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1 **Epigenome-wide SRC-1 mediated gene silencing represses cellular**
2 **differentiation in advanced breast cancer**

3 Ward E^{1†}, Varešlija D^{1†}, Charmsaz S¹, Fagan A¹, Browne AL¹, Cosgrove N¹, Cocchiglia S¹, Purcell S¹,
4 Hudson L¹, Das S², O'Connor D², O'Halloran PJ³, Sims A⁴, Hill AD¹, Young LS^{1*}

5 [†]These authors contributed equally to this study

6 ¹Endocrine Oncology Research Group, Department of Surgery, Royal College of Surgeons in Ireland,
7 Dublin, Ireland

8 ²Molecular and Cellular Therapeutics, Royal College of Surgeons in Ireland, 123 St Stephen's Green,
9 Dublin, Ireland

10 ³Department of Neurosurgery, National Neurosurgical Center, Beaumont Hospital, Dublin, Ireland

11 ⁴ Applied Bioinformatics of Cancer Group, University of Edinburgh Cancer Research UK Centre, MRC
12 Institute of Genetics & Molecular Medicine, Western General Hospital, Edinburgh, UK

13 ***Corresponding author:** Leonie Young, Endocrine Oncology Research Group, Department of Surgery,
14 Royal College of Surgeons in Ireland, Dublin 2, Ireland. Tel: +353 1 4028576. lyoung@rcsi.ie

15 **Running Title:** SRC-1 mediates repressive DNA methylation in breast cancer

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17 dependent cancer, estrogen receptor, metastasis

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1 **Translational Relevance**

2 Aberrant DNA methylation-mediated gene silencing frequently occurs in cancer. While
3 substantial effort has been devoted to the elucidation of methylome changes associated
4 with the development of breast cancer, comparatively little is known about the methylome
5 alterations that accompany treatment resistance and their contribution to the metastatic
6 phenotype. In this study we addressed this gap by generating a comprehensive epigenomic
7 map of endocrine treatment resistance and identified a key potentiator, its effectors and
8 their mechanistic and functional output. From this study, we established a methylation
9 molecular marker set of 5 genes whose silencing mediated tumor aggressiveness.
10 Subsequently these markers were confirmed to predict metastatic survival from a cohort of
11 endocrine treated breast cancer patients. These novel insights provide vital clues to the
12 epigenetic basis of on-treatment progression in endocrine resistant breast cancer and could
13 advance the management of resistant disease.

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1 **Abstract**

2 **Purpose:** Despite the clinical utility of endocrine therapies for estrogen receptor positive
3 (ER) breast cancer, up to 40% of patients eventually develop resistance, leading to disease
4 progression. The molecular determinants that drive this adaptation to treatment remain
5 poorly understood. Methylation aberrations drive cancer growth yet the functional role and
6 mechanism of these epimutations in drug resistance are poorly elucidated.

7 **Experimental design:** Genome-wide multi-omics sequencing approach identified a
8 differentially methylated hub of pro-differentiation genes in endocrine resistant breast
9 cancer patients and cell models. Clinical relevance of the functionally validated methyl-
10 targets was assessed in a cohort of endocrine treated human breast cancers and patient-
11 derived *ex vivo* metastatic tumours.

12 **Results:** Enhanced global hypermethylation was observed in endocrine treatment resistant
13 cells and patient metastasis relative to sensitive parent cells and matched primary breast
14 tumor respectively. Using paired methylation and transcriptional profiles we found that SRC-
15 1-dependent alterations in endocrine resistance lead to aberrant hyper-methylation which
16 resulted in reduced expression of a set of differentiation genes. Analysis of ER positive
17 endocrine treated human breast tumors (n=669) demonstrated that low expression of this
18 pro-differentiation gene set significantly associated with poor clinical outcome (p=0.00009).
19 We demonstrate that the re-activation of these genes *in vitro* and *ex vivo* reverses the
20 aggressive phenotype.

21 **Conclusion:** Our work demonstrates that SRC-1-dependent epigenetic remodeling is a 'high
22 level' regulator of the poorly differentiated state in ER -positive breast cancer. Collectively
23 these data revealed an epigenetic reprogramming pathway, whereby concerted differential
24 DNA methylation is potentiated by SRC-1 in the endocrine resistant setting.

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1 Introduction

2 Breast cancer (BC) develops through the accumulation of genetic and epigenetic
3 abnormalities to chief regulators of cell proliferation, differentiation and apoptosis.
4 Estrogen receptor (ER) is a key driver of hormone-dependent BC and its expression is
5 indicative of good prognosis. Despite the efficacy of endocrine treatment, including
6 tamoxifen and aromatase inhibitors (AIs) in ER-positive BC, acquired therapy resistance is
7 common and it remains a major clinical challenge (1). Mechanisms underlying this resistance
8 are complex, highly adaptive and heterogeneous and can vary from patient to patient, from
9 primary to metastatic tissue and even amongst different endocrine treatments. Recent
10 studies of metastatic tissues from patients that have failed AIs revealed a number of
11 mutations, including those activating ESR1 (2), as a feature of resistance. On the other hand,
12 loss of ER function/expression can be found in 20% of metastatic tumors highlighting the
13 dynamic nature of therapeutic resistance (3,4).

14 Endocrine treatment-resistant cancer cells activate pathways co-operating and
15 interacting with ER, its co-regulators and transcription factors providing survival advantage
16 and therapeutic escape. One such ER regulator, SRC-1, has been shown to be central to the
17 ability of ER tumors to adapt and facilitate metastatic disease progression (5,6). Typically,
18 SRC-1 binds to and co-activates nuclear receptors such as ER to regulate a network of
19 proliferation- and differentiation-associated genes critical to BC progression (7). Notably,
20 aberrant up-regulation of SRC-1 has been implicated in the development of endocrine
21 treatment resistance in BC, where high protein levels correlate with endocrine resistance
22 and poor clinical outcome (8-10). Modulations of these endocrine resistant pathways can be
23 driven by genomic, epigenetic or tumor microenvironment influences.

