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The Metabolic Regulator ERRα, a Downstream Target of HER2/IGF-1R, as a Therapeutic Target in Breast Cancer

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SUMMARY

A genomic signature designed to assess the activity of the estrogen-related receptor alpha (ERRα) was used to profile more than 800 breast tumors, revealing a shorter disease-free survival in patients with tumors exhibiting elevated receptor activity. Importantly, this signature also predicted the ability of an ERRα antagonist, XCT790, to inhibit proliferation in cellular models of breast cancer. Using a chemical genomic approach, it was determined that activation of the Her2/IGF-1R signaling pathways and subsequent C-MYC stabilization upregulate the expression of peroxisome proliferator-activated receptor gamma coactivator-1 beta (PGC-1β), an obligate cofactor for ERRα activity. PGC-1β knockdown in breast cancer cells impaired ERRα signaling and reduced cell proliferation, implicating a functional role for PGC-1β/ERRα in the pathogenesis of breast cancers.

INTRODUCTION

The estrogen-related receptor alpha (ERRα) is an orphan member of the nuclear hormone receptor superfamily of transcription factors for which an endogenous ligand has yet to be defined (Giguère et al., 1988). The high degree of structural similarity between ERRα and the estrogen receptor (ER), particularly in the DNA binding domain, has led to the speculation that the transcriptomes of these two receptors may overlap. Given the established role of estrogens in breast cancer, it was not surprising that there is considerable interest in determining the extent to which ERRα impinges on ER signaling and contributes to the pathogenesis of breast cancer. It was significant, therefore, that the expression of ERRα, in breast cancer was shown to correlate with unfavorable clinical outcomes in a manner that was independent of ER status (Ariazi et al., 2002; Suzuki et al., 2004). Further, higher expression of ERRα was observed in ER-negative breast cancers and its expression is also associated with that of Human Epidermal Growth Factor Receptor 2 (Her2). These observations suggest that ERRα is not merely a substitute for, or a modulator of, ER function but rather has distinct activities that may contribute to the pathogenesis of breast cancer. This conclusion was reinforced by the results of recently published chromatin immunoprecipitation combined with microarray (ChIP-chip) analyses of breast cancer cells that revealed relatively minor overlap in the transcriptomes of ER and ERRα. Indeed, these studies indicated that the majority of the genes that are regulated by ERRα are distinct from those controlled by ER (Deblois et al., 2009; Dufour et al., 2007).

ERRα functions downstream of the peroxisome proliferator-activated receptor gamma coactivator-1 alpha and beta (PGC-1α and PGC-1β) and controls the expression of genes involved in the tricarboxylic acid (TCA) cycle, oxidative phosphorylation (OXPHOS), and lipid metabolism (for review, see Lin et al., 2005). By regulating these genes, ERRα controls energy metabolism in the liver, skeletal muscle, heart, and adipose tissue (Huss et al., 2004, 2007; Kamei et al., 2003; Mootha et al., 2004; Villena et al., 2007). Although it is unclear whether ERRα-dependent regulation of cellular metabolism plays a role in the pathogenesis of breast cancer, it is worth noting that enzymes involved in glycolysis, TCA cycle, and OXPHOS have been found to be upregulated in models of breast cancer brain metastases (Chen et al., 2007). Aside from the ability to regulate energy metabolism, ERRα regulates VEGF expression in breast cancer

Significance

Overexpression of ERRα has been correlated with progression of breast and ovarian cancers in several small studies. Using a genomic approach, we defined specific aspects of the activity of this receptor that track with shorter disease-free survival in multiple cohorts of breast cancer patients. Importantly, cellular models of breast cancer exhibiting high ERRα activity are more sensitive to growth inhibition by an ERRα antagonist. This finding highlights a promising treatment strategy for those aggressive tumors that currently have limited therapeutic options.

