of tumors were identified that were positive for total SRC3, but negative for SRC3-pS543. Furthermore, no tumors could be identified that were positive for the phospho-SRC3 but negative for total SRC3, again highlighting specificity of the antibody (Supplementary Figure S12).

While no association on IHC between total SRC3 and ERα was found, there was a strong association for SRC3-pS543 with ERα (p=<0.0001) and high expression of ERα-associated proteins (progesterone receptor; p=<0.0001 and Bcl2; p=<0.0001). Total SRC3 expression was significantly associated with HER2 (p=<0.0001) and HER3 (p=0.023), and correlated the absence of basal-like (p=0.021) and triple negative phenotypes (p=0.005). (Table 1). SRC3-pS543 was significantly associated with well-differentiated (p=<0.0001), low proliferative (p=<0.0001) tumours as well as androgen receptor (p=<0.0001) expression. In addition, it was associated with the presence of p53 (p=0.025) and the absence of MDM4 (p<0.001) and MDM2 (p<0.001), as well as the absence of both basal like and triple negative phenotypes (p<0.0001). The absence of SRC3-pS543 was significantly associated with the overexpression of HER4 (p<0.0001), the presence of lympho-vascular invasion (p=<0.0001) and loss of expression of the key DNA repair proteins including BRCA1 (p<0.0001), ATM (p<0.0001) and TOP2A (p<0.0001), (Table 1). Apart from being phosphorylated by estrogen treatment, SRC3-S543 is also phosphorylated by JNK and p38 MAPK (16).
line with these previous observations, we find a significant correlation between the phosphorylated JNK and p38 MAPK with SRC3-pS543 (p<0.0001) (table 1). Additional tumour biomarkers and their correlation with SRC3 and SRC3-pS543 are shown in supplementary table S7.

**Association of total SRC3 and SRC3-pS543 expression with outcome**

With regard to outcome, no association of total SRC3 with outcome was observed in the whole cohort (Supplemental Figure S11A), the ERα positive group in total (Supplemental Figure S11B), or on the basis of tamoxifen treatment (Supplemental Figure S11D, E, F). Furthermore, no association of total SRC3 with outcome was observed in the ERα negative cohort (Supplemental Figure S11C). However, expression of SRC3-pS543 in the whole cohort was associated with significantly longer disease-free survival (DFS) (p<0.00001) and breast cancer specific survival (BCSS) (p=0.0001) (Figure 4A). SRC3-pS543 expression in the ERα positive cohort correlated with a longer DFS (p=0.003) and BCSS (p=0.001), (Figure 4B) while SRC3-pS543 did not associate with survival in ERα-negative breast cancers (Figure 4C). Furthermore, in low risk ERα positive cancers, no correlation of SRC3-pS543 with outcome was found (Figure 4D).

Importantly, SRC3-pS543 was associated with a longer DFS (p=0.035) and BCSS (p=0.005) in ER positive high risk BC not treated with tamoxifen (Figure 4E). In high risk patients who did receive tamoxifen, SRC3-pS543 did not associate with a longer DFS (p=0.57) or BCSS (p=0.21) (Figure 4F). For ER positive high risk patients with low SRC3-pS543, exposure to tamoxifen reduced the risk of death from BC (HR (95% CI) = 0.68 (0.49-0.94), p=0.019)
by 32% (Figure 4G), whereas for those with high pS543, there was no effect (Figure 4H), the interaction was statistically significant at (p=0.001). Similarly, exposure to tamoxifen reduced risk of recurrence by 42% (HR (95% CI) = 0.58 (0.44-0.77), p=0.0001) in ER+ high risk patients with low pS543 whereas no effect on those with high pS543 patients, the interaction was statistically significant at (p=0.0001). Tamoxifen-treated patients with HER2-positive tumours that expressed high levels of SRC3 had a significantly shorter disease free and breast cancer specific survival. This was not observed for the patients who did not receive endocrine treatment (Supplementary Figure S14).