24 Although current emphasis for tumor profiling is on mutation-level alterations, these
25 approaches have failed to uncover the molecular determinants that drive adaptation to
26 treatment. Conversely, transcriptional and epigenetic reprogramming develops with higher
27 frequency and has been observed to functionally affect oncogenes and related signalling
28 pathways (11-13). Increasing evidence suggests that aberrant DNA methylation of tumor
29 suppressors and differentiation/developmental genes may represent a major mechanism
30 underlying tumor progression (11). The discovery of hypo and hyper-methylation (14-16) in

1 cancer has led to major advancements towards uncovering novel epigenetic drivers in
2 tumor initiation and progression. Aberrant DNA hypermethylation is the most prominent
3 epi-alteration reported in cancer, originating in regions marked with repressive histone
4 marks (e.g. H3K27me3) (17) ,guided by DNA-methyl transferases (DNMTs) (18) and carefully
5 regulated by transcriptional influencers such as polycomb-repressive complexes (PRCs)
6 (17,19) and methyl-CpG-binding domain proteins (MBDs) (20-23). These methylome
7 changes are potentially reversible making them prime candidates as novel targets for
8 diagnosis and treatment strategies. Indeed, altered DNA methylation events in BC have
9 been used to identify potential biomarkers (24,25), whilst DNA methylation signatures can
10 be used to classify tumor subtypes (26-28) or inform endocrine response (29,30). Although,
11 several studies have reported shifts in the epigenetic profile of endocrine resistant cell line
12 models (29,31,32), the role of epigenetic dysregulation in endocrine treatment resistance is
13 still poorly understood, as are the key potentiators of its function.

14 In the current study, we have investigated the methylome profile of endocrine
15 resistant tumors and we report on extensive global epigenomic remodeling events unique
16 to treatment resistant disease. Our investigation places SRC-1 in a critical position in
17 controlling the methylation reprogramming in endocrine treatment resistant models and
18 identifies it as a necessary component in the core regulatory circuitry. SRC-1's role as a
19 transcriptional repressor was further validated as SRC-1-dependent events mediate
20 aberrant methylation leading to reduced expression of a set of differentiation genes. We
21 demonstrate that SRC-1 is pivotal in recruiting the co-repressor complex to a hub of pro-
22 differentiation genes, thus remodeling BC cells to promote a more aggressive endocrine
23 treatment resistant phenotype. Our data support a model where epigenetic reprogramming
24 towards a poorly differentiated cell profile, driven by an oncogenic co-regulator, is a crucial
25 step in endocrine treatment resistance.

26

27 **Methods**

28 **Cell culture**

29 The endocrine sensitive MCF7 cells (ATCC, USA) were cultured in Minimum Essential
30 Medium Eagle (MEM) (M2279, Sigma) supplemented with 10 % fetal calf serum (FCS)

1 (F7524, Sigma). The endocrine resistant LY2 breast cancer cells were a gift from Robert
2 Clarke (Georgetown, USA) and were cultured as previously described (33). Each cell line was
3 tested for mycoplasma (LT07-118, Lonza), genotyped (SourceBioScience) and authenticated
4 according to ATCC guidelines. The T347x brain metastatic primary cell line was derived from
5 an ER+ PR- HER2+ patient tumor, which was expanded in NOD-SCID mice (34). The tumor
6 was resected, dissociated and cultured in human breast epithelial cell (HBEC) media as
7 described previously (34) for *in-vitro* experiments.

8 **CRISPR/Cas9, Lentiviral transduction, siRNA transfection**

9 The LY2 SRC-1 knockout (KO) cell line was created using CRISPR/CCas9 technology (Santa
10 Cruz) details of the transfection procedure are provided in supplementary information. The
11 LY2 luciferase (LY2-Luc), LY2 shSRC-1 KD (knockdown) and LY2 shNT (non-targeting) cells
12 were created by transducing LY2 cells with viral particles as previously described (35). Gene
13 silencing was carried out using predesigned siRNAs directed against NTRK2 (144201,
14 Ambion), non-targeting siRNA control (NT siRNA) (AM4611, Ambion), NR2F2 (J-003422-06-
15 0002), CTDP1 (J-009326-080002), SETBP1 (J-013930-18-0002), POU3F2 (J-020029-06-0002)
16 and NT siRNA (J.Human-xx-002) (Dharmacon, USA) and transfection was carried out using
17 Lipofectamine 2000 (11668-019, Invitrogen) as per manufacturer's instructions.

18 **Gene expression**

19 RNA extractions were performed using the RNeasy Mini Kit (74106, Qiagen) as per
20 manufactures instructions and SuperScript III (18080400, Invitrogen) was utilized for cDNA
21 conversion. Gene expression was confirmed by semi qPCR using pre-designed Taqman
22 assays (Thermo Fischer Scientific) for β -actin (401846), NTRK2 (Hs00178811), NR2F2
23 (Hs00819630), CTDP1 (Hs00364467), SETBP1 (Hs01098447), POU3F2 (Hs00271595), DNMT1
24 (Hs0094587) and DMNT3A (Hs01027162) on the StepOnePlus Real Time System (Applied
25 Biosystems). The comparative C_T ($\Delta\Delta C_T$) method was applied to analyze relative mRNA
26 expression.

27

28 **Flow cytometry**

29 Fluorescence-activated cell sorting (FACS) was performed on the FACS ARIA II (BD
30 Biosciences). The LY2 CRISPR/Cas9 cells (clone 7c and 9c) were sorted for both CRISPR HDR
31 plasmid (Red Florescent Protein) and luciferase (Green Florescent Protein). Stem cell

1 analysis was carried out on the BD FACS Canto II (BD Biosciences). Cell lines which
2 underwent gene silencing were analyzed by flow cytometry 48 hrs post siRNA transfection
3 for with NTRK2, NR2F2, CTD1P1, SETBP1, and POU3F2. Cells were stained with CD24
4 (555428; BD Biosciences), CD44 (555478; BD Biosciences), EpCam (12-9326-42,
5 ThermoFisher) and CD49f (17-0495-82, ThermoFisher) antibodies. Data were analyzed using
6 FlowJo Software (FlowJo, USA).

