Inhibition of DNMT1 and ERRα crosstalk suppresses breast cancer via derepression of IRF4

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Abstract
DNA methylation is implicated in the acquisition of malignant phenotypes, and the use of epigenetic modulating drugs is a promising anti-cancer therapeutic strategy. 5-aza-2’deoxycytidine (decitabine, 5-azadC) is an FDA-approved DNA methyltransferase (DNMT) inhibitor with proven effectiveness against hematological malignancies and more recently triple-negative breast cancer (BC). Herein, genetic or pharmacological studies uncovered a hitherto unknown feedforward molecular link between DNMT1 and the estrogen related receptor α (ERRα), a key transcriptional regulator of cellular metabolism. Mechanistically, DNMT1 promotes ERRα stability which in turn couples DNMT1 transcription with that of the methionine cycle and S-adenosylmethionine synthesis to drive DNA methylation. In vitro and in vivo investigation using a pre-clinical mouse model of BC demonstrated a clear therapeutic advantage for combined administration of the ERRα inhibitor C29 with 5-azadC. A large-scale bisulfite genomic sequencing analysis revealed specific methylation perturbations fostering the discovery that reversal of promoter hypermethylation and consequently derepression of the tumor suppressor gene, IRF4, is a factor underlying the observed BC suppressive effects. This work thus uncovers a critical role of ERRα in the crosstalk between transcriptional control of metabolism and epigenetics and illustrates the potential for targeting ERRα in combination with DNMT inhibitors for BC treatment and other epigenetics-driven malignancies.

Introduction
Methylation of DNA is an evolutionarily conserved mechanism that allows control of gene expression by modulating chromatin accessibility to specific transcription factors (TFs) [1, 2]. This epigenetic process is crucial for proper mammalian development, essential for cellular differentiation and plays a determinant role in maintaining genomic stability [3–5]. Aberrant DNA methylation patterns have been observed in a large variety of diseases including obesity, diabetes, neurodegeneration, and cancer, thus prompting intense research to fully discern its regulatory modes and fuel the development of new therapeutic approaches [6–8].

In mammals, DNA methylation occurs at the fifth position of cytosine to produce 5-methylcytosine (5-mC). Maintenance of genomic methylation is ensured by the DNA methyltransferase DNMT1 which copies the DNA methylation pattern from the parental DNA strand onto the newly synthesized daughter strand during replication [9]. DNA methylation is also responsive and adaptive to environmental changes by modulating the expression of genes [10]. Two
other methyltransferases, DNMT3A and 3B, have been described to methylate DNA de novo. Conversely, the ten–eleven translocation (TET) enzymes 1, 2, and 3 have the capacity to demethylate the genome [11–13].

The methyl group needed for DNA methylation is typically derived from dietary methionine, which is metabolized through the methionine cycle upon entering the cell. In this pathway, a molecule of ATP is transferred to methionine for S-adenosylmethionine (SAM) synthesis, the primary methyl donor for most biological methylation reactions [14]. After the methyl group is donated, the resulting S-adenosylhomocysteine (SAH) is either recycled or metabolized into cysteine, a nonessential amino acid that serves as a key building block for glutathione synthesis. The demethylation process catalyzed by the TET enzymes is also dependent on metabolite levels, and requires α-ketoglutarate (αKG), which can be generated from the tricarboxylic acid (TCA) cycle and glutaminolysis [12]. While DNA methylation and demethylation are clearly intrinsically linked to metabolism, the mechanisms that coordinate these programs remain poorly understood.

In the context of breast cancer (BC), several aspects of cellular metabolism are controlled by members of the estrogen related receptor (ERR) family [15]. The ERRs are orphan nuclear receptors that regulate a large variety of metabolic gene networks implicated in glycolysis, glutaminolysis, mitochondrial biogenesis, and cellular respiration [16, 17]. Recently, we have shown that ERRα is also a transcriptional regulator of the folate cycle, a metabolic pathway closely related to the methionine cycle [18]. ERRα also plays a central role in regulating the adaptive metabolic processes used by breast tumors to thrive in conditions of fluctuating nutrient availability [19]. Furthermore, high ERRα transcript levels are associated with the HER2 positive and triple-negative (TN) molecular subtypes known to be among the most aggressive forms of the disease [20].

