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Translational Relevance

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

Abstract

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

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

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

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

Introduction

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

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

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

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

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

Methods

Cell culture

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

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

CRISPR/Cas9, Lentiviral transduction, siRNA transfection

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

Gene expression

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

Flow cytometry

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

analysis was carried out on the BD FACS Canto II (BD Biosciences). Cell lines which underwent gene silencing were analyzed by flow cytometry 48 hrs post siRNA transfection for with NTRK2, NR2F2, CTDP1, SETBP1, and POU3F2. Cells were stained with CD24 (555428; BD Biosciences), CD44 (555478; BD Biosciences), EpCam (12-9326-42, Thermofisher) and CD49f (17-0495-82, Thermofisher) antibodies. Data were analyzed using FlowJo Software (FlowJo, USA).

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

Functional assays were performed in the LY2 luc control cell line, LY2 SRC-1 CRISPR/Cas9 KO cells (clones 7c and 9c), and LY2 shSRC-1 cells 24 hrs post gene silencing with NTRK2, NR2F2, CTDP1, SETBP1, POU3F2. All functional assays were carried out with cells treated with 4-OHT [10^{-7} M].

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

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

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

Chromatin Immuno-precipitation (ChIP)

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

Immunohistochemistry

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

Explant studies

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

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

Sequencing Acquisition

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

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

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

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

Bioinformatic Analysis

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

Affymetrix microarray analysis

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

Statistical analysis

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

Data availability

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

Ethics

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

Results

SRC-1 global gene methylation signature in endocrine resistant breast cancer. Perturbations in DNA methylation profiles may influence tumor initiation, metastatic progression and resistance to treatment. To investigate global aberrant DNA methylation as

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

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

suppressed by SRC-1-dependent DNA methylation (Fig. 1f, Table 1). From these 9 genes NTRK2, NR2F2, CTDP1, SETBP1 and POU3F2 have described roles in cellular development and differentiation. ChIP and qPCR analysis confirmed these genes as direct SRC-1 targets (Fig. S3a, b).

Functional role of SRC-1 in disease progression in endocrine resistance is mirrored by the roles of NTRK2, NR2F2, CTDP1, SETBP1 and POU3F2 in tumor suppression. In endocrine treatment resistant cells SRC-1 is a known mediator of drug resistance phenotype (36,38). Loss of SRC-1 expression can lead to re-sensitization of endocrine resistant cell lines to tamoxifen treatment (Fig S4a,b).To assess the impact of SRC-1 and its suppressed pro-differentiation target genes on tumor progression we investigated the role of the coregulatory protein and each of the individual pro-differentiation genes in classic mechanisms of tumor aggression including stemness and migration. Expression levels of each of the 5 target genes are elevated in the absence of SRC-1 (LY2 shSRC-1) in comparison to the parental resistant cells (LY2 shNT) (Fig. S3b).

In endocrine resistant breast cancer cells CRISPR/Cas9 gene editing was used to specifically knockout SRC-1 (clone 9 (9c) and clone 7 (7c)) confirmed with mRNA (Fig S4a) and protein expression of SRC-1 (Fig. S4b). No effect on protein expression levels of SRC-2 or SRC-3 was observed (Fig S5a). Given relative importance of SRC-3 in breast cancer drug resistance and metastasis we further confirmed no upregulation of phosphorylated SRC-3 protein when SRC-1 is suppressed (Fig. S5c). SRC-1 CRISPR/Cas9 KO cells demonstrated enhanced differentiated $CD24^{+}/44^{-}$ cell populations in comparison to their control. siRNA was used in LY2 shSRC-1 cells to transiently silence the SRC-1 pro-differentiation target genes NTRK2, NR2F2, CTDP1, SETBP1 and POU3F2 (Fig. S5b). Silencing of NTRK2 and POU3F2 significantly reduced the number of $CD24^{+}/44$ differentiated cells (Fig. 2a). In these endocrine resistant cells, knockout of SRC-1 resulted in reduced self-renewal capacity (2^{nd} generation mammosphere), colony formation, cell organization and migration (Fig. 2b-e). In contrast, silencing of each of the SRC-1 target genes displayed loss of cell differentiation through increased self-renewal capacity and colony formation along with loss of cellular organization (Fig. 2b-d). Furthermore, silencing of the pro-differentiation genes elevated migratory capacity of the endocrine resistant breast cancer cells (Fig. 2e).