7 **Mammosphere forming, anchorage independence, 3D acini and motility assays**

8 Functional assays were performed in the LY2 luc control cell line, LY2 SRC-1 CRISPR/Cas9 KO
9 cells (clones 7c and 9c), and LY2 shSRC-1 cells 24 hrs post gene silencing with NTRK2, NR2F2,
10 CTD1P1, SETBP1, POU3F2. All functional assays were carried out with cells treated with 4-
11 OHT [10^{-7} M].

12 Mammosphere culture and analysis was performed as previously described.
13 Anchorage independence was analyzed using the agarose colony formation assay as
14 previously described (36).

15 3D Acini assays were performed to assess cellular polarization/organization. Cells were
16 cultured for 21 days, then fixed and stained as previously described (8).

17 Cell migration was carried out using the Cellomics Cell Motility Kit (K0800011, Thermo
18 Scientific) as previously described (8).

19 **Chromatin Immuno-precipitation (ChIP)**

20 ChIP was performed on the LY2 cells, LY2-luc and LY2 CRISPR/Cas9 SRC-1 KO cell line (clone
21 7c) as previously described (4). Full details can be found in Supplementary Methods.

22 **Immunohistochemistry**

23 Immunohistochemistry (IHC) was performed on 5 μ M formalin fixed paraffin embedded
24 (FFPE) tumor sections as previously described using DAKO envision+ HRP kit (K400611-2,
25 Agilent Technologies) (4). Full details on antibodies and protocols can be found in
26 Supplementary Methods.

27 **Explant studies**

28 An LY2 luciferase cell line xenograft and patient breast cancer brain metastatic tumors
29 (T347x and T638x) were expanded in NOD-SCID mice. The primary tumors were resected,
30 grown on gelatin sponges (Spongostan, Johnson and Johnson) as previously described (37)
31 and treated with estrogen combined with vehicle, 4-OHT, RG108 and a combination of 4-

1 OHT and RG108 for 72 hrs. Following treatment tumor pieces were formalin fixed and
2 paraffin embedded for IHC analysis. LY2 cell line-derived xenograft results shown are a
3 representative of 3 individual experiments, T347x and T638x PDX results are individual
4 experiments. The viability of the tumors was evaluated by screening for necrosis of the
5 tissue and using proliferation markers to confirm viable, proliferating cells.

6 **Sequencing Acquisition**

7 **SeqCap Epi targeted bisulfite methylation sequencing** (Roche) was performed on breast
8 cancer cell lines MCF7 (n=2) and LY2 (N=3) cells and in FFPE breast cancer primary and
9 matched metastatic patient tumor samples (RCS_4). DNA was extracted using the DNA/RNA
10 FFPE extraction kit (80234, Qiagen) as per manufacturer's instructions. Further details are
11 available in Supplementary Information.

12 **MeDIP sequencing** was carried out in the LY2 shNT (n=2) and LY2 shSRC-1
13 knockdown (n=2) cells following 4-OHT treatment for 3 hrs. DNA extraction, MeDIP library
14 construction and sequencing (50PE) were all performed by Beijing Genomics Institute (BGI,
15 Hong Kong) following standard protocols on the Illumina platform.

16 **ChIP sequencing** was performed in LY2 cells treated with vehicle or 4-OHT for 45 min
17 and immunoprecipitated with SRC-1 antibody, as previously described (38).

18 **RNA sequencing** was performed on 4 technical replicates of LY2 shNT shRNA and LY2
19 shSRC-1 cells treated with 4-OHT for 8 hrs. RNA extraction and sequencing was carried out
20 by Beijing Genomics Institute (BGI, Hong Kong) as per standard protocols (3).

21 **Bioinformatic Analysis**

22 Full details of the bioinformatic analysis undertaken for each of the sequencing methods is
23 available in Supplementary Methods.

24 **Affymetrix microarray analysis**

25 Data from four published data sets (GSE66532, GSE9195, GSE17705 and GSE12093) (39)
26 were utilized to generate the ranked gene expression heatmap for the SRC-1 target genes in
27 ER+ve tamoxifen treated patient tumors (n=669). Data is summarized with Ensemble
28 alternative CDF and normalized with Robust Multi-array Average (RMA), before integration
29 using ComBat to remove dataset-specific bias.

30 **Statistical analysis**

1 Gene expression, *In-vitro* assays and ki67 scores are shown as mean \pm SEM. The student
2 paired *t* test was used for two group comparisons and results for each assay are
3 representative of 3 individual experiments unless otherwise stated and expressed as mean \pm
4 SEM, * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$. Treatment groups were compared to vehicle or
5 parental cell line unless otherwise stated. With respect to randomization, for *ex vivo*
6 experiments, similar sized tumors were equally divided into the control and experimental
7 groups for subsequent drug treatment which was not blinded. The investigators were not
8 blinded to allocation for *ex vivo* and immunohistochemical analyses. No statistical method
9 was used to predetermine sample size. Gene Expression-Based Outcome for Breast Cancer
10 Online (GOBO) was applied to analyze expression of the SRC-1 target genes (NTRK2, NR2F2,
11 CTDP1, SETBP1 and POU3F2) in the Pam50 breast cancer tumor subtype (Basal, ERBB2,
12 Luminal A, Luminal B, and Normal like). Kaplan-Meier plots were used as an estimate of
13 Distant Metastatic Free Survival (DMFS) in SRC-1 target genes in ER+ve patients (n=669) and
14 Recurrence Free Survival (RFS) in untreated patients (n=343) (39).

15 **Data availability**

16 RNA-seq, MeDIP-seq and SeqCap Epi targeted bisulfite sequencing data files were deposited
17 and are available on Gene Expression Omnibus GSE99649. Data from tamoxifen treated
18 SRC-1 CHIP-seq in LY2 cells is available on Gene Expression Omnibus GSE28987.

19 **Ethics**

20 Written and informed consent was acquired prior to collection of patient tumor tissue
21 under The Royal College of Surgeons Institutional Review Board approved protocol
22 (#13/09:ICORG 09/07). Mouse experiments were performed in accordance with the
23 European Communities Council Directive 2010/63/EU and were reviewed and approved by
24 Research Ethics Committee under license from the HPR (Health Products and Research
25 Authority).