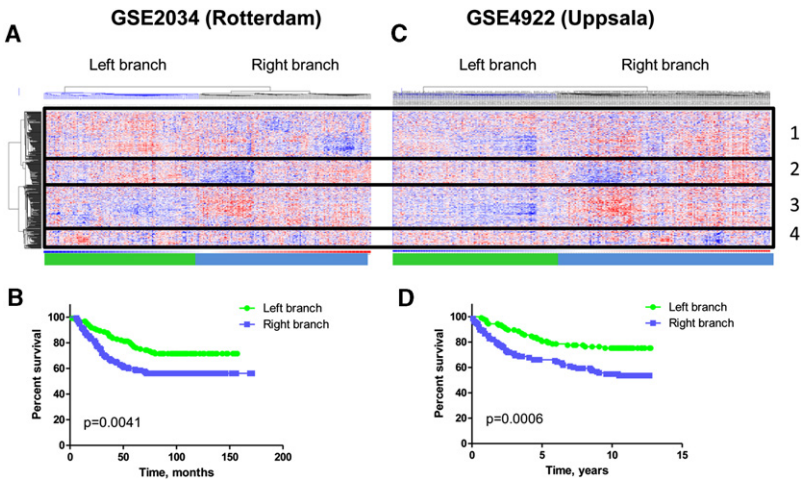

Figure 1. Expression of ERRα-Regulated Genes Clusters Tumor Samples into Clinically Distinct Groups

(A and C) Unsupervised hierarchical clustering of tumor samples in two clinical data sets using 448 ERRα-regulated probe sets. The order of probe sets in the Uppsala data set was fixed to match that in the Rotterdam clustering diagram to reveal common patterns of gene regulation in these two clinical data sets. Hierarchical clustering revealed that the expression pattern of ERRα-regulated genes in clinical samples separates the genes into 4 different clusters (boxed and labeled 1, 2, 3, and 4).

(B and D) Relapse-free survival was plotted for patients in the right branch versus patients in the left branch. Kaplan-Meier curves were generated using GraphPad Prism and p value was determined by log rank test.

See also Figure S1.

and skeletal muscle cells, interacts with and modulates HIF1 activity, and is required for the migration of MDA-MB-231 breast cancer cells in vitro (Ao et al., 2008; Arany et al., 2008; Chinsomboon et al., 2009; Stein et al., 2008, 2009). Together, these data suggest that ERRα is a key regulator of several ER-independent processes of importance in breast cancer. It is not clear, however, which specific aspects of ERRα biology are most relevant to the pathogenesis of breast cancer.

Recent studies using siRNAs and small-molecule antagonists have demonstrated that ERRα is required for the growth of both ERα-positive and ERα-negative breast cancer cells when assayed in vitro or when propagated as xenografts (Ao et al., 2008; Bianco et al., 2009; Chisamore et al., 2009; Stein et al., 2008). Whereas these findings provide the rationale for the exploitation of ERRα as a therapeutic target, it remains unclear, absent evidence for the existence of a bone fide small molecule ligand, how the transcriptional activity of this receptor is regulated in cancer. Thus, the primary objectives of this study were to define the processes that impinge upon and regulate ERRα transcriptional activity in breast cancer cells and to define the processes downstream of the receptor that contribute to breast cancer pathogenesis.

RESULTS

The Expression of a Subset of ERRα-Regulated Genes Correlates with Relapse-Free Survival in Breast Cancer Patients

The expression of ERRα has been shown to correlate with an unfavorable clinical prognosis in breast cancer (Ariazi et al., 2002; Suzuki et al., 2004). However, the role(s) of this receptor in the pathogenesis of this disease or its potential utility as a therapeutic has not been established. To address these issues, we first examined whether *ERRα* mRNA expression itself is a robust predictor of patient survival in six clinical breast tumor microarray data sets. Surprisingly, only one data set showed a statistically significant correlation between high *ERRα* expression and shorter disease survival (data not shown). Because ERRα is a transcription factor and its activity is regulated at multiple levels (i.e., expression of its required coactivators), we reasoned that a genomic signature derived from ERRα-regulated genes might be a more appropriate way to evaluate the pathological significance of ERRα activity in clinical samples.