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

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

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

SRC-1 in combination with a complex of methylators represses expression of NTRK2, NR2F2, CTDP1, SETBP1 and POU3F2. We wanted to further delineate the mechanistic pathway involved. We interrogated the DNA methylation of the SRC-1 pro-differentiation genes by analyzing methyl sites from Seq Cap Epi data from endocrine resistant LY2 cells and matched primary and metastatic tumor from an endocrine resistant patient. The percentage methylation indicating the proportion of cytosine's methylated at each CpG probe is reported for the LY2 cells and metastatic brain tissue (Fig. 4a). Differential methylated regions were analyzed from the resistant metastatic tumor and the matched primary. Hypermethylated regions were identified upstream of NTRK2, CTDP1, SETBP1 and POU3F2 and a hypomethylated region upstream of NR2F2 (Fig. 4a). These regions corresponded to SRC-1 peaks as determined from global ChIP-seq analyses in LY2 cells (Fig. 4a).

The expression of the maintenance and *de novo* methyltransferases, DNMT1 and DMMT3a, respectively were found to be reduced in the absence of SRC-1 (LY2 shSRC-1) compared to the control (LY2 shNT) (Fig. 4b). As DNA and histone lysine methylation systems are highly interrelated and rely mechanistically on each other, we investigated histone methylation at the SRC-1 target genes. Consistent with this, elevated recruitment of the histone repression marker H3K27me3, a known mediator of *de novo* DNA hypermethylation, to the pro-differentiation genes was observed (Fig. 4c).

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

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

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

Discussion

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

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

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

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

expression of the maintenance and *de novo* methyltransferases, DNMT1 and DMMT3a, were seen in the presence of SRC-1. Moreover the presence of the lysine methylator, H3k27me is consistent with the established mechanistic link between DNA- and histone-methylation (17) and is indicative of the epigenetic activity at these regions.

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

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

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

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

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

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Figure Legends

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

Figure 2. SRC-1 tumorigenic potential, mirrored by NTRK2, NR2F2, CTDP1, SETBP1 and POU3F2 functional role in tumorigenic suppression. (a) In SRC-1 CRISPR/Cas9 KO cells (clone 9c and 7c) the $CD24^{+}CD44^{-}$ differentiated cell population is significantly increased compared to control endocrine resistant LY2 luc cell lines (n=3). In contrast $CD24^{+}CD44^{-}$ and $CD49f^{-}Epcam^{+}$differentiated cell population is significantly decreased after siRNA knock down of NTRK2 and POU3F2 and NTRK2, NR2F2, CTDP1 and POU3F2 respectively in LY2

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

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

Figure 3. NTRK2, NR2F2, CTDP1, SETBP1, and POU3F2 expression in breast cancer patients. **(a)** GOBO analysis of combined expression of NTRK2, NR2F2, CTDP1, SETBP1 and POU3F2, in PAM50 subtypes of breast cancer tumors, showed significantly higher expression in Luminal A subtype, (p<0.00001). Analysis of combined expression of NTRK2, NR2F2, CTDP1, SETBP1 and POU3F2 stratified by estrogen receptor status in all tumors, showed significantly higher expression of these pro-differentiation genes in the ER positive tumors compared to ER negative tumors, (p<0.00001). **(b)** Ranked SRC-1 target gene set expression in 669 primary breast tumors from ER positive 4-OHT-treated patients (39). Colors are log2 mean-centered values, Red=high, Green=low. Data is from four published Affymetrix microarray datasets (GSE6532, GSE9195, GSE17705, GSE12093) summarized with Ensembl alternative CDF and