SRC3-pS543 was confirmed as an independent prognostic factor in breast cancers after adjustment for endocrine therapy and other validated prognostic factors; and absence of SRC3-pS543 signal correlated with recurrence (HR=1.4, CI 95%; 1.1-2.5) and death from breast cancer (HR=1.6, CI 95%; 1.1-2.5), (Table 2).
Discussion

Coactivators for ERα have been the focus of many clinical and cell biological studies. SRC3 is of particular interest, since the SRC3 gene is frequently amplified in breast cancer (7), and its expression was found to correlate with HER2 expression levels and poor outcome (11). SRC3 is a member of the p160 coregulator protein family (along with SRC1 and SRC2), which all have shared and preferred genome-wide chromatin binding patterns, which are strongly induced by ERα activation (15).

Here, we focus on one distinct phosphorylation event on SRC3, Serine 543 phosphorylation. This phosphorylation has spatial selectivity on a genomic scale, where chromatin binding was selectively enriched at active promoter regions. This genomic preference at promoters is in contrast to total SRC3, which is mainly found at enhancers. It is likely that SRC3 and SRC3-pS543 antibodies have different sensitivities, and the level of overlap detected between these two signals is possibly an underestimate of the actual proportion of SRC3 that is phosphorylated. Relative expression levels and antibody sensitivity are likely to alter the threshold of detection in ChIP-seq experiments, forming a major source for false-negative signal. Since the strongest total SRC3 sites are enhancer-selective, while the strongest SRC-pS543 were mostly enriched at promoters, this genomic selectivity of phosphorylated SRC3 to promoter regions cannot be merely explained by relative expression levels or antibody sensitivity.

Since ERα typically binds enhancers (30), the determination of which ERα binding events are functionally orchestrating what genes has remained a
challenge in the field. Since most ERα binding events are observed within a 20kb window from the transcription start sites of responsive genes (33), this spacing around the promoter is typically applied to provide an indication of functionality. All p160 coactivators, including SRC3, show comparable genomic features as compared to ERα and are typically found at enhancers as well (15). Since the SRC3-pS543 phosphorylation is promoter-enriched, commonly shared with ERα and induced by estradiol, this phosphorylation event could be considered as a novel ERα functionality probe, highlighting ERα complexes that are actively involved in transcriptional events at gene promoters.

Smaller previous studies on SRC3 expression and its correlations with ERα and members of the EGFR/HER family have reported varying results (11-13). We find no correlation of SRC3 with ERα but do find a correlation with HER2 and HER3. However, SRC3-pS543 does significantly correlate with ERα, potentially reflecting the role of E2/ERα in phosphorylation of SRC3. Consistent with these results was the observed correlation of SRC3-pS543 with known estrogen-regulated genes, including PR. While previous studies have explored the influence of SRC3 expression on patient outcome as well how it may influence endocrine therapy (11-13,34-39), the current study is the largest and most well-characterised series to date, with a long median follow-up. We find no statistically significant association between SRC3 levels and DFS and BCSS in patients with ERα positive breast cancer treated with tamoxifen, consistent with other reports (12,13). Of note, in those women with ERα positive tumours not treated with tamoxifen, there is a trend to an improved outcome and survival for SRC3-positive tumours, analogous
to the first report relating SRC3 and response to endocrine therapy (11). The poor outcome seen in HER2-positive/SRC3-high tumours for patients treated with tamoxifen is consistent with previously published data (11,12). One previous study on ERα negative tumours, which utilised automated quantitative analysis (AQUA) in a sub-group of 133 patients, found SRC3 associated with a poorer overall survival (38). However, in the current study of 377 ERα negative tumours no correlations with recurrence or outcome were found for SRC3. Of note, we show a significant correlation of p-JNK and p-p38 with SRC3-pS543. These kinases have previously been described to phosphorylate SRC3-S543 (16), and activated (phosphorylated) JNK has previously be linked to endocrine therapy resistance (37) (40), as well as poor outcome in breast cancer (41). The current study linking pJNK with high SRC3-pS543 and a good prognosis group is not comparable with these previous studies for technical and methodological reasons. In the previous study which investigated the expression of pJNK in human breast cancer (39), differing reagents, scoring system as well as a smaller number of cases (n=68) of cases was used. Furthermore, 40% of the cases were ER negative, and in those that were ER positive no clinicopathological correlation was presented (39). While the other studies either investigated the activity of JNK and not its expression in the context of breast cancers exposed to tamoxifen (37) or where levels of pJNK were assessed via immunoblotting in the context of a MCF-7 breast cancer xenograft model (38).