As a first step in the development of a genomic predictor of ERRα activity, we defined the spectrum of ERRα-regulated genes in human primary mammary epithelial cells (hMECS), a nontransformed cellular background, to avoid introduction of bias by other dysregulated oncogenic pathways. Unlike most other nuclear receptors whose transcriptional activity can be regulated by small molecule ligands, the activity of ERRα appears to be controlled at the level of coactivator availability and/or posttranslational modifications of the ERRα protein itself. Thus, in order to generate a robust genomic signature reflecting the activated state of ERRα, we used the best characterized ERRα coregulator, PGC-1α, as a protein ligand to activate ERRα for this analysis. Because PGC-1α coactivates a number of transcription factors, we used the previously described ERR-selective derivative of PGC-1α (PGC-1α 2x9) and a mutant form unable to interact with the NRs (PGC-1α L2L3M) to capture the gene expression profiles representing "high ERRα activity" and "low ERRα activity" states (Gaillard et al., 2006). The experiment was performed ten times to generate independent biological replicates. The ERRα-regulated gene set was defined using the following cutoffs: fold change (1.4-fold), p value in pairwise t tests (0.001 before adjustment for multiple comparisons) and proportion of "present" calls (20% in each treatment group). The resulting ERRα-regulated gene set consisted of 448 probe sets, corresponding to 354 unique genes.

To assess the clinical relevance of this gene set, we explored the expression patterns of these genes in available breast cancer clinical data sets using unsupervised hierarchical clustering. As shown in Figure 1A, patient samples within the GSE2034 data set partitioned into two branches of approximately the same size based on the expression patterns of these ERRα-regulated genes. Interestingly, patients in the right branch have shorter relapse-free survival (RFS) compared with patients in the left branch (Figure 1B). A similar observation was made when we analyzed an additional data set, GSE4922 (Figures 1C and 1D). We next wanted to know if the clinically distinct groups of patient samples shared a coordinate regulation of defined sets of genes. Thus, we performed unsupervised clustering of probe sets in GSE2034 and fixed the order of probe sets in GSE4922 to match that in GSE2034 to identify the common regulatory patterns

