



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

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

### Citation for published version:

Ward, E, Vareslija, D, Charmsaz, S, Fagan, A, Browne, AL, Cosgrove, N, Cocchiglia, S, Purcell, S, Hudson, L, Das, S, O'Connor, D, O'Halloran, PJ, Sims, AH, Hill, AD & Young, LS 2018, 'Epigenome-wide SRC-1 mediated gene silencing represses cellular differentiation in advanced breast cancer', *Clinical Cancer Research*. <https://doi.org/10.1158/1078-0432.CCR-17-2615>

### Digital Object Identifier (DOI):

[10.1158/1078-0432.CCR-17-2615](https://doi.org/10.1158/1078-0432.CCR-17-2615)

### Link:

[Link to publication record in Edinburgh Research Explorer](#)

### Document Version:

Peer reviewed version

### Published In:

Clinical Cancer Research

### General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

### Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact [openaccess@ed.ac.uk](mailto:openaccess@ed.ac.uk) providing details, and we will remove access to the work immediately and investigate your claim.



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

3   Ward E<sup>1†</sup>, Varešlija D<sup>1†</sup>, Charmsaz S<sup>1</sup>, Fagan A<sup>1</sup>, Browne AL<sup>1</sup>, Cosgrove N<sup>1</sup>, Cocchiglia S<sup>1</sup>, Purcell S<sup>1</sup>,  
4   Hudson L<sup>1</sup>, Das S<sup>2</sup>, O'Connor D<sup>2</sup>, O'Halloran PJ<sup>3</sup>, Sims A<sup>4</sup>, Hill AD<sup>1</sup>, Young LS<sup>1\*</sup>

5   <sup>†</sup>These authors contributed equally to this study

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

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

10   <sup>3</sup>Department of Neurosurgery, National Neurosurgical Center, Beaumont Hospital, Dublin, Ireland

11   <sup>4</sup> 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

16   **Key words:** breast cancer, DNA methylation, SRC-1, endocrine resistant breast cancer, hormone-  
17   dependent cancer, estrogen receptor, metastasis

18   **Conflict of interest:** The authors declare there is no conflict of interest.

19   **Word Count:** 4959

20   **Total number of figures and/or tables:** 5

21  
22  
23  
24

## 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.

14

15

16

17

18

19

20

21

22

23

24

25

26

## 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.

25

26

27

28

## 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), CTD1 (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  $\beta$ -actin (401846), NTRK2 (Hs00178811), NR2F2  
23 (Hs00819630), CTD1 (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  $\mu$ 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 HPRC (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, CTD1, 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, CTD1, 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<sup>+</sup>/44<sup>-</sup> 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, CTD1, SETBP1 and POU3F2 (Fig. S5b). Silencing of NTRK2 and  
24 POU3F2 significantly reduced the number of CD24<sup>+</sup>/44 differentiated cells (Fig. 2a). In these  
25 endocrine resistant cells, knockout of SRC-1 resulted in reduced self-renewal capacity (2<sup>nd</sup>  
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, CTDP1, 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, CTDP1, 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 H3K27me<sub>3</sub>, 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

9 **Acknowledgements:** We kindly acknowledge the funding support from Irish Cancer Society  
10 Collaborative Cancer Research Centre grant, CCRC13GAL, Breast Cancer Research  
11 Foundation, Science Foundation Ireland and Breast Cancer Ireland

12 **Author Contributions:** Study Concept and Design (E.W, D.V.,A.F, L.S.Y.); Acquisition,  
13 analysis, or interpretation of data (E.W, D.V.,S.C.,A.F, A.L.B, N.C, S.C, S.P,A.S, L.S.Y.),  
14 Bioinformatic analyses (E.W,,A.F.,N.C.,A.S); Provision of administrative, technical, or  
15 material support (S.C.,S.P., L.H.,S.D.,D.P.O’C.,P.J.O’H,A.D.K.H.); Drafting of the manuscript  
16 (E.W., D.V., S.C., S.C., L.S.Y.), Critical revision of the manuscript (All Authors); Study  
17 Supervision (A.D.H,L.S.Y.).

18 **Competing financial interests:** The authors declare there are no related competing financial  
19 interests.