26

27 **Results**

28 **SRC-1 global gene methylation signature in endocrine resistant breast cancer.**

29 Perturbations in DNA methylation profiles may influence tumor initiation, metastatic
30 progression and resistance to treatment. To investigate global aberrant DNA methylation as

1 a function of treatment resistant BC we undertook targeted bisulfite sequencing in
2 endocrine sensitive and resistant samples. Density distribution of events examining
3 differential methylation revealed increased hypermethylation in endocrine resistant cells
4 and an ER+ve metastatic patient tumor, relative to parent sensitive cells and matched
5 primary tumor tissue, respectively (Fig. 1a). Moreover genome wide CpG methylation was
6 observed in the metastatic tumor in comparison to the matched primary tissue (Fig 1a).
7 Having established the role of differential DNA methylation in acquired resistance the
8 resulting changes to ER binding was examined as differential ER-binding is observed in
9 tumors from patients with poor outcome (40). ER occupancy of CpG islands in endocrine
10 resistant LY2 cells is greater than in the endocrine sensitive MCF7 cells. Furthermore,
11 tamoxifen driven ER/SRC-1 co-occupancy at these sites suggests a role for the steroid
12 receptor/coactivator complex in mediating these enhanced DNA-hypermethylation events
13 (Fig 1b). This further supports the role of SRC-1 in transcriptional silencing (35). To
14 understand the contribution of SRC-1 to global methylation, comprehensive genome-wide
15 MeDIP-seq was undertaken in the presence and absence of SRC-1 (shNT and shSRC-1; Fig.
16 S1a) in endocrine resistant cells. Consistent with its role as a coactivator protein,
17 hypermethylation was enriched in the absence of SRC-1 (Fig. 1c,). However, SRC-1
18 dependent hypermethylation events were also observed throughout the genome (Fig. 1c).
19 Of interest, from SRC-1-ChIP-seq analysis in endocrine resistant cells (38) we observed an
20 over-representation of methyl marks at CpG islands within 2Kb of an SRC-1 peak (Fig. 1d).

21 The initial methylome data suggests that SRC-1-dependent events result in an
22 altered methylome profile and may in fact suppress specific gene sets in the resistant
23 setting. Before attempting to dissect the underlying mechanism it was important to
24 determine the identity of these suppressed genes and if such genes were likely to contribute
25 to the resistant state. RNA-seq identified 736 genes down-regulated in the presence of SRC-
26 1 (Fig. 1e). Correspondence analysis and heatmap displayed separation between shNT and
27 shSRC-1 gene expression (Fig. S1.b,c). Pathway analysis of the SRC-1 suppressed genes
28 revealed a preponderance of genes pertinent to cell development and pro-differentiation
29 (Fig. S2). This is particularly poignant since a significant function of DNA methylation is in
30 modulating differentiation and developmental pathways. We integrated data from the SRC-
31 1- RNA-seq, ChIP-seq and MeDIP-seq assays to identify putative genes that were directly

1 suppressed by SRC-1-dependent DNA methylation (Fig. 1f, Table 1). From these 9 genes
2 NTRK2, NR2F2, CTD1P1, SETBP1 and POU3F2 have described roles in cellular development
3 and differentiation. CHIP and qPCR analysis confirmed these genes as direct SRC-1 targets
4 (Fig. S3a, b).

5 **Functional role of SRC-1 in disease progression in endocrine resistance is mirrored by the**
6 **roles of NTRK2, NR2F2, CTD1P1, SETBP1 and POU3F2 in tumor suppression.** In endocrine
7 treatment resistant cells SRC-1 is a known mediator of drug resistance phenotype (36,38).
8 Loss of SRC-1 expression can lead to re-sensitization of endocrine resistant cell lines to
9 tamoxifen treatment (Fig S4a,b). To assess the impact of SRC-1 and its suppressed pro-
10 differentiation target genes on tumor progression we investigated the role of the
11 coregulatory protein and each of the individual pro-differentiation genes in classic
12 mechanisms of tumor aggression including stemness and migration. Expression levels of
13 each of the 5 target genes are elevated in the absence of SRC-1 (LY2 shSRC-1) in comparison
14 to the parental resistant cells (LY2 shNT) (Fig. S3b).

15 In endocrine resistant breast cancer cells CRISPR/Cas9 gene editing was used to
16 specifically knockout SRC-1 (clone 9 (9c) and clone 7 (7c)) confirmed with mRNA (Fig S4a)
17 and protein expression of SRC-1 (Fig. S4b). No effect on protein expression levels of SRC-2 or
18 SRC-3 was observed (Fig S5a). Given relative importance of SRC-3 in breast cancer drug
19 resistance and metastasis we further confirmed no upregulation of phosphorylated SRC-3
20 protein when SRC-1 is suppressed (Fig. S5c). SRC-1 CRISPR/Cas9 KO cells demonstrated
21 enhanced differentiated CD24⁺/44⁻ cell populations in comparison to their control. siRNA
22 was used in LY2 shSRC-1 cells to transiently silence the SRC-1 pro-differentiation target
23 genes NTRK2, NR2F2, CTD1P1, SETBP1 and POU3F2 (Fig. S5b). Silencing of NTRK2 and
24 POU3F2 significantly reduced the number of CD24⁺/44⁻ differentiated cells (Fig. 2a). In these
25 endocrine resistant cells, knockout of SRC-1 resulted in reduced self-renewal capacity (2nd
26 generation mammosphere), colony formation, cell organization and migration (Fig. 2b-e). In
27 contrast, silencing of each of the SRC-1 target genes displayed loss of cell differentiation
28 through increased self-renewal capacity and colony formation along with loss of cellular
29 organization (Fig. 2b-d). Furthermore, silencing of the pro-differentiation genes elevated
30 migratory capacity of the endocrine resistant breast cancer cells (Fig. 2e).