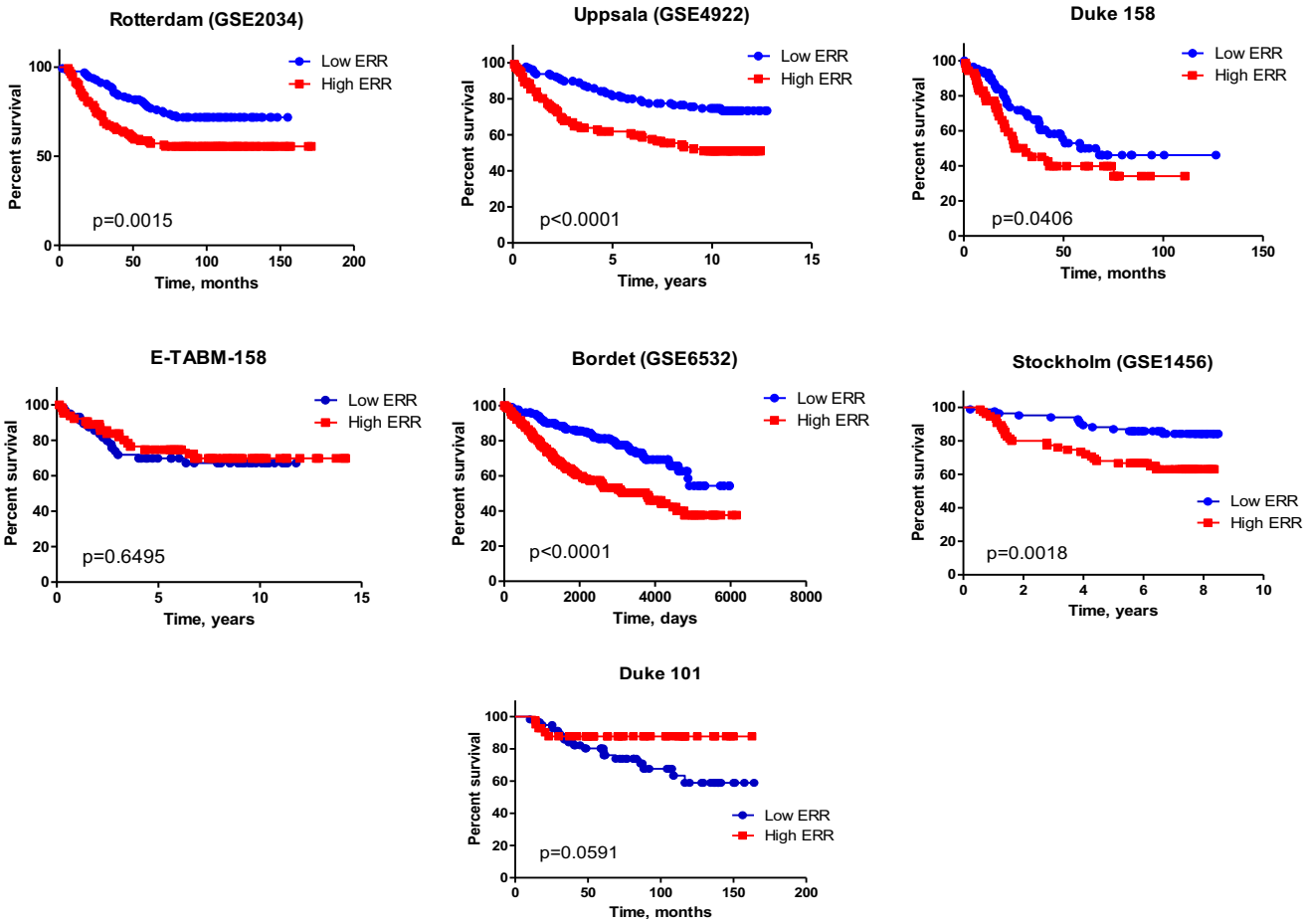

Figure 2. Correlations between Posterior Probabilities of ERRα Activation (as predicted by the cluster 3 classifier) and Relapse-Free Survival in Seven Clinical Data Sets

"Low ERR" and "High ERR" groups represent tumor samples with the posterior probability of ERRα activation lower than the mean and higher than the mean for that data set. Significance was determined by log rank test. The predictability of cluster 3 ERRα classifier was validated in two independent data sets (Figure S2). See also Tables S1 and S2.

across data sets. Four major clusters of coregulated genes were identified, with some groups of genes demonstrating clear coregulation in distinct patient groups. We next determined whether certain groups of genes carry more weight in partitioning the patient samples into clinically distinct groups than others. To this end, an unsupervised clustering of the samples was performed to determine the ability of the genes within each cluster to classify patient samples into groups with different clinical outcomes. Only cluster 3 was found to retain the full capacity of the larger, 448 probe set signature to distinguish between patients with better or worse prognosis (Figure S1, available online), while gene clusters 1, 2, and 4 do not have predictive values (data not shown). Based on these observations, we hypothesized that the genes within cluster 3 are potentially the most relevant to patient outcome.

Although the genes from cluster 3 could be used to classify patients into two separate groups, this analysis does not specify whether tumors in the right branch, which represent worse prognosis, have high or low ERRα activity. In order to address this question, we utilized binary regression modeling to ascribe a numerical value reflecting the estimated ERRα activity (using the cluster 3 genes) to each patient sample and then tested the correlation between this estimated activity and available clinical endpoints. The details of this method have been described elsewhere (Bild et al., 2006; Pittman et al., 2004) and the list of probe sets comprising the cluster 3 is shown in Table S1. Briefly, binary regression is a statistical method for classifying each sample within the test (clinical) data set into one of the following two phenotypic classes: "low ERRα activity" and "high ERRα activity." The phenotypic classes are defined by the training data set, in this case, the expression values of ERRα-regulated cluster 3 genes obtained from hMEC cells overexpressing PGC-1α L2L3M or PGC-1α 2x9, providing the low (p = 0) and high (p = 1) boundaries, respectively. It should be noted that for this analysis, the estimated probability does not reflect the overall activity of ERRα, but rather, the aspect of ERRα activity manifest by the genes included in the classifier. For comparative purposes, we also generated classifiers using the probe sets within clusters 1, 2, and 4. The results of this analysis demonstrated that the tumors with elevated ERRα activity, as defined by cluster 3 genes, correlated with shorter RFS in 5 out of 7 clinical data sets tested (Figure 2). Analysis of two additional data sets produced