Our study is the first to explore the clinical significance of a phosphorylated form of SRC3. SRC3-pS543 correlates with an improved DFS and BCSS for the whole cohort and is a predictor of outcome in ERα positive
breast cancer. Furthermore, in high risk ER positive disease it is demonstrated that SRC3-pS543 is predictive of benefit with adjuvant tamoxifen. With no benefit being seen with adjuvant tamoxifen in the presence of high SRC3-pS543, while those tumours with low SRC3-pS543 had improved outcomes with tamoxifen treatment. The lack of benefit observed in high SRC3-pS543 tumours treated with tamoxifen indicates that the transcriptional events driven by at SRC3-pS543 at gene promoters of ER responsive result in a phenotype with an excellent clinical outcome, this would be consistent with the clinicopathological features associated with SRC3-pS543. While insufficiency or inability to enrich at these promoters as a result of low or absent SRC3-pS543 results in a more aggressive phenotype which can be abrogated by tamoxifen.

The patient group studied here is a well-characterised historical cohort treated with or without tamoxifen. However, estrogen withdrawal therapy using aromatase inhibitors (AIs) is now standard clinical practice in the adjuvant setting for postmenopausal breast cancer, and correlations of response to AIs with (phosphorylated) SRC3 levels need to be assessed within this setting, particularly since the oncogenicity of SRC3 appears modulated by the hormonal milieu in mouse models (42). Currently, the translational arm of the Intergroup Exemestane Study (so called Path-IES) is exploring the potential role of SRC3 and pS543 in this context (43).

In summary, we have found a novel biomarker for ERα positive breast cancer and provide mechanistic insights in its functional involvement in the ERα transcription complex. SRC3-pS543 phospho-specific antibodies could aid in identifying patients with a functional ERα signalling pathway, which
predicts for a favorable outcome in absence of adjuvant therapy and where tamoxifen might better be withheld.
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Conflict of interest: The authors declare no conflict of interest
References


13. Dihge L, Bendahl PO, Grabau D, Isola J, Lovgren K, Ryden L, et al. Epidermal growth factor receptor (EGFR) and the estrogen receptor modulator amplified in breast cancer (AIB1) for predicting clinical


27. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a


Table 1: Patient Characteristics and tumour biomarkers and correlation SRC3 and SRC3-pS543

<table>
<thead>
<tr>
<th>Variables</th>
<th>SRC3</th>
<th>p value</th>
<th>SRC3-pS543</th>
<th>p value</th>
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<tbody>
<tr>
<td></td>
<td>Low (n=689)</td>
<td>High (n=699)</td>
<td>Low (n=680)</td>
<td>High (n=836)</td>
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<tr>
<td><strong>Tumour size</strong></td>
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<td></td>
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<tr>
<td>T1 a+ b (=&lt;1.0)</td>
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<td>55 (8.8)</td>
<td>95 (9.0)</td>
<td>24 (10.5)</td>
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<td>341 (54.6)</td>
<td>517 (49.2)</td>
<td>130 (56.8)</td>
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<tr>
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<td>271 (34.8)</td>
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<td>68 (29.7)</td>
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<td><strong>Tumour grade</strong></td>
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<td>316 (30.1)</td>
<td>103 (45.0)</td>
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<td><strong>Mitotic index</strong></td>
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<td>M1 (low; mitoses &lt;10)</td>
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<td>318 (30.5)</td>
<td>117 (51.1)</td>
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<td>$p$-value</td>
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<td>278 (60.7)</td>
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<td><strong>Epithelial Mesenchymal transition</strong></td>
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<td><strong>Triple Negative phenotype</strong></td>
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<td>520 (84.4)</td>
<td>817 (79.4)</td>
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<td>543 (90.0)</td>
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Table 2: Multivariate analysis using Cox regression analysis confirms that SRC3-pS543 protein expression is independent prognostic factor for both Disease Free Survival and Breast cancer Specific Survival.