1 **NTRK2, NR2F2, CTDP1, SETBP1 and POU3F2 expression in breast cancer patients.** The
2 clinical relevance of the SRC-1 pro-differentiation genes was examined in a cohort of breast
3 cancer patients. We used GOBO to analyze transcript expression of the gene set in
4 published clinically annotated primary tumors (39). The genes stratified according to PAM50
5 subtype ($p < 0.0001$) with the highest expression levels observed in luminal A and normal-like
6 tumors (Fig.3a). Gene set expression also associated with ER+ve primary breast cancers
7 ($p < 0.00001$) (Fig.3a). In a cohort of ER+ve tamoxifen treated patients ($n=669$), ranked sum
8 of SRC-1 suppression genes transcript significantly associated with reduced distant
9 metastatic disease free survival ($p=0.00009$) (Fig.3b, c). The association of dysregulated gene
10 set with good outcome strongly aligns with its pro-differentiation role and suggests that its
11 suppression can be detected in a relatively large subset of human BC and could contribute
12 to risk assessment for endocrine treatment resistance. This relationship would appear to be
13 treatment dependent as the reverse relationship was observed in ER+ve untreated patients
14 ($n=343$) where high transcript expression of the gene set associated with reduced
15 recurrence free survival ($p < 0.05$) (Fig. 3c, S6).

16 To enhance the translational value of our findings and further reinforce the role of
17 aberrant DNA methylation in this dysregulated pathway we employed an *ex vivo* model of
18 endocrine resistant metastatic tumors to evaluate the effect of DNA methylation disruption
19 (Fig. 3d). These models recapitulate tissue heterogeneity, morphology and architecture and
20 create a unique opportunity for drug efficacy studies and pose a good platform for
21 mechanistic studies. Given our findings that SRC-1 target genes were regulated through
22 inappropriate methylation-dependent silencing, we first used a DNA methyltransferase
23 inhibitor, RG108, to confirm its capacity to re-express the SRC-1 target genes in endocrine
24 resistant LY2 cells and those derived from endocrine resistant T347 metastatic tumor (Fig.
25 3d; Fig. S7a). Silencing of the SRC-1 pro-differentiation target genes NTRK2, NR2F2, CTDP1,
26 SETBP1 and POU3F2 resulted in an increase in proliferative capacity of LY2 shSRC-1 model
27 (Fig. S7b). Additionally, in endocrine resistant metastatic-competent cell-line derived
28 xenograft tumor (LY2) and endocrine resistant metastatic tumors (T347x and T638x, Fig
29 S7c), cultured *ex vivo*, DNA methyltransferase treatment over 72 hours had a substantial
30 anti-tumor effect as demonstrated by a significant decrease in proliferating cells (ki67+)
31 compared to vehicle treated tumors (Fig. 3e and f). This proof of concept study further

1 supports methylation as a necessary and reversible mechanism promoting tumorigenesis in
2 multiple models of endocrine treatment resistance.

3 **SRC-1 in combination with a complex of methylators represses expression of NTRK2,**
4 **NR2F2, CTD1, SETBP1 and POU3F2.** We wanted to further delineate the mechanistic
5 pathway involved. We interrogated the DNA methylation of the SRC-1 pro-differentiation
6 genes by analyzing methyl sites from Seq Cap Epi data from endocrine resistant LY2 cells
7 and matched primary and metastatic tumor from an endocrine resistant patient. The
8 percentage methylation indicating the proportion of cytosine's methylated at each CpG
9 probe is reported for the LY2 cells and metastatic brain tissue (Fig. 4a). Differential
10 methylated regions were analyzed from the resistant metastatic tumor and the matched
11 primary. Hypermethylated regions were identified upstream of NTRK2, CTD1, SETBP1 and
12 POU3F2 and a hypomethylated region upstream of NR2F2 (Fig. 4a). These regions
13 corresponded to SRC-1 peaks as determined from global ChIP-seq analyses in LY2 cells (Fig.
14 4a).

15 The expression of the maintenance and *de novo* methyltransferases, DNMT1 and
16 DNMT3a, respectively were found to be reduced in the absence of SRC-1 (LY2 shSRC-1)
17 compared to the control (LY2 shNT) (Fig. 4b). As DNA and histone lysine methylation
18 systems are highly interrelated and rely mechanistically on each other, we investigated
19 histone methylation at the SRC-1 target genes. Consistent with this, elevated recruitment of
20 the histone repression marker H3K27me₃, a known mediator of *de novo* DNA
21 hypermethylation, to the pro-differentiation genes was observed (Fig. 4c).

22 Network of nuclear receptors and co-regulator proteins is highly complex,
23 interconnected and regulates many transcriptionally active regions in the cistrome that are
24 co-ordinately occupied by multiple nuclear receptors including ER, PR and AR (41,42). To
25 further dissect the nuclear receptors' contribution to the SRC-1 regulated processes we
26 investigated ER, AR and PR binding to the target genes. Enhanced recruitment of ER and its
27 coactivator protein SRC-1 were observed at each of the pro-differentiation genes (Fig. 4d).
28 Interestingly, we found an enhanced, but non-significant, recruitment of PR to most of the
29 target genes, but not AR (Fig. 4d, S8a). Treatment with antagonists against ER and PR
30 produced no reduction in the recruitment of SRC-1 to each of the pro-differentiation genes

1 (Fig. S8b). Consistent with the specificity of SRC-1's contribution in this process, no binding
2 of SRC-3 was detected at any of the target genes (Fig. S8c).

3 To unravel the complex that may regulate methylation at these SRC-1 target genes
4 we examined the recruitment of MBD proteins to the pro-differentiation target genes. MBD
5 proteins bind methylated DNA and are believed to participate in DNA methylation-mediated
6 transcriptional repression (43). DNA binding of MECP2 and MBD2, two MBD family
7 members, to each of the target genes was confirmed by ChIP and additionally with ChIP-re-
8 ChIP qPCR indicating co-occupancy (Fig. 4e, f). To determine if SRC-1 is essential for the
9 recruitment of the methylation regulatory module we examined recruitment of the methyl
10 proteins to the pro-differentiation genes in the absence of SRC-1 using the LY2 SRC-1
11 CRISPR/Cas9 7c KO. Loss of MBD protein-DNA binding at each of the targets was observed in
12 these cells in comparison to the luciferase control endocrine resistant cells (Fig.4g).
13 Moreover, SRC-1-dependent recruitment of the histone deacetylase protein, HDAC2, a
14 known complex partner of the MBD methyl proteins, was also observed in each of the
15 target genes, in which loss of recruitment was again observed after SRC-1 KO (Fig. 4i).
16 Interestingly, we detected no such consistent significant binding of the methyl complex to
17 target genes in endocrine sensitive MCF7 cells (Fig. S8d-g). This finding is in line with our
18 previous reports of a reduction of SRC-1-DNA binding in steroid depleted endocrine
19 sensitive breast cancer cells in comparison to the endocrine resistant phenotype (35) .
20 Variable occupancy of the methyl complex and subsequent loss following SRC-1 KO suggests
21 a central role for the transcriptional regulatory protein in the management of the
22 methylome. Together, these data suggest that SRC-1 plays a regulatory role in orchestrating
23 the operational methyl complex at the DNA to bring about successful functional repression
24 of pro-differentiation target genes to enable tumor progression in the context of endocrine
25 resistance (Fig. 4i).