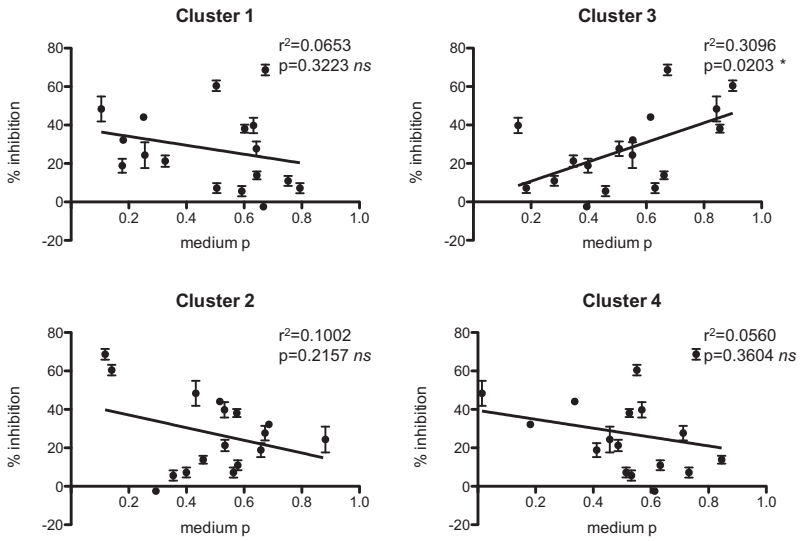

Figure 3. The Sensitivity of Breast Cancer Cells to ERRα Antagonist Correlates with Their Intrinsic Cluster 3 ERRα Activity

Seventeen breast cancer cell lines with predicted high, medium, and low levels of ERRα activity were treated with an ERRα antagonist XCT790 and their growth inhibitory responses (% inhibition) to these compounds were plotted against the ERRα activity (average med p) of each cell line predicted using gene expression data derived from each cluster defined in Figure 1. Linear regression and p values were determined using the GraphPad Prism software. The percent inhibition was presented as mean ± SEM from four independent experiments.

See also Tables S3 and S4 and Figure S3.

discordant results; we hypothesize that this is due to unusually high RFS rates for patients with advanced (G3) disease in these data sets compared with G1-G2 patients. Our analysis indicates that Cluster 3 ERRα activity tends to be elevated in higher grade tumors, which usually carry more negative prognosis (data not shown). Aspects of ERRα activity predicted by classifiers derived from clusters 1, 2, and 4 did not track with RFS (data not shown). Also of interest, gene set enrichment analysis based on expression correlation networks indicated that both the expression of the cluster 3 signature, and individual genes within this signature most strongly correlate with the expression of numerous markers of proliferation, as well as gene sets characteristic of undifferentiated tumor type, and c-MYC and EZH2 activation (Table S2).

Based on these observations, we concluded that the regulation of genes within cluster 3 by ERRα is likely to reflect the specific aspect of its activity that is most relevant to tumor pathology.

The Intrinsic ERRα Activity of Breast Cancer Cells Correlates with Their Sensitivity to ERRα Antagonists

Given that ERRα activity, as determined by the cluster 3-based classifier, predicts cancer progression, it was of interest to see if (1) the same activity was manifest in cellular models of breast cancer and (2) ERRα activity could be used to predict phenotypic responses to treatment with currently available antagonists of this receptor. To this end, we determined the intrinsic ERRα activity using the cluster 3 gene signature in a panel of breast cancer cell lines using two published microarray data sets (Table S3)(Bild et al., 2006; Neve et al., 2006). Seventeen cell lines that were randomly selected from low, medium, and high ERRα activities were evaluated for their sensitivity to the well-characterized ERRα antagonist XCT790 (Willy et al., 2004). In this manner, it was determined that the sensitivity of breast cancer cell lines to XCT790 correlates with their intrinsic ERRα activity, in that cells with higher "cluster 3" ERRα activity are more sensitive to XCT790-induced inhibition of cell growth (r^2 = 0.3096, p = 0.0203) (Figure 3). Notably, ERRα activity predicted using cluster 1, 2, and 4 genes did not correlate with sensitivity to XCT790 (Figure 3, Figure S3, and Table S4). Taken together, these data indicate that the specific aspect of ERRα activity reflected in the expression of the cluster 3 genes is important in breast cancer pathology and provides a strong rationale for therapeutic targeting of the ERRα signaling axis in breast cancer.