In this study, we identify ERRα as a direct link between cellular metabolism and DNA methylation. We first show that inhibition of ERRα activity diminishes the expression of methionine cycle enzymes and markedly reduces DNMT1 transcription resulting in a global loss of cellular DNA methylation. In a feedforward regulatory loop, DNMT1 elevated ERRα protein, and levels of DNMT1 mRNA correlated with high ERRα activity in BC patients. Importantly, pharmacological inhibition of ERRα further sensitized BC cells in vitro and in vivo to the anti-neoplastic effects of the DNMT inhibitor 5-aza-2'-deoxycytidine (decitabine, 5-AzaD). The clinical significance of our findings is further supported by genome-wide bisulfite sequencing, revealing that co-administration of ERRα and DNMT1 inhibitors leads to promoter demethylation and re-expression of IRF4-encoding Interferon Regulatory Factor-4 and found herein to exhibit tumor-suppressor activity in BC cells.

Results

**ERRα regulates the expression of enzymes of the methionine cycle and DNA methylation**

DNA methylation is dependent on cellular metabolic activity, specifically the methionine cycle (Fig. 1a), and considering that the ERRs are key transcriptional regulators of cell metabolism, we investigated whether ERRα is directly implicated in this process. To this end, we interrogated ERRα ChIP-seq datasets obtained in the BC cell lines BT474 and SKBR3 [21]. These cells are characterized by high expression of the receptor tyrosine kinase HER2, known to drive ERRα function [22, 23]. Consequently, BT474 and SKBR3 cells possess high ERRα activity and represent ideal models for our investigation. ERRα-binding sites were found near the transcriptional start sites of the DNA methyltransferase genes DNMT1 and DNMT3A, the DNA demethylase enzyme genes TET2, TET3, and TDG, and the AHCY gene of the methionine cycle in both cell lines (Supplementary Fig. 1a). ERRα was found specifically bound to regulatory regions near MAT1A in BT474 cells and MAT2A in SKBR3 cells suggesting cell-specific differences in isoform expression (Supplementary Fig. 1a). ChIP-qPCR validated the recruitment of ERRα to these sites which was lost when cells were treated with the specific ERRα inhibitor C29 [24] (Fig. 1b). ERRα inhibition with C29 led to a significant induction of the DNA-demethylating genes TET3 and TDG along with an observed inverse regulation of DNA methylating genes with downregulation of DNMT1 and upregulation of DNMT3A (Fig. 1c, d). Further, targeting ERRα diminished the expression of AHCY, as well as that of MAT1A and MAT2A, respectively, in BT474 and SKBR3 cells (Fig. 1c, d), the latter in line with ERRα ChIP-seq binding profiles. Immunoblot analysis confirmed similar effects on the protein levels of these genes following ERRα knockdown or inhibition by C29 in both HER2+ cell lines (Fig. 1e–h). Although, HER2 can positively regulate ERRα activity, ERRα is also expressed in the ER+ and TN BC subtypes. To verify whether ERRα regulation of DNA methylating enzyme expression is subtype-specific, we looked at protein levels of DNMT1 after knockdown of ERRα by RNA interference in MCF7 cells, an ER+ BC cell line, as well as in the three TNBC cell lines MDA-MB-231, MDA-MB-436, and MDA-MB-468. In each case, impairment of ERRα function reduced DNMT1 protein levels (Supplementary Fig. 1b–e).

Importantly, this specific involvement of ERRα in the regulation of DNA methylation is not restricted to human cancer cells. Drug-induced inhibition of ERRα by C29 in the mouse cell lines NIC-5231 and NIC-5257, derived from ErbB2-driven mammary tumors [25], also led to a stark reduction in DNMT1 protein (Fig. 1i, j). This mechanism is
also conserved in normal cells whereby ERRα knockout mouse embryonic fibroblasts (ERRα KO MEFs) exhibited similar alterations in DNMT isoforms and TET expression as compared to BC cells, a phenotype reversed by ectopic expression of ERRα (Fig. 1k, l).

**ERRα controls DNA methylation**

To investigate the influence of ERRα on the methionine cycle and DNA methylation programs, we measured the steady-state levels of methionine cycle intermediates in
SKBR3 cells after pharmacological inhibition of ERRα with C29 for 24 h. Impeding ERRα resulted in a significant accumulation of all intermediates of this metabolic process (Fig. 2a). Moreover, C29-mediated ERRα inhibition led to a marked decrease in global DNA methylation in both SKBR3 and BT474 cell lines, exemplified by a significant reduction in total 5-methylcytosine levels (Fig. 2b, c). Importantly, ERRα KO MEFs also displayed a decreased level of DNA methylation compared to WT MEFs (Supplementary Fig. 2).