26

27 Discussion

28 A growing body of evidence suggests that breast cancer cells can develop resistance to
29 endocrine therapy, not only through clonal selection of pre-existing progenitor/stem cell like
30 populations and genetic mutations, but also via aberrant epigenetic regulation of gene

1 expression. Altered DNA methylation during early carcinogenesis has been associated with
2 dysregulation of key transcriptional regulators including p53 (44). Further epigenetic
3 remodeling occurs with disease progression and treatment resistance (45). Aberrant
4 methylation has been associated with activation of cholesterol biosynthesis (32) and
5 decreased gene expression of classic ER targets in endocrine resistant cell line models (30).
6 Consistent with these studies we observed differential methylation patterns between
7 sensitive and treatment resistant breast cancer cell lines and patient tumors and uncovered
8 extensive hypermethylation and hypomethylation events unique to treatment resistant
9 disease. To date the mechanism of communication between the key transcriptional
10 mechanics of the resistant cell with the methylation process to drive the phenotype and
11 promote disease progression has not been elucidated. We examined the global differential
12 methylation observed between endocrine sensitive and resistant models and patient
13 tumors, defining a role for the ER coregulatory protein SRC-1 in mediating gene repression
14 which is both functional and clinically relevant.

15 Nuclear receptor gene repression is regulated, at least in part, through interactions
16 with coregulatory proteins. The glucocorticoid receptor can utilize SRC-2 to activate and
17 repress target gene expression depending on the transcriptional target (46). More recently,
18 an amphipathic role for the ER co-regulator protein SRC-1 has also been described, where
19 SRC-1 the classic steroid receptor coactivator protein, has been shown to transcriptionally
20 repress the differentiation marker CD24 and the apoptotic protein PAWR (35). In this study,
21 employing integrated multi-omics approach, we found specific global SRC-1 dependent
22 hypermethylation, corresponding to regions of transcriptional repression, which were
23 confirmed as direct SRC-1 targets. Though the complex interplay between nuclear receptors
24 and coregulatory proteins is known to play a significant role in the development of breast
25 cancer, data reported here suggest that SRC-1 repression of these target genes is not
26 dependent on multiple nuclear receptor interactions. Importantly, pathway analysis
27 revealed an over representation of these genes in development and differentiation
28 processes, suppression of which are essential for tumor development.

29 In this study we defined five genes with described roles in cellular development and
30 differentiation that are direct suppression targets of SRC-1. Aberrant methylation of these
31 genes in endocrine resistant models and in patient tumors was observed. Increased

1 expression of the maintenance and *de novo* methyltransferases, DNMT1 and DNMT3a,
2 were seen in the presence of SRC-1. Moreover the presence of the lysine methylator,
3 H3K27me is consistent with the established mechanistic link between DNA- and histone-
4 methylation (17) and is indicative of the epigenetic activity at these regions.

5 To elucidate the mechanism of repression and the regulatory link between
6 methylation and the steroid receptor transcriptional system we investigated the methylome
7 pertinent to the target genes. MBD2 and MECP2, members of the methyl binding domain
8 (MBD) protein family which deciphers the DNA methylation code (47), were both found to
9 be recruited to the target genes. Full suppression capacity of the MBD protein complex is
10 dependent however on histone deacetylation (20). Recruitment to the target genes of the
11 histone deacetylator, HDAC2, a known MECP2 binding partner (20), was also observed. The
12 dependence of this regulatory methylome on SRC-1 provides evidence of the central role of
13 this transcriptional protein in coordinating these epigenetic events.

14 De-repression of the SRC-1 epi-silenced genes influences a number of key functional
15 pathways whose deregulation is a facet of endocrine treatment resistant phenotype.
16 Integration with existing patient datasets and patient survival data (39) revealed that
17 reduced expression of this gene set associated with poor outcome in tamoxifen-treated
18 population. This was not true for treatment naïve populations suggesting that this is a
19 feature of long-term endocrine treatment. Therefore, SRC-1 dependent methylated genes
20 identified here underline key molecular features that distinguish between good outcome
21 and poor outcome in endocrine treated ER+ve patients. Still, these interpretations warrant
22 further clinical investigation in larger independent cohorts as methylation of specific genes
23 have the power to be a valuable tool in the management of breast cancer (48).

24 In contrast to mutational modifications, epigenetic alterations are potentially
25 reversible (49). Demethylating agents have demonstrated therapeutic benefit at low dose
26 long-term treatments in solid tumors (50). However, these agents can have broad impacts
27 on gene expression and also the number of tumor-associated methylated genes could
28 impact its efficacy. To demonstrate methylation as a crucial mechanism of the aggressive
29 phenotype observed in our models, we show that DNA demethylator treatment re-
30 expressed SRC-1 suppression genes and significantly inhibited proliferation of *ex vivo*

1 endocrine resistant metastatic tumors. Promising observations reported here, warrant
2 further studies using a larger cohort of patient tumor samples, providing full clinical
3 relevance of these mechanisms in breast cancer patients.

4 Taken together data presented here link, for the first time, the key transcriptional
5 machinery of the endocrine resistant cell with global methyl-dependent gene suppression.
6 We demonstrate that SRC-1 is one of the key orchestrators of the endocrine resistant
7 methylome which has consequences that are both functionally and clinically relevant.