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

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

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

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

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

Figure 1

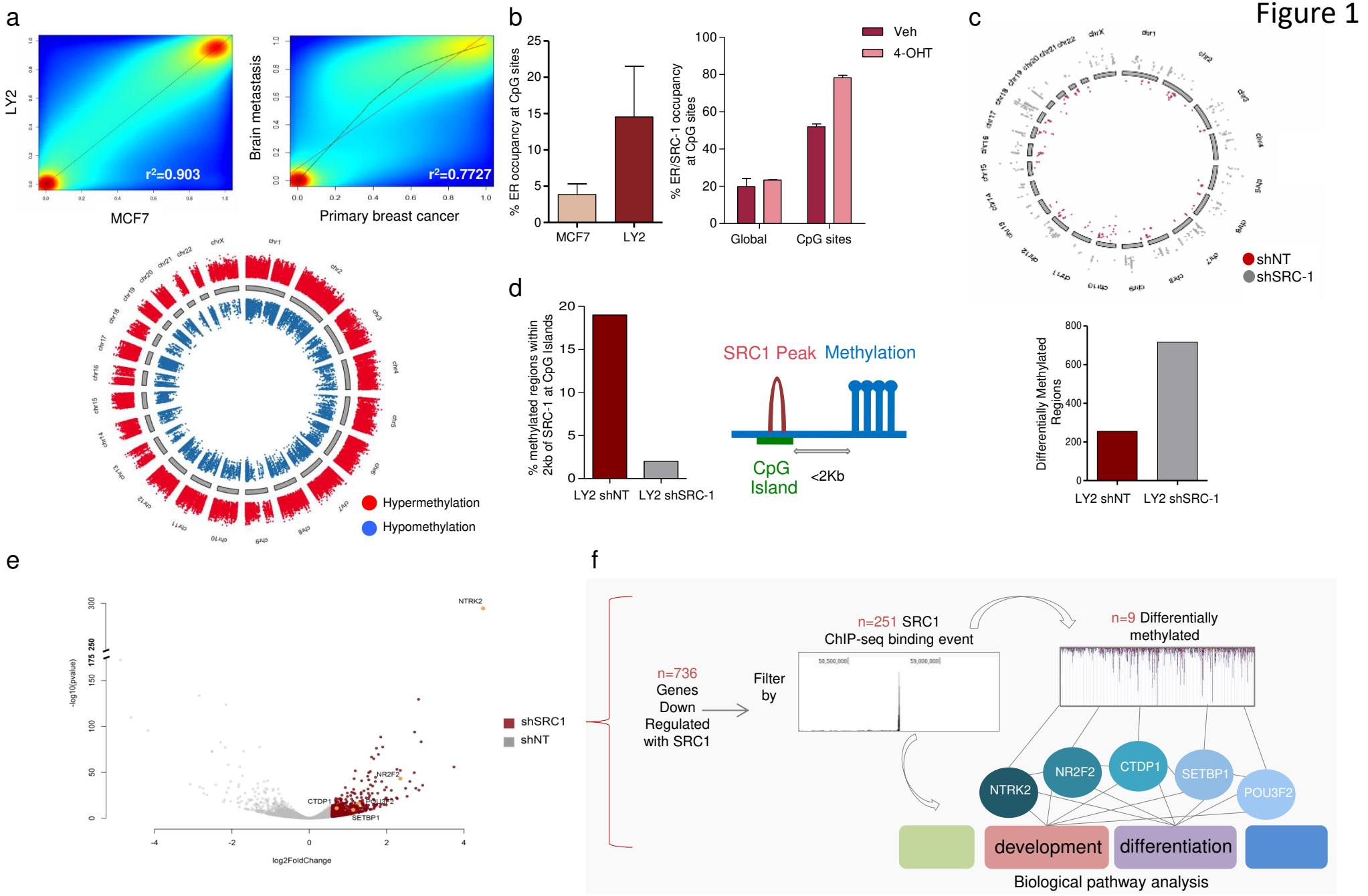

a
LY2
MCF7
r²=0.903
Brain metastasis
Primary breast cancer
r²=0.7727
Hypermethylation
Hypomethylation
b
% ER occupancy at CpG sites
MCF7
LY2
% ER/SRC-1 occupancy at CpG sites
Global
CpG sites
Veh
4-OHT
c
shNT
shSRC-1
d
% methylated regions within 2kb of SRC-1 at CpG Islands
LY2 shNT
LY2 shSRC-1
SRC1 Peak
Methylation
CpG Island
<2Kb
Differentially Methylated Regions
LY2 shNT
LY2 shSRC-1
e
-log10(pvalue)
log2FoldChange
NTRK2
NR2F2
CTDP1
SETBP1
shSRC1
shNT
f
n=736 Genes Down Regulated with SRC1
Filter by
n=251 SRC1 ChIP-seq binding event
n=9 Differentially methylated
NTRK2
NR2F2
CTDP1
SETBP1
POU3F2
development
differentiation
Biological pathway analysis