The accumulation of methionine cycle intermediates might be the consequence of a reduced metabolic rate due to the decreased expression of the methionine cycle enzymes we observed (Fig. 1c–h) or a bottleneck downstream in the transfer of a methyl group from SAM to DNA. To measure the metabolic rate of the methionine cycle, we designed an isotope tracer experiment whereby SKBR3 cells were first treated with C29 for 24 h, followed by incubation with labeled methionine (\(^{15}\text{C}_2^{15}\text{N}\)-methionine) for 2 h. Given that methionine is an essential amino acid, we could follow the incorporation of labeled atoms from dietary methionine into SAM, SAH, and homocysteine by liquid chromatography coupled to mass spectrometry (LC/MS). Interestingly, while we could not detect homocysteine in this setting, C29-treated cells displayed an increase of labeled methionine (Fig. 2d). Note, C29 had no significant impact on SAM and SAH levels, suggesting a similar rate of the methionine cycle compared to control (Fig. 2d).

We then quantified the levels of labeled 5-methylcytosine arising from labeled methionine. BC cells were first treated with C29 for 24 h and then incubated with labeled methionine for another 24 h to allow for labeled methyl incorporation prior to genomic DNA isolation. As expected, diminished levels of labeled 5-methylcytosine were observed when ERRα activity was impaired (Fig. 2e, f). Notably, this effect was largely rescued by exogenous expression of DNMT1 (Fig. 2g, h), highlighting that DNMT1 is the rate-limiting enzyme and is critical for driving DNA methylation in this context.

### ERRα activity correlates with DNMT1 expression in BC patients

We next re-analyzed publicly available gene expression datasets from cohorts of BC patient tumors of mixed molecular subtypes to determine whether ERRα activity correlates with the expression of DNA methylation regulators. For this, we utilized a previously established 121-gene ERRα signature shown to cluster BC patients into groups of low or high ERRα activity independent of their BC molecular subtype [23]. To this gene list, we added key ERRα-targeted genes identified in this study namely DNMT1, DNMT3A, TET2, TET3, AHCY, MAT1A, and MAT2A, as they were not included in the original dataset (Supplementary Table 1). Unsupervised hierarchical clustering successfully partitioned the tumor profiles into 2 groups distinguished by having either low or high ERRα activity across three independent cohorts obtained from Gene Omnibus and ArrayExpress (GSE2034, GSE24450 and E-TABM-158), thus confirming the validity of the ERRα signature (Fig. 3). Next, we tested for a significant association between the expression of our genes of interest and ERRα activity. Of the genes examined, only DNMT1 transcript levels showed a consistent and significant correlation with BC tumors bearing high ERRα activity across the three independent patient cohorts (Fig. 3 and Supplementary Fig. 3). This raises the possibility that simultaneous inhibition of DNMT1 and ERRα activity may offer a therapeutic advantage for the treatment of BC patients.

### Dual inhibition of ERRα and DNMT1 suppresses BC cell growth in vitro

While human cancer cells generally harbor global DNA hypomethylation profiles, they also specifically display hypermethylation of promoters of tumor suppressor genes leading to their silencing [26]. Accordingly, inhibition of DNMTs correlates with reduced tumorigenicity often related to re-expression of tumor suppressors [27]. Recently, the FDA-approved DNMT inhibitor 5-azadC (decitabine) was proven effective in treating TNBC [28]. Given our identification of an association between ERRα activity and DNMT1 expression in BC patients, we tested whether ERRα inhibition could further sensitize BC cells to 5-azadC. Unexpectedly, 5-azadC alone robustly reduced ERRα protein levels, an effect also observed following the specific knockdown of DNMT1 by RNA interference in...
both SKBR3 and BT474 cells (Fig. 4a, b). Further analysis determined that the DNA-demethylating agent 5-azadC induces BC cell autophagy, as indicated by increased levels of the autophagy marker LC3B-II (Fig. 4c), and that blockade of autophagy with bafilomycin A is sufficient to rescue 5-azadC-induced ERRα degradation (Fig. 4d). 5-azadC has also been shown to promote autophagy in ovarian cancer cells [29].
Next, we examined the effect of co-treatment with the ER\(\alpha\) inhibitor C29 and 5-azadC on BC cellular proliferation. While 5-azadC alone significantly diminished the growth of both the HER2 human SKBR3 and mouse NIC-5231 cell lines, C29 amplified the neoplastic effects of the DNMT inhibitor (Fig. 4e, f). Similar beneficial anti-tumor effects of combined ER\(\alpha\) and DNMT inhibitors were observed in the ER+ BC cell line MCF7 and TNBC cell lines MDA-MB-231, MDA-MB-436, and MDA-MB-468 (Fig. 4g–j and Supplementary Fig. 4a–d).