8

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20

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4

5

1 **Figure Legends**

2 **Figure 1. SRC-1 global gene methylation signature. (a)** Colorimetric density plot of the
 3 correlation between the global methylation profile of endocrine sensitive MCF7 (n=2) and
 4 endocrine resistant LY2 (n=3) cell lines and patient primary breast tumor with a matched
 5 brain metastatic tumor (RCS_4) using Roche SeqCap Epi targeted bisulfite methylation
 6 sequencing. The correlation plot demonstrates an asymmetric density distribution: LY2 cells
 7 and the brain metastatic tumor have increased hyper-methylation relative to the MCF7 cells
 8 and matched primary tumor sample, respectively. Circos plot of differentially methylated
 9 CpGs detected in brain metastasis compared to primary tumor. **(b)** Analysis of ER and SRC-1
 10 ChIP-seq in MCF7 (n=2) and LY2 (n=2) cells. Bar plot demonstrates a greater percentage of
 11 ER binding at CpG islands in the resistant LY2 cells in comparison to the sensitive MCF7 cells.
 12 ER/SRC-1 DNA binding shows that 74% of ER peaks have SRC-1 co-bound at CpG islands in
 13 LY2 treated cells in comparison to 45% in untreated cells. **(c)** The circos plot demonstrates
 14 the distribution of the differentially methylated regions of shNT (n=2) and shSRC-1 (n=2)
 15 MeDIP-seq in LY2 cells treated with 4-OHT across all chromosomes using Log2 fold change
 16 difference. A bar plot illustrates the differences in differentially methylated regions in shNT
 17 and shSRC-1. **(d)** A higher proportion of MeDIP-seq hypermethylated regions located
 18 adjacent to SRC-1 ChIP-seq peaks (<2Kb) are observed in LY2 shNT cells in comparison to LY2
 19 shSRC-1 cells. **(e)** Volcano plot illustrating the differentially expressed genes between shNT
 20 and shSRC-1 RNA-seq from 4-OHT treated LY2 cells (fold change > 1, adjusted p-value <0.05,
 21 n=4). **(f)** The 736 genes down regulated in shNT (from RNA-seq analysis, Table S1) were
 22 filtered and 251 genes were identified within 5kb upstream of the transcription start site
 23 (ChIP-seq analysis, Table S2), from 251 genes, nine genes were found with adjacent
 24 differentially methylated regions (MeDIP-seq analysis). The chart illustrates the work flow
 25 from which nine genes were identified with known role in development and differentiation.

26 **Figure 2. SRC-1 tumorigenic potential, mirrored by NTRK2, NR2F2, CTDPI, SETBP1 and**
 27 **POU3F2 functional role in tumorigenic suppression. (a)** In SRC-1 CRISPR/Cas9 KO cells
 28 (clone 9c and 7c) the CD24⁺CD44⁻ differentiated cell population is significantly increased
 29 compared to control endocrine resistant LY2 luc cell lines (n=3). In contrast CD24⁺CD44⁻ and
 30 CD49f⁻Epcam⁺ differentiated cell population is significantly decreased after siRNA knock
 31 down of NTRK2 and POU3F2 and NTRK2, NR2F2, CTDPI and POU3F2 respectively in LY2

1 shSRC-1 cells (n=3). **(b)** LY2 SRC-1 CRISPR/Cas9 knock out clones 9c and 7c have significantly
 2 less mammosphere forming efficiency compared to control LY2 luc cell line (n=3), whilst the
 3 siRNA knock down of NTRK2, NR2F2, CTDP1, SETBP1 and POU3F2 in LY2 shSRC-1 cells
 4 significantly increases the cell lines mammosphere forming efficiency compared to LY2
 5 shSRC-1 control (n=3). **(c)** Anchorage independent growth in LY2 cell lines was significantly
 6 reduced in the absence of SRC-1 (LY2 SRC-1 CRISPR/Cas9 KO 9c and 7c) (n=3). Anchorage
 7 independent growth was significantly increased in all pro-differentiation genes, NTRK2,
 8 NR2F2, CTDP1, SETBP1 and POU3F2, after siRNA in LY2 shSRC-1 cells compared to scramble
 9 control (n=3) **(d)** Bar plot and representative images of acini formation from LY2 SRC-1
 10 CRISPR/Cas9 KO (clone 9c and 7c) showed more organized acini with superior apico-
 11 basolateral structure compared to wild-type LY2 cells (n=3). In contrast, siRNA knock down
 12 of the pro-differentiation genes, NTRK2, NR2F2, CTDP1, SETBP1 and POU3F2, showed
 13 decreased level of organization relative to the SRC-1 control cell line (LY2 shSRC-1) (n=3) .
 14 Phalloidin 594 (pink color) stains F-actin and DAPI (blue color) stains the nucleus. Organised
 15 acini structures were defined based upon presence of hollow lumen and structured apico-
 16 basolateral layer.

17 **(e)** Scratch assay showed that LY2 SRC-1 CRISPR/Cas9 KO (clone 9c and 7c) were
 18 significantly less motile in comparison to wild type LY2 luc cells (n=3). Knock down of NTRK2,
 19 NR2F2, CTDP1, SETBP1 and POU3F2 in the absence of SRC-1 (LY2 shSRC-1) migratory
 20 capacity of the cells compared to siNT cells (fluorescent bead assay, n= 3). Results are
 21 expressed as mean \pm SEM, *p<0.05, **p<0.01, ***p<0.001.