Figure 2

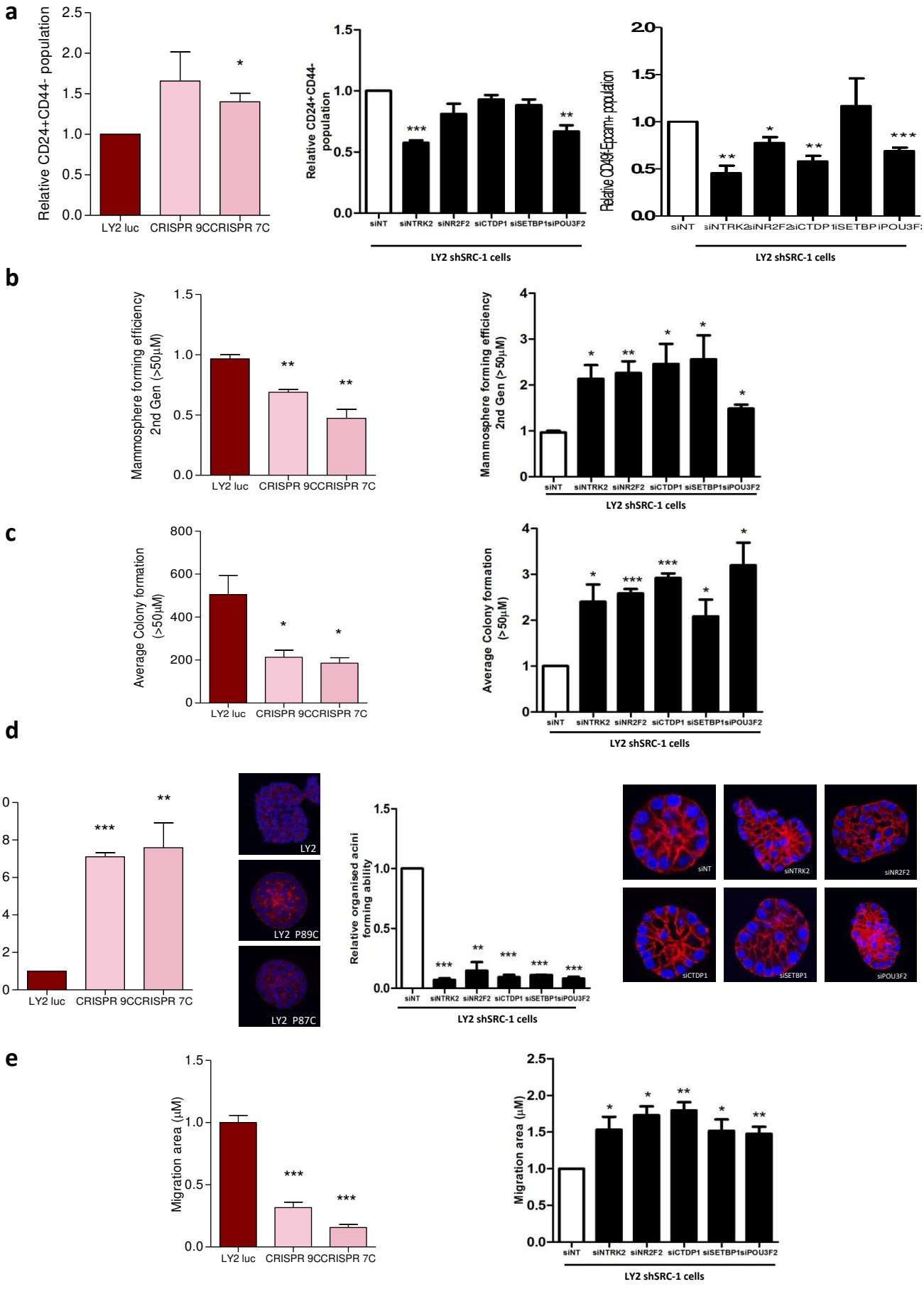

a
Relative CD24+CD44- population
LY2 luc
CRISPR 9C
CRISPR 7C
Relative CD24+CD44- population
siNT
siNTRK2
siNR2F2
siCTDP1
siSETBP1
siPOU3F2
LY2 shSRC-1 cells
Relative CD49f+Epcam+ population
b
Mammosphere forming efficiency 2nd Gen (>50μM)
c
Average Colony formation (>50μM)
d
Relative organised acini forming ability
LY2
LY2 P89C
LY2 P87C
e
Migration area (μM)