Since ER\(\alpha\) activity is regulated by growth factors and HER2 [30], we wondered whether classical anti-HER2 therapy would also sensitize SKBR3 cells to the DNMT inhibitor 5-azadC. Similar to ER\(\alpha\) loss-of-function in BC cells (Fig. 1e–j), SKBR3 cells treated with lapatinib, a dual epidermal growth factor receptor (EGFR)/human EGFR-2 (HER2) tyrosine kinase inhibitor approved for patients with...
HER2-amplified breast tumors, decreased both ERRα and DNMT1 protein levels (Supplementary Fig. 4e). However, lapatinib was also found to reduce DNMT3a levels (Supplementary Fig. 4e), while ERRα inhibition seemingly stabilized it (Fig. 1e–h, k). Co-treatment of SKBR3 cells with lapatinib and 5-azadC did not further increase the antitumoral effects of each drug alone (Supplementary Fig. 4f), suggesting that the effects of anti-HER2 therapy beyond ERRα inhibition impede the benefit of co-targeting ERRα and DNMT simultaneously.
C29 enhances the efficacy of 5-azadC on impeding BC tumor development in vivo

To determine the efficacy of the therapeutic agent 5-azadC in combination with C29 at suppressing BC tumor growth in vivo, compared to 5-azadC therapy alone, we used a pre-clinical mouse cell line-derived xenograft (CDX) model. NIC-5231 cells were injected into the mammary fat pad of NSG mice, and primary tumors were treated with either C29 (10 mg/kg) and/or 5-azadC (1 mg/kg), or vehicle control (Fig. 5a). As anticipated, treatment with the DNA-demethylating drug 5-azadC alone significantly attenuated tumor growth (Fig. 5b, c). Importantly, as observed in vitro, C29 potentiated the tumor suppressive effect of 5-azadC in vivo, thus validating the utility of combining ERRα and DNA methyltransferase inhibitors in the pharmacological intervention of BC (Fig. 5b, c). Immunoblot analysis of end-point tumors confirmed the positive molecular link between ERRα and DNMT1 expression whereby ERRα loss of function by C29 decreased DNMT1 protein and treatment with 5-azadC reduced ERRα levels (Fig. 5d). Surprisingly, while C29 and 5-azadC independently decreased intra-tumoral levels of 5-methylcytosine, there was no further significant decline in global DNA methylation upon co-treatment compared to the individual drug regimens (Fig. 5e). We therefore hypothesized that C29 and 5-azadC may have differential effects on promoter-specific DNA methylation that may be otherwise masked by the evaluation of global DNA methylation states. To address this, we performed reduced representation bisulfite sequencing (RRBS) on tumor DNA from these mice. RRBS is a high-throughput technique that offers a large-scale high-resolution mapping of DNA methylation across the genome that enriches for regions with high CpG content such as promoters and repeated sequences [31]. Overall, lower methylated CpGs were found in all three drug treatment groups compared to controls, and the repartitions of the differentially methylated CpGs between introns, exons, promoters and intergenic regions were almost identical across treatment groups (Supplementary Fig. 5a–f). Pathway enrichment analysis of the genes with differentially methylated regions (DMRs) showed no major differences between treatment groups (Supplementary Fig. 5g–i). We next devised a pipeline as outlined in Fig. 5f to focus our attention on more precise features of the tumor RRBS datasets using several filtering criteria. First, since cancer cells possess specific promoter hypermethylation of tumor suppressor genes, we restricted our analysis to DMRs located within promoter regions (Supplementary Table 2). Given that promoters are under the control of TFs, we looked specifically for TFs targeting these regions and for which C29 and 5-azadC co-treatment induced promoter hypomethylation. Promoter methylation status was analyzed in silico using the SMARTapp [32], which allowed us to identify TFs possessing hypomethylated CpG sites that correlate with bad prognosis in BC patients. Those presenting hypomethylated CpG sites following the combinatorial drug regimen were retained, given that they offer a therapeutic benefit by allowing for re-expression of tumor suppressor genes. Third, to identify candidate TFs regulating these genes, Homer (Hypergeometric Optimization of Motif EnRichment) software was used for analysis of TF motif enrichment on gene promoters harboring DMRs [33]. In our analysis, 890 genes with promoter DMRs were found uniquely modified in tumors co-treated with C29 and 5-azadC—this included 51 TFs based on a recent article referencing all known human TFs (Fig. 5g and Supplementary Table 3) [34]. Among these 51 TFs, 9 were characterized in the SMARTapp as having promoter CpG hypermethylation and a significant association with bad prognosis in BC. Dual inhibition of ERRα and DNMT specifically induced promoter hypomethylation of six of these nine prognostic TFs: IRF4, FOXE1, VDR, TEAD2, POU3F3, and ZBTB20 (Fig. 5g and Supplementary Table 3). Both VDR and ZBTB20 were previously shown to operate as tumor suppressors in cancer [35, 36]. IRF4 has also been found to have both oncogenic and tumor suppressive activities in hematological cancer [37, 38]. Further examination with TF motif enrichment analysis revealed strong differences in potential TFs implicated in the response to each treatment (Fig. 5h). Intriguingly, the IRF4 motif was specifically enriched after co-treatment with C29 and 5-azadC (Fig. 5h), a condition found to demethylate the IRF4 promoter (Fig. 5g). Thus, the computational analysis highlights IRF4 as a potential active participant in mediating the anti-tumor effects observed. Alongside this implication, we noted that the strongest motif enriched in the combined treatment group was for ETV1, a member of the ETS family of TFs and well-known as an oncogene in several cancer types including BC [39]. Considering that a role for IRF4 in BC has never been explored, we next sought to investigate its potential tumor suppressor activity in this context.