20

## 21 **References**

- 22 1. Clarke R, Tyson JJ, Dixon JM. Endocrine resistance in breast cancer--An overview and  
23 update. *Molecular and cellular endocrinology* **2015**;418 Pt 3:220-34 doi  
24 10.1016/j.mce.2015.09.035.
- 25 2. Jeselsohn R, Buchwalter G, De Angelis C, Brown M, Schiff R. ESR1 mutations as a  
26 mechanism for acquired endocrine resistance in breast cancer. *Nature reviews*  
27 *Clinical oncology* **2015**;12(10):573-83 doi 10.1038/nrclinonc.2015.117.

- 1 3. McBryan J, Fagan A, McCartan D, Bane FT, Vareslija D, Cocchiglia S, *et al.*  
2 Transcriptomic Profiling of Sequential Tumors from Breast Cancer Patients Provides a  
3 Global View of Metastatic Expression Changes Following Endocrine Therapy. *Clinical*  
4 *cancer research : an official journal of the American Association for Cancer Research*  
5 **2015**;21(23):5371-9 doi 10.1158/1078-0432.CCR-14-2155.
- 6 4. Vareslija D, McBryan J, Fagan A, Redmond AM, Hao Y, Sims AH, *et al.* Adaptation to  
7 AI Therapy in Breast Cancer Can Induce Dynamic Alterations in ER Activity Resulting  
8 in Estrogen-Independent Metastatic Tumors. *Clinical cancer research : an official*  
9 *journal of the American Association for Cancer Research* **2016**;22(11):2765-77 doi  
10 10.1158/1078-0432.CCR-15-1583.
- 11 5. Qin L, Liu Z, Chen H, Xu J. The steroid receptor coactivator-1 regulates twist  
12 expression and promotes breast cancer metastasis. *Cancer Res* **2009**;69(9):3819-27  
13 doi 0008-5472.CAN-08-4389 [pii] 10.1158/0008-5472.CAN-08-4389.
- 14 6. Xu J, Qiu Y, DeMayo FJ, Tsai SY, Tsai MJ, O'Malley BW. Partial hormone resistance in  
15 mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene. *Science*  
16 **1998**;279(5358):1922-5.
- 17 7. Xu J, Wu RC, O'Malley BW. Normal and cancer-related functions of the p160 steroid  
18 receptor co-activator (SRC) family. *Nature reviews Cancer* **2009**;9(9):615-30 doi  
19 10.1038/nrc2695.
- 20 8. McBryan J, Theissen SM, Byrne C, Hughes E, Cocchiglia S, Sande S, *et al.* Metastatic  
21 progression with resistance to aromatase inhibitors is driven by the steroid receptor  
22 coactivator SRC-1. *Cancer Res* **2012**;72(2):548-59 doi 10.1158/0008-5472.CAN-11-  
23 2073.
- 24 9. Redmond AM, Bane FT, Stafford AT, McIlroy M, Dillon MF, Crotty TB, *et al.*  
25 Coassociation of estrogen receptor and p160 proteins predicts resistance to  
26 endocrine treatment; SRC-1 is an independent predictor of breast cancer recurrence.  
27 *Clinical cancer research : an official journal of the American Association for Cancer*  
28 *Research* **2009**;15(6):2098-106 doi 10.1158/1078-0432.CCR-08-1649.
- 29 10. Al-azawi D, Ilroy MM, Kelly G, Redmond AM, Bane FT, Cocchiglia S, *et al.* Ets-2 and  
30 p160 proteins collaborate to regulate c-Myc in endocrine resistant breast cancer.  
31 *Oncogene* **2008**;27(21):3021-31 doi 10.1038/sj.onc.1210964.