Figure 3

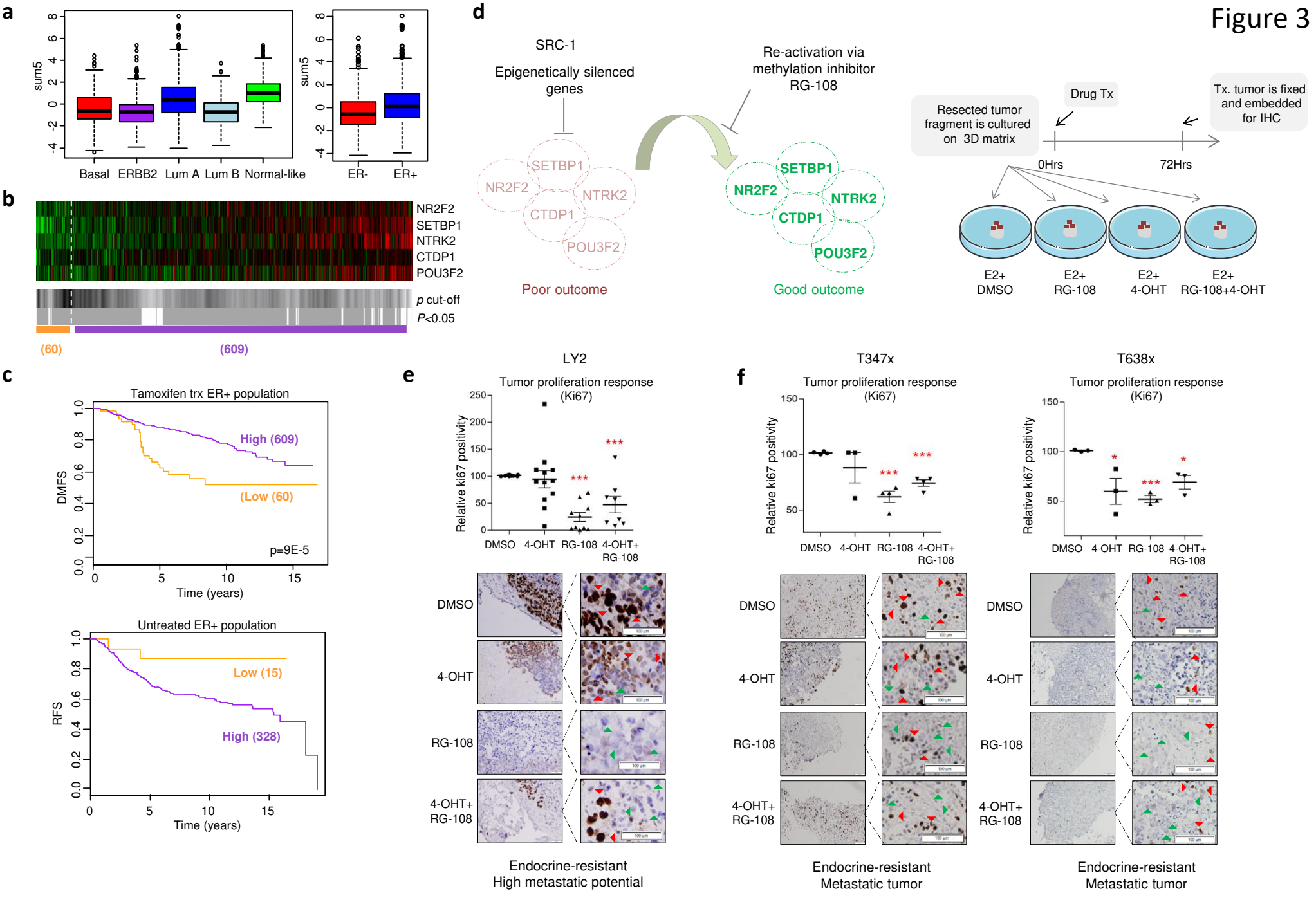