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<th>BCSS at 10 years</th>
<th>DFS at 10 years</th>
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<td>Lymph node stage</td>
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<td>9.6x10^-11**</td>
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<td>Positive (&gt;3 nodes)</td>
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<td>Chemotherapy (No)</td>
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<td>Bcl2 expression (positive)</td>
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<td>Ki67 expression (High)</td>
<td>1.5 (1.1-2.2)</td>
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<tr>
<td>HER2 (overexpression)</td>
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** Statistically significant

BCSS; BC specific survival, DFS; disease free survival, HR; hazard ratio, CI; confident interval
Figure and table legends

Table 1: Patient Characteristics and tumour biomarkers and correlation SRC3 and SRC3-pS543.

Table 2: Multivariate analysis using Cox regression analysis on the entire cohort confirms that SRC3-pS543 protein expression is independent prognostic factor for both Disease Free Survival and Breast cancer Specific Survival.

Figure 1: Validation of phosphostate-specific antibody

A. CHO cells were transfected with pCDNA3, Flag-tagged wild-type (WT) SRC3 or a Flag-tagged SRC3 mutant in which serine at residue 543 has been substituted by alanine. MCF-7 cells were used as control. The lysates were immunoprecipitated with SRC-3 antibody. Immunoblotting was performed for SRC-3 and phopsho-S543. Note that Flag-tagged SRC3 migrates slower on western blot as compared to endogenous SRC3.

B. MCF-7 cell lysate was immunoprecipitated with SRC-3 antibody. The beads were then divided into two halves, one untreated and the other treated with λ-phosphatase. Immunoblotting was performed for SRC-3 and phosphorylation state-specific antibody.

C. CHO cells were grown in DMEM with dextran-coated charcoal stripped FCS for 72hrs and then transfected with vector (pCDNA3) or SRC-3 for 24hrs using. Cells were then treated with vehicle or increasing
concentrations of 17ß-estradiol (E2) for 1hr before making lysates for IP. Western blotting was carried out with the antibodies indicated. MCF-7 cells were used as control.

D. Representative tumour sections following pre-incubation with phospho peptide and non-phospho peptide and phosphatase treatment prior to staining with SRC3-pS543 in breast cancers with negative, low/medium, high expression of SRC3-pS543.

**Figure 2:** SRC3-pS543, SRC3 and ERα chromatin binding patterns in MCF-7 cells.

A. Genome browser snapshot of ChIP-seq samples for ERα (red), SRC3 (blue) and SRC3-pS543 (green) in proliferating MCF-7 cells. Y bar shows tag count. Genomic coordinates are indicated.

B. Venn diagram of ERα (red), SRC3 (blue) and SRC3-pS543 (green). Numbers of shared and unique peaks are shown.

C. Heatmap visualisation of ERα (red), SRC3 (blue) and SRC3-pS543 (green).

D. Motif analyses of ERα/SRC3-pS543 shared and SRC3-pS543 enriched binding sites. Z score for each motif is shown in a Motif Radar Plot (MRP). Motif enrichment is shown for chromatin binding sites shared between ERα and SRC3-pS543 (red) or selectively enriched for SRC3-pS543 (green). The radial data points represent the absolute value of Z-score.

E. Genomic distributions of ERα, SRC3 and SRC3-pS543 sites
F. Percentage of ERα-regulated genes with a SRC3-pS543 binding event at the promoter, compared to all refseq genes. Chi-square test: p<0.0001.