- 1 11. Zhao SS, Geybels MS, Leonardson A, Rubicz R, Kolb S, Yan QX, *et al.* Epigenome-Wide  
2 Tumor DNA Methylation Profiling Identifies Novel Prognostic Biomarkers of  
3 Metastatic-Lethal Progression in Men Diagnosed with Clinically Localized Prostate  
4 Cancer. *Clinical Cancer Research* **2017**;23(1):311-9 doi 10.1158/1078-0432.CCR-16-  
5 0549.
- 6 12. Visvanathan K, Fackler MS, Zhang Z, Lopez-Bujanda ZA, Jeter SC, Sokoll LJ, *et al.*  
7 Monitoring of Serum DNA Methylation as an Early Independent Marker of Response  
8 and Survival in Metastatic Breast Cancer: TBCRC 005 Prospective Biomarker Study. *J*  
9 *Clin Oncol* **2017**;35(7):751-+ doi 10.1200/Jco.2015.66.2080.
- 10 13. Priedigkeit N, Hartmaier RJ, Chen Y, Vareslija D, Basudan A, Watters RJ, *et al.* Intrinsic  
11 Subtype Switching and Acquired ERBB2/HER2 Amplifications and Mutations in Breast  
12 Cancer Brain Metastases. *JAMA oncology* **2016** doi 10.1001/jamaoncol.2016.5630.
- 13 14. Eden A, Gaudet F, Waghmare A, Jaenisch R. Chromosomal instability and tumors  
14 promoted by DNA hypomethylation. *Science* **2003**;300(5618):455 doi  
15 10.1126/science.1083557.
- 16 15. Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW, *et al.*  
17 Induction of tumors in mice by genomic hypomethylation. *Science*  
18 **2003**;300(5618):489-92 doi 10.1126/science.1083558.
- 19 16. Ting AH, McGarvey KM, Baylin SB. The cancer epigenome--components and  
20 functional correlates. *Genes & development* **2006**;20(23):3215-31 doi  
21 10.1101/gad.1464906.
- 22 17. Cedar H, Bergman Y. Linking DNA methylation and histone modification: patterns  
23 and paradigms. *Nature reviews Genetics* **2009**;10(5):295-304 doi 10.1038/nrg2540.
- 24 18. Rountree MR, Bachman KE, Baylin SB. DNMT1 binds HDAC2 and a new co-repressor,  
25 DMAP1, to form a complex at replication foci. *Nature genetics* **2000**;25(3):269-77 doi  
26 10.1038/77023.
- 27 19. Schlesinger Y, Straussman R, Keshet I, Farkash S, Hecht M, Zimmerman J, *et al.*  
28 Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de  
29 novo methylation in cancer. *Nature genetics* **2007**;39(2):232-6 doi 10.1038/ng1950.
- 30 20. Nan X, Ng HH, Johnson CA, Laherty CD, Turner BM, Eisenman RN, *et al.*  
31 Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a  
32 histone deacetylase complex. *Nature* **1998**;393(6683):386-9 doi 10.1038/30764.

- 1 21. Liu MY, DeNizio JE, Schutsky EK, Kohli RM. The expanding scope and impact of  
2 epigenetic cytosine modifications. *Current opinion in chemical biology* **2016**;33:67-  
3 73 doi 10.1016/j.cbpa.2016.05.029.
- 4 22. Mellen M, Ayata P, Dewell S, Kriaucionis S, Heintz N. MeCP2 binds to 5hmC enriched  
5 within active genes and accessible chromatin in the nervous system. *Cell*  
6 **2012**;151(7):1417-30 doi 10.1016/j.cell.2012.11.022.
- 7 23. Bogdanovic O, Veenstra GJ. DNA methylation and methyl-CpG binding proteins:  
8 developmental requirements and function. *Chromosoma* **2009**;118(5):549-65 doi  
9 10.1007/s00412-009-0221-9.
- 10 24. Chimonidou M, Tzitzira A, Strati A, Sotiropoulou G, Sfikas C, Malamos N, *et al.* CST6  
11 promoter methylation in circulating cell-free DNA of breast cancer patients. *Clinical*  
12 *biochemistry* **2013**;46(3):235-40 doi 10.1016/j.clinbiochem.2012.09.015.
- 13 25. Xu Z, Bolick SC, DeRoo LA, Weinberg CR, Sandler DP, Taylor JA. Epigenome-wide  
14 association study of breast cancer using prospectively collected sister study samples.  
15 *Journal of the National Cancer Institute* **2013**;105(10):694-700 doi  
16 10.1093/jnci/djt045.
- 17 26. Fackler MJ, Umbricht CB, Williams D, Argani P, Cruz LA, Merino VF, *et al.* Genome-  
18 wide methylation analysis identifies genes specific to breast cancer hormone  
19 receptor status and risk of recurrence. *Cancer Res* **2011**;71(19):6195-207 doi  
20 10.1158/0008-5472.CAN-11-1630.
- 21 27. Fang F, Turcan S, Rimner A, Kaufman A, Giri D, Morris LG, *et al.* Breast cancer  
22 methylomes establish an epigenomic foundation for metastasis. *Science*  
23 *translational medicine* **2011**;3(75):75ra25 doi 10.1126/scitranslmed.3001875.
- 24 28. Holm K, Hegardt C, Staaf J, Vallon-Christersson J, Jonsson G, Olsson H, *et al.*  
25 Molecular subtypes of breast cancer are associated with characteristic DNA  
26 methylation patterns. *Breast cancer research : BCR* **2010**;12(3):R36 doi  
27 10.1186/bcr2590.
- 28 29. Fan M, Yan PS, Hartman-Frey C, Chen L, Paik H, Oyer SL, *et al.* Diverse gene  
29 expression and DNA methylation profiles correlate with differential adaptation of  
30 breast cancer cells to the antiestrogens tamoxifen and fulvestrant. *Cancer Res*  
31 **2006**;66(24):11954-66 doi 10.1158/0008-5472.CAN-06-1666.