22 **Figure 3. NTRK2, NR2F2, CTDP1, SETBP1, and POU3F2 expression in breast cancer patients.**

23 **(a)** GOBO analysis of combined expression of NTRK2, NR2F2, CTDP1, SETBP1 and POU3F2, in
 24 PAM50 subtypes of breast cancer tumors, showed significantly higher expression in Luminal
 25 A subtype, (p<0.00001). Analysis of combined expression of NTRK2, NR2F2, CTDP1, SETBP1
 26 and POU3F2 stratified by estrogen receptor status in all tumors, showed significantly higher
 27 expression of these pro-differentiation genes in the ER positive tumors compared to ER
 28 negative tumors, (p<0.00001). **(b)** Ranked SRC-1 target gene set expression in 669 primary
 29 breast tumors from ER positive 4-OHT-treated patients (39). Colors are log2 mean-centered
 30 values, Red=high, Green=low. Data is from four published Affymetrix microarray datasets
 31 (GSE6532, GSE9195, GSE17705, GSE12093) summarized with Ensembl alternative CDF and

1 normalized with Robust Multi-array Average (RMA), before integration using ComBat to
2 remove dataset-specific bias. White–gray–black bars indicate significance of all possible cut
3 points from $P = 1$ to 0.001. **(c)** Kaplan–Meier analysis of distant metastatic free survival
4 (DMFS) according to expression of the SRC-1 pro-differentiation target genes in ER positive
5 4-OHT-treated patients (n=669) and Kaplan-Meier analysis of recurrence free survival (RFS)
6 in untreated patients (n=343). **(d)** Schematic of the *ex vivo* experimental set up. **(e)** Tumors
7 extracted from LY2 xenografts were assessed for proliferation by immunohistochemical
8 analysis of Ki67 quantified using the Aperio digital pathology imaging, shows significantly
9 less Ki67 in RG108 and RG108/4-OHT treated groups compared to DMSO control, (n=10
10 images/group). **(f)** Patient breast cancer brain metastatic tumor explants (T347x, T638x)
11 were assessed for Ki67 expression and quantified using the Aperio digital pathology imaging.
12 The results shows significantly less Ki67 positivity in the RG-108 treated group and in
13 RG108/4-OHT treated groups in both T347x and T638x tumor explant compared to DMSO
14 control (n=10 images/group). Ki67 positive cells indicated with red triangles and negative
15 cells indicated with a green triangle. Results are expressed as mean \pm SEM, * $p < 0.05$,
16 ** $p < 0.01$, *** $p < 0.001$.

17 **Figure 4. SRC-1 in combination with a complex of methylators drives repressive state of**
18 **NTRK2, NR2F2, CTDP1, SETBP1 and POU3F2.** **(a)** Differentially methylated regions (DMR) of
19 SRC-1 pro-differentiation genes were identified with SeqCap Epi sequencing by comparing
20 primary breast tumor with matched brain metastatic tumor (case RCS_4). Plot shows
21 regions of hypermethylation (red) and hypomethylation (blue) (meth.diff 30%; q-value <
22 0.01) found in NTRK2, NR2F2, CTDP1, SETBP1 and POU3F2 genes. Tracks show: CpGs probed
23 (purple), percentage of Methylation in LY2 cell line (green), differential methylation in brain
24 metastatic patient over primary (case RCS_4) (grey), % Methylation in case RCS_4 prior to
25 differential analysis (light grey), SRC-1 Chipset peaks in 4-OHT treated LY2 cells (yellow),
26 SRC-1 ChIP-seq peaks in untreated LY2 cells (orange) and RefSeq HG19 gene model. **(b)**
27 mRNA expression levels of DNA methyl transferases in the presence of SRC-1 (LY2 shNT)
28 compared to its absence (shSRC-1). Expression of de novo transferases DNMT1 and
29 DNMT3A are significantly increased in the presence of SRC-1 (shNT) in comparison to
30 silenced (shSRC-1) cells (n=3). **(c)** ChIP assay showed significantly higher recruitment of
31 histone repression marker H3K27me3 to pro-differentiation genes in 4-OHT treated LY2 cells

1 over IgG. **(d)** CHIP assays showed significantly higher recruitment of transcription regulators,
2 SRC-1 and ER over IgG. CHIP assay of PR recruitment to the target genes is included. **(e)**
3 Recruitment of methylators, MBD2 and MECP2, to NTRK2, NR2F2, CTDP1, SETBP1 and
4 POU3F2, in 4-OHT treated LY2 cells over IgG (n=3). **(f)** CHIP-re-CHIP assay of SRC-1-MBD2 and
5 SRC-1 MECP2 occupancy over SRC-1-IgG control at each of the target genes. **(g)** MBD2 and
6 MECP2 recruitment to pro-differentiation genes in LY2 SRC-1 CRISPR/Cas9 KO (clone 7c)
7 relative to LY2-luc parental cell line (n=3). **(h)** CHIP assays shows significantly higher
8 recruitment of HDAC2 to the NTRK2, NR2F2, CTDP1, and SETBP1 in LY2 cells over IgG, which
9 is significantly reduced in LY2 SRC-1 CRISPR/Cas9 KO (clone 7c) cells (n=3) compared to LY2
10 luc in NR2F2, CTDP1, SETBP1 and POU3F2. **(i)** Heatmap demonstrating relative DNA
11 recruitment of ER, SRC-1 and methyl proteins to the SRC-1 pro-differentiation genes in LY2
12 cells. Cartoon illustrating complex recruitment of regulatory proteins driving methylation
13 and subsequent repression to SRC-1 pro-differentiation genes. Results are expressed as
14 mean \pm SEM, *p<0.05, **p<0.01, ***p<0.001.

Table 1. Pro-differentiation genes repressed by SRC-1- dependent DNA methylation

<i>Gene</i>	<i>Ensemble ID</i>	<i>Gene Function</i>	<i>Methyl marks</i>
<i>NTRK2</i>	ENSG00000148053	Regulation of neuron survival, proliferation, migration and differentiation	Intronic
<i>NR2F2</i>	ENSG00000185551	Nuclear receptor involved in neuronal differentiation	Downstream (4kb) of TSS and upstream (380kb)
<i>CTDP1</i>	ENSG00000060069	Regulates RNA polymerase II, cellular organisation and differentiation	Intronic near TSS/intergenic
<i>SETBP1</i>	ENSG00000152217	DNA replication, differentiation	Intronic
<i>POU3F2</i>	ENSG00000184486	Differentiation	2.5kb upstream of promoter
<i>MMP16</i>	ENSG00000156103	Involved in the breakdown of extracellular matrix in normal physiological processes	Intergenic, intronic and exon
<i>NELL2</i>	ENSG00000184613	Cell growth regulation	Intronic
<i>RASD1</i>	ENSG00000108551	Negatively regulates the transcription regulation activity of the APBB1/FE65-APP complex via its interaction with APBB1/FE65	Intergenic
<i>SDK1</i>	ENSG00000146555	Adhesion molecule that promotes lamina-specific synaptic connections in the retina	Intronic and exon