Combined C29 and 5-azadC treatment reverses IRF4 promoter hypermethylation in BC

We first aimed to confirm in silico the methylation status of IRF4 in cancer. The SMARTapp revealed the existence of 16 CpGs with available methylation data for this gene. Aggregating the mean methylation levels of all CpGs, IRF4 is significantly hypermethylated in BC as well as in almost every other cancer type within the TCGA collection (Fig. 6a and Supplementary Fig. 6a). Specifically, 10 out of 16 CpGs are localized in the IRF4 promoter (Fig. 6b). According to SMARTapp, these ten sites are all
hypermethylated in BC with nine displaying a significant positive correlation with poor overall survival (Fig. 6b–e and Supplementary Fig. 6b). Moreover, their methylation status negatively correlated with gene-level expression, confirming that hypermethylation of the IRF4 promoter would lead to IRF4 silencing (Fig. 6f–h and Supplementary Fig. 6c). To validate this, we selected one CpG site within each of the three CpG islands found within the IRF4 promoter (Fig. 6b), and performed methylation-specific quantitative PCR (MS-qPCR), a technique that allows direct
evaluation of the methylation status of a specific CpG site [40]. Compared to control SKBR3 cells treated with vehicle, the level of methylation at all three CpG sites tested was significantly reduced by the addition of either C29 or 5-azadC. Remarkably, these effects were additive, as co-treatment of the drugs effectively abrogated IRF4 promoter CpG methylation (Fig. 6i–k).

The suppressive effects of concomitant ERRα and DMNT inhibition on BC growth is dependent on IRF4 derepression

To verify IRF4 expression, we performed RT-PCR on SKBR3 cells treated with C29 and/or 5-azadC, or vehicle control, using primers designed to amplify a 200 bp cDNA region of IRF4. In accordance with the state of IRF4 promoter methylation (Fig. 6i–k), we could not detect IRF4 in SKBR3 cells under basal conditions or in response to C29 or 5-azadC treatment alone (Fig. 7a). However, co-administration of both drugs resulted in amplification of the IRF4 cDNA (Fig. 7a), thus confirming that concurrent inhibition of ERRα and DMNT can successfully derepress the IRF4 gene that was silenced by promoter hypermethylation. We next sought to provide evidence for a tumor suppressive action of IRF4 re-activation underlying the therapeutic benefit to a combined ERRα and DMNT drug therapy in BC. Accordingly, SKBR3 cells were first infected with either a non-specific shRNA (shNTC) or with one of two different shRNAs targeting IRF4 (Fig. 7b).