- 1 30. Stone A, Zotenko E, Locke WJ, Korbie D, Millar EK, Pidsley R, *et al.* DNA methylation  
2 of oestrogen-regulated enhancers defines endocrine sensitivity in breast cancer.  
3 Nature communications **2015**;6:7758 doi 10.1038/ncomms8758.
- 4 31. Badia E, Duchesne MJ, Semlali A, Fuentes M, Giamarchi C, Richard-Foy H, *et al.* Long-  
5 term hydroxytamoxifen treatment of an MCF-7-derived breast cancer cell line  
6 irreversibly inhibits the expression of estrogenic genes through chromatin  
7 remodeling. Cancer Res **2000**;60(15):4130-8.
- 8 32. Nguyen VT, Barozzi I, Faronato M, Lombardo Y, Steel JH, Patel N, *et al.* Differential  
9 epigenetic reprogramming in response to specific endocrine therapies promotes  
10 cholesterol biosynthesis and cellular invasion. Nature communications **2015**;6:10044  
11 doi 10.1038/ncomms10044.
- 12 33. Bronzert DA, Greene GL, Lippman ME. Selection and characterization of a breast  
13 cancer cell line resistant to the antiestrogen LY 117018. Endocrinology  
14 **1985**;117(4):1409-17 doi 10.1210/endo-117-4-1409.
- 15 34. Vareslija D, Cocchiglia S, Byrne C, Young L. Patient-Derived Xenografts of Breast  
16 Cancer. Methods in molecular biology **2017**;1501:327-36 doi 10.1007/978-1-4939-  
17 6475-8\_17.
- 18 35. Walsh CA, Bolger JC, Byrne C, Cocchiglia S, Hao Y, Fagan A, *et al.* Global Gene  
19 Repression by the Steroid Receptor Coactivator SRC-1 Promotes Oncogenesis.  
20 Cancer Research **2014**;74:2533-44 doi 10.1158/0008-5472.CAN-13-2133.
- 21 36. Browne AL, Charmsaz S, Varešlija D, Fagan A, Cosgrove N, Cocchiglia S, *et al.* Network  
22 analysis of SRC-1 reveals a novel transcription factor hub which regulates endocrine  
23 resistant breast cancer. Oncogene **2018** doi 10.1038/s41388-017-0042-x.
- 24 37. Charmsaz S, Hughes E, Bane FT, Tibbitts P, McIlroy M, Byrne C, *et al.* S100beta as a  
25 serum marker in endocrine resistant breast cancer. BMC medicine **2017**;15(1):79 doi  
26 10.1186/s12916-017-0836-2.
- 27 38. McCartan D, Bolger JC, Fagan A, Byrne C, Hao Y, Qin L, *et al.* Global characterization  
28 of the SRC-1 transcriptome identifies ADAM22 as an ER-independent mediator of  
29 endocrine-resistant breast cancer. Cancer Res **2012**;72(1):220-9 doi 10.1158/0008-  
30 5472.CAN-11-1976.
- 31 39. Simoes BM, O'Brien CS, Eyre R, Silva A, Yu L, Sarmiento-Castro A, *et al.* Anti-estrogen  
32 Resistance in Human Breast Tumors Is Driven by JAG1-NOTCH4-Dependent Cancer