Figure 3: SRC3-pS543 binding events in MCF-7 cells and breast tumours.

A. Genome browser snapshot, ChIP-seq for SRC3-pS543 on MCF-7 cells (green), tumour sample #1 (blue) and tumour sample #2 (purple). Y bar shows tag count. Genomic coordinates are indicated.

B. Venn diagram of SRC3-S543P ChIP-seq for MCF-7 cells (green), tumour sample #1 (blue) and tumour sample #2 (purple). Number of shared and unique sites is illustrated.

C. Heatmap visualisation of SRC3-S543P ChIP-seq for MCF-7 cells (green), tumour sample #1 (blue) and tumour sample #2 (purple). Number of shared and enriched sites is illustrated.

D. Genomic distributions of shared and unique SRC3-pS543 binding events for MCF-7 cells, tumour sample #1 and tumour sample #2.

E. Genome browser snapshot, ChIP-seq on a primary breast tumor for ERα (red) and SRC3-pS543 (blue).

F. Venn diagram of ERα (red) and SRC3-pS543 (blue) binding events.

G. Heatmap analysis, depicting all shared and enriched binding events for ERα (red) and SRC3-pS543 (blue). Shown are all binding events with a window of 5kb around the binding site.

H. Genomic distributions of the shared and enriched binding events of ERα and SRC3-pS543.
I. Motif analyses of the shared and enriched binding events of ERα and SRC3-pS543. Z score for each motif is shown in a Motif Radar Plot (MRP). Motif enrichment is shown for chromatin binding sites enriched for ERα (red), SRC3-pS543 (green) or shared between ERα and SRC3-pS543 (blue). The radial data points represent the absolute value of Z-score.

**Figure 4:** Disease Free Survival and breast cancer specific survival in breast cancer patients based on SRC3-pS543 protein expression.

A. Entire Nottingham cohort
B. ERα positive Cohort
C. ERα negative Cohort NPI <3.4
D. ERα positive patients NPI <3.4 who did not receive tamoxifen
E. ERα positive patients NPI ≥3.4 who did not receive tamoxifen
F. ERα positive patients NPI ≥3.4 who received tamoxifen
G. Breast cancer specific survival based on SRC3-pS543 protein expression and tamoxifen treatment in ERα positive high risk group with low pSRC3-pS543
H. Breast cancer specific survival based on SRC3-pS543 protein expression and tamoxifen treatment ERα positive high risk group with high pSRC3-pS543
Figure 3

A

SRC3 pS543

chr21:30,357,497-30,451,412

tumor #1

tumor #2

B

SRC3 pS543

MCF7

2523

456

194

2670

1816

2509

4631

tumor #1

tumor #2

C

MCF-7

Tumor 1

Tumor 2

D

SRC3 pS543

tumor #1

tumor #2

MCF7

shared

E

tumor #1

chr18:23,428,922-23,470,813

SRC3 pS543

F

SRC3 pS543

ERα

5537

1727

15060

G

SRC3 pS543

ERα

H

ERα unique

SRC3-pS543 shared

SRC3-pS543 unique

promoter
downstream
5'UTR
3'UTR
coding exon
intron
distal intergenic

I

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Figure 4

A  Nottingham Whole Series (n=1282)

B  ER+ (n=924)

C  ER- high risk (NPI>3.4) subgroup (n=294)

D  ER+ Low risk (NPI<3.4) subgroup (n=317)

E  ER+ high risk (NPI>3.4) subgroup who did not received Tamoxifen (n=263)

F  ER+ high risk (NPI>3.4) subgroup who received tamoxifen (n=269)

G  ERα +/- high risk with Low pSRC3-pS543

H  ERα +/- high risk with High pSRC3-pS543

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Clinical Cancer Research

SRC3 phosphorylation at Serine 543 is a positive independent prognostic factor in ER positive breast cancer

Wilbert Zwart, Koen D Flach, Bharath Rudraraju, et al.

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