Subsequently, the cells were exposed to either a combined C29 and 5-azadC drug regimen or vehicle control and the impact on cellular growth was evaluated (Fig. 7c and Supplementary Fig. 7). As expected, knockdown of IRF4 had no impact on cell growth in the vehicle condition, a context in which IRF4 is already silenced (Supplementary Fig. 7). In stark contrast, while C29 and 5-azadC inhibited cell proliferation, this effect was demonstrated to be IRF4-dependent as shRNA-mediated suppression of IRF4 could significantly restore cell growth capabilities, underscoring an anti-proliferative function of IRF4 in BC cells (Fig. 7c).

Finally, we interrogated the PRECOG website (https://pre cog.stanford.edu/) [41] to investigate the correlation between IRF4 expression and patient overall survival in BC. Our analysis confirmed a positive correlation between IRF4 expression and favorable patient outcome, thus validating our findings that IRF4 plays a tumor suppressor role in BC (Fig. 7d, e).

Discussion

In this study, we identified a new ERRα-dependent regulatory pathway conserved across species linking cell metabolism and DNA methylation. We show that ERRα, a major regulator of cellular metabolism, coordinates SAM synthesis through the methionine cycle while driving DNMT1 expression to promote DNA methylation. Genetic or pharmacological inhibition of ERRα repressed DNMT1 expression, the activity of the methionine cycle and, ultimately, global DNA methylation. Reciprocally, we uncovered that inhibiting DNMT1 diminishes ERRα levels, suggesting that DNMT1 directly influences cell metabolism. ERRα activity and DNMT1 expression were found to positively correlate in BC patients independent of BC subtype, reinforcing the molecular link between these two genomic regulators. Of clinical relevance, targeting ERRα with the specific inhibitor C29 significantly increased the sensitivity of BC cells to the DNMT inhibitor decitabine both in vitro and in vivo. A large-scale analysis of DNA methylation further revealed that co-treatment with both drugs alters promoter methylation of a specific set of genes, leading to the identification and functional characterization of IRF4, found to possess tumor suppressor activity in BC and derepressed in this context.

While DNA methylation is tightly bound to the metabolic state of the cell, active DNA demethylation also occurs and depends on the availability of specific metabolites. Indeed, we observed that ERRα inhibition induced the expression of the demethylase TET3, suggesting that ERRα could also be involved in active DNA demethylation (Fig. 1). However, we speculate that loss of DNA methylation linked to ERRα inhibition is more likely due to a lack
of DNMT1 activity than to an increase in demethylase activity. This hypothesis is supported by our recent report demonstrating that ERRα inhibition depletes the available pool of αKG [21], a required cofactor of dioxygenase enzymes such as the TET DNA demethylases. αKG is mostly synthesized through glutaminolysis in cancer, a pathway regulated by ERRα in BC [42], and is an important intermediate of the TCA cycle involved in many cellular functions such as anti-oxidation, protein, and lipid synthesis, as well as cellular respiration [43].

The methionine cycle is central to several essential metabolic pathways. Notably, methionine combines with...
the expression of several methionine cycle genes, in understanding. Here, we show that ERRα methionine cycle enzymes are regulated must be well understood. Methionine in cancer, the mechanisms through which elevating levels of intermediate metabolites. These can have promising results in improving metabolism, investigation as a potential anti-cancer therapy and has been proven to be low in cancer cells, rendering them highly dependent on exogenous methionine [14, 46]. However, regeneration of methionine from homocysteine has been shown to be low in cancer cells, rendering them highly dependent on exogenous methionine [45].

ATP to produce SAM, the principal methyl donor for methylation of proteins, DNA, RNA, and lipids [44]. By donating one carbon for methylation, SAM generates SAH, the precursor of homocysteine which can ultimately produce glutathione, the main cellular antioxidant [45]. How-ever, regeneration of methionine from homocysteine has been shown to be low in cancer cells, rendering them highly dependent on exogenous methionine [45].