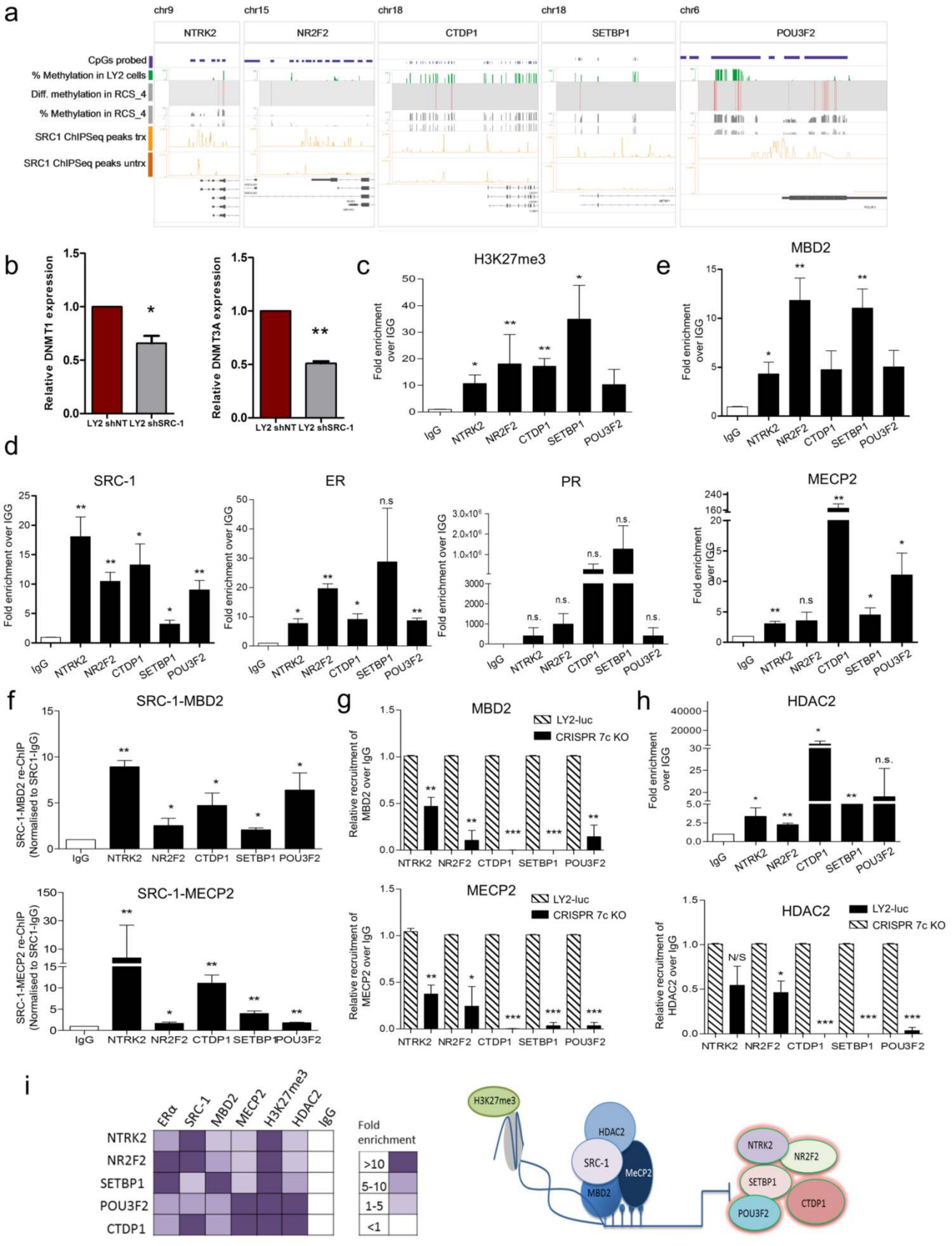