- 1 Stem Cell Activity. Cell reports **2015**;12(12):1968-77 doi  
2 10.1016/j.celrep.2015.08.050.
- 3 40. Ross-Innes CS, Stark R, Teschendorff AE, Holmes KA, Ali HR, Dunning MJ, *et al.*  
4 Differential oestrogen receptor binding is associated with clinical outcome in breast  
5 cancer. Nature **2012**;481(7381):389-93 doi nature10730 [pii] 10.1038/nature10730.
- 6 41. Kittler R, Zhou J, Hua S, Ma L, Liu Y, Pendleton E, *et al.* A comprehensive nuclear  
7 receptor network for breast cancer cells. Cell reports **2013**;3(2):538-51 doi  
8 10.1016/j.celrep.2013.01.004.
- 9 42. Park S, Koo J, Park HS, Kim JH, Choi SY, Lee JH, *et al.* Expression of androgen  
10 receptors in primary breast cancer. Ann Oncol **2010**;21(3):488-92 doi  
11 10.1093/annonc/mdp510.
- 12 43. Shen H, Laird PW. Interplay between the cancer genome and epigenome. Cell  
13 **2013**;153(1):38-55 doi 10.1016/j.cell.2013.03.008.
- 14 44. Tishkov I, Mushmov D, Milkov V, Lazarov V, Rapundzhieva A. The treatment of  
15 patients with idiopathic membranous glomerulonephritis with cyclosporin A.  
16 Vutreshni bolesti **1989**;28(4):104-6.
- 17 45. Lin X, Li J, Yin G, Zhao Q, Elias D, Lykkesfeldt AE, *et al.* Integrative analyses of gene  
18 expression and DNA methylation profiles in breast cancer cell line models of  
19 tamoxifen-resistance indicate a potential role of cells with stem-like properties.  
20 Breast cancer research : BCR **2013**;15(6):R119 doi 10.1186/bcr3588.
- 21 46. Uhlenhaut NH, Barish GD, Yu RT, Downes M, Karunasiri M, Liddle C, *et al.* Insights  
22 into negative regulation by the glucocorticoid receptor from genome-wide profiling  
23 of inflammatory cistromes. Molecular cell **2013**;49(1):158-71 doi  
24 10.1016/j.molcel.2012.10.013.
- 25 47. Fatemi M, Wade PA. MBD family proteins: reading the epigenetic code. Journal of  
26 cell science **2006**;119(Pt 15):3033-7 doi 10.1242/jcs.03099.
- 27 48. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev  
28 Genet **2002**;3:415-28.
- 29 49. West AC, Johnstone RW. New and emerging HDAC inhibitors for cancer treatment.  
30 The Journal of clinical investigation **2014**;124(1):30-9 doi 10.1172/JCI69738.

- 1 50. Jones PA, Issa JP, Baylin S. Targeting the cancer epigenome for therapy. *Nature*
- 2 *reviews Genetics* **2016**;17(10):630-41 doi 10.1038/nrg.2016.93.

3

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, CTD1, SETBP1 and**  
 27 **POU3F2 functional role in tumorigenic suppression. (a)** In SRC-1 CRISPR/Cas9 KO cells  
 28 (clone 9c and 7c) the CD24<sup>+</sup>CD44<sup>-</sup> differentiated cell population is significantly increased  
 29 compared to control endocrine resistant LY2 luc cell lines (n=3). In contrast CD24<sup>+</sup>CD44<sup>-</sup> and  
 30 CD49<sup>f</sup>Epcam<sup>+</sup> differentiated cell population is significantly decreased after siRNA knock  
 31 down of NTRK2 and POU3F2 and NTRK2, NR2F2, CTD1 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

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