Hence, dietary methionine restriction is under intense investigation as a potential anti-cancer therapy and has shown promising results in improving metabolism, increasing lifespan and preventing cancer cell growth in numerous contexts [47–52]. Given the clear importance of methionine in cancer, the mechanisms through which methionine cycle enzymes are regulated must be well understood. Here, we show that ERRα positively regulates the expression of several methionine cycle genes, influencing the levels of intermediate metabolites. These findings are significant in the light of our recent work showing that ERRα represses the folate cycle and that ERRα inhibition leads to an increase in purine biosynthesis [18]. Considering the intimate relationship between these two pathways as the recycling of methionine links the methionine and the folate cycles, nucleotide synthesis and NADH/NADPH production, it raises the question as to why ERRα would regulate these programs differently [53–55]. One possible reason stems from a recent study demonstrating that the methionine and folate cycles compete for metabolites involved in DNA methylation, nucleotide synthesis, and anti-oxidation [56].

This suggests that ERRα might act as a switch or sensor to balance these processes [57], which could be particularly important in cancer cells where high nucleotide synthesis, elevated oxidative states, and DNA hypomethylation are often observed.

Cancer cell DNA is characterized by promoter hypermethylation of tumor suppressor genes that induces their silencing. Thus, demethylating agents such as 5-azadC have been proposed as anti-cancer therapies with the intention of re-establishing tumor suppressor expression. 5-azadC is currently used clinically for the treatment of myelodysplastic syndrome and other leukemias, where the drug has received FDA approval [58]. However, clinical development of this drug is still prohibited in solid tumors due to
Fig. 7 IRF4 is a tumor suppressor in BC re-expressed upon co-treatment with C29 and 5-azadC. a Agarose gel electrophoresis showing PCR product amplification of IRF4 cDNA following treatment with C29 (5 μM), 5-azadC (3 μM) or a combination of both drugs for 7 days in SKBR3 cells. b Relative mRNA levels of IRF4 in SKBR3 cells infected with shRNAs against IRF4 after treatment with C29 (5 μM), 5-azadC (3 μM) or a combination of both drugs for 7 days. c Normalized cell index curves representing proliferation of SKBR3 cells infected with either a control shRNA (shNTC) or two different shRNAs against IRF4 in the presence of C29 (5 μM) and 5-azadC (3 μM). Data represent one experiment performed with five replicates. d, e Kaplan–Meier survival curves representing the positive correlation between IRF4 mRNA expression and overall survival of BC patients in two independent cohorts consisting of 158 patients (GSE3143) (d) and 155 patients (GSE7390) (e). f Model illustrating how pharmacological inhibition of the inter-connected factors ERα and DNMT1 can halt BC progression by simultaneously repressing methionine cycle metabolism and DNA methylation. Consequent epigenetic modulation impinges on the newly attributed tumor suppressor gene IRF4 in BC with C29 and 5-azadCo-treatment promoting IRF4 derepression. Data shown in b and c represent means ± SEM. *p < 0.05, ***p < 0.001; Student’s t test.

Detailed methods

Materials and methods

Details of all methods are found in Supplementary Information.

Accession numbers

RRBS datasets from mouse tumor xenographs have been deposited in the NCBI Gene Expression Omnibus (GEO; https://ncbi.nlm.nih.gov/geo/) under the accession number GSE149603.

Acknowledgements

We acknowledge contributions from the Metabolomics Core Facility (MCF) of the GCRC, as well as technical assistance from Dr. Daina Avizionis, Mariana De Sa Tavares Russo, Gáeille Bridon, and Luc Choinière. We thank all members of the V.G. laboratory for discussions, Xiaojing Li for technical assistance and Dr. David J. Papadopoli for guidance with metabolic studies. This work was supported by a Terry Fox Research Institute Program Project Team Grant on Oncometabolism (VG and JS-P), a Foundation Grant from the Canadian Institutes of Health Research (CIHR) to VG, and an operating grant from CIHR to JS-P (MOP-106603). MV was a recipient of a post-doctoral fellowship from CIHR. SM was supported by a Vanier Canada Graduate Scholarship–CIHR. EAW was a recipient of a post-doctoral fellowship from CIHR.

Author contributions

Conception and design: MV, VG. Development of methodology: MV, SM, CRD, JS-P. Acquisition of data: MV, SW, EA-W. Data analysis and interpretation: MV, SM, CRD, JS-P, VG. Writing of the manuscript: MV, CRD, VG. Study supervision: VG

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

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