a
sum5
Basal
ERBB2
Lum A
Lum B
Normal-like
ER-
ER+
b
NR2F2
SETBP1
NTRK2
CTDP1
POU3F2
p cut-off
P<0.05
(60)
(609)
c
Tamoxifen trx ER+ population
DMFS
High (609)
(Low (60)
p=9E-5
Time (years)
Untreated ER+ population
RFS
Low (15)
High (328)
Time (years)
d
SRC-1
Epigenetically silenced genes
Re-activation via methylation inhibitor RG-108
SETBP1
NR2F2
NTRK2
CTDP1
POU3F2
Poor outcome
Good outcome
Resected tumor fragment is cultured on 3D matrix
Drug Tx
Tx. tumor is fixed and embedded for IHC
0Hrs
72Hrs
E2+ DMSO
E2+ RG-108
E2+ 4-OHT
E2+ RG-108+4-OHT
e
LY2
Tumor proliferation response (Ki67)
Relative ki67 positivity
DMSO
4-OHT
RG-108
4-OHT+ RG-108
Endocrine-resistant
High metastatic potential
f
T347x
T638x
Tumor proliferation response (Ki67)
Relative ki67 positivity
DMSO
4-OHT
RG-108
4-OHT+ RG-108
Endocrine-resistant
Metastatic tumor
Endocrine-resistant
Metastatic tumor

Figure 4


a
chr9
chr15
chr18
chr18
chr6
NTRK2
NR2F2
CTDP1
SETBP1
POU3F2
CpGs probed
% Methylation in LY2 cells
Diff. methylation in RCS_4
% Methylation in RCS_4
SRC1 ChIPSeq peaks trx
SRC1 ChIPSeq peaks untrx
b
Relative DNMT1 expression
LY2 shNT
LY2 shSRC-1
Relative DNMT3A expression
c
H3K27me3
Fold enrichment over IGG
IgG
NTRK2
NR2F2
CTDP1
SETBP1
POU3F2
e
MBD2
d
SRC-1
ER
PR
MECP2
f
SRC-1-MBD2
SRC-1-MBD2 re-ChIP (Normalised to SRC1-IgG)
SRC-1-MECP2
SRC-1-MECP2 re-ChIP (Normalised to SRC1-IgG)
g
MBD2
LY2-luc
CRISPR 7c KO
Relative recruitment of MBD2 over IgG
MECP2
Relative recruitment of MECP2 over IgG
h
HDAC2
Relative recruitment of HDAC2 over IgG
N/S
n.s.
i
ERα
SRC-1
MBD2
MECP2
H3K27me3
HDAC2
IgG
Fold enrichment
>10
5-10
1-5
<1
H3K27me3
HDAC2
SRC-1
MeCP2
MBD2
NTRK2
NR2F2
SETBP1
POU3F2
CTDP1