PGC-1β Is an Important ERRα Coregulator in Breast Cancer Cells

It is generally believed that posttranslational modifications and/or cofactor availability constitute the main regulatory mechanisms by which ERRα activity is modulated in cells. Specifically, it has been demonstrated that both PGC-1α and PGC-1β robustly enhance ERRα activity when assayed on reporter genes and regulate overlapping repertoires of endogenous ERRα target genes. To determine which of these cofactors are important for ERRα activity in breast cancer cells, we assessed the expression profiles of *PGC-1α*, *PGC-1β,* and *PPRC1*, a third member of the *PGC-1* subfamily of coregulators in a panel of breast cancer cell lines. These studies revealed that *PGC-1α* mRNA expression was limited to a few cell lines but that all of the cell lines examined express both *PGC-1β* and *PPRC1*. Furthermore, it was noted that the expression of *PGC-1β*, but not *PGC-1α* or *PPRC1*, correlated with the expression of *ERRα* (Figure 4A). These data suggest that within the context of breast cancer, PGC-1β is likely to be the most relevant ERRα coactivator. However, *PGC-1α* expression can be acutely regulated by nutrient status and environmental stimuli (e.g., fasting, cold, and exercise), and thus we cannot rule out the possibility that this coactivator may be induced and regulate ERRα activity in breast cancer cells under certain conditions. Hence, we next determined if the expression of PGC-1β is important for ERRα activity in breast cancer cells. Four different siRNAs targeting *PGC-1β* and one targeting *ERRα*, as well as mock and relevant negative control siRNAs were transfected into SKBR3 cells, a cell line exhibiting elevated *PGC-1β* expression and high ERRα activity. The knockdown efficacy of the respective genes was verified at both the RNA (not shown) and protein levels (Figure 4B, bottom panel). Using this approach, we demonstrated that knockdown of *PGC-1β* led to a decreased expression of the cluster 3 ERRα target genes (Figure 4B, top panel). Furthermore, knockdown of either *ERRα* or *PGC-1β* leads to significant retardation of cell growth (Figure 4C). The effects of *PGC-1β* knockdown on the proliferation of additional cell lines were analyzed (Figure S4D and Table S5).

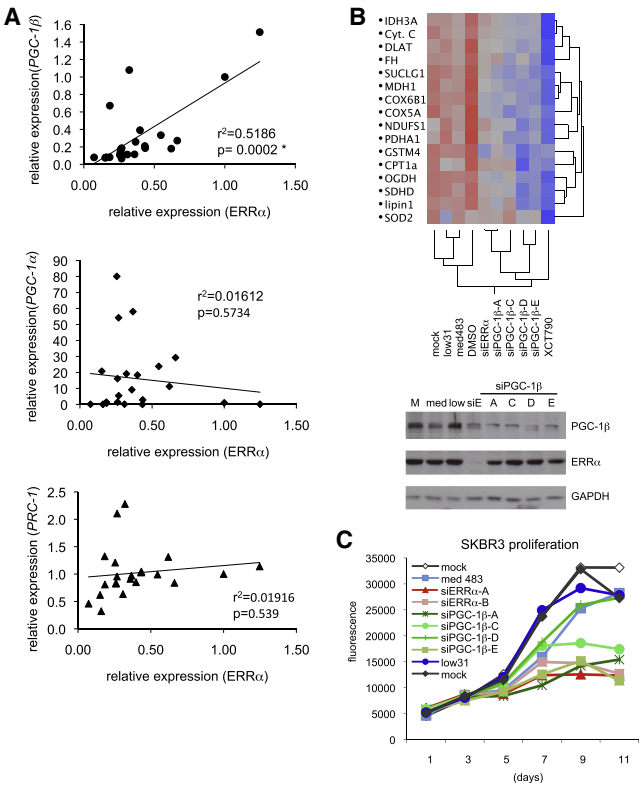

Figure 4. PGC-1β Is Important for Maintaining ERRα Activity in Breast Cancer Cells

(A) RNA from 19 breast cancer cell lines was harvested and the expression of *ERRα*, *PGC-1β*, *PGC-1α*, and *PRC-1* was assessed by qPCR. The relative expression values of each gene of interest in SKBR3 cells were set as 1. The data shown are representative of two independent experiments. Correlation analysis was performed using GraphPad Prizm software.

(B) The expression of ERRα target genes in SKBR3 was assessed following the knockdown of *ERRα* (siERRα) or *PGC-1β* (siPGC1β A, C, D, and E). Control siRNA (low31, med483), mock transfection (mock) and DMSO treatment were included as negative controls. XCT790 treatment (10 μM) was used as a positive control. The data shown are representative of three independent experiments. The right panel shows the western blot analysis demonstrating the knockdown of ERRα and PGC-1β at the protein levels. GAPDH was used as a loading control. "siE" denotes siERRα.

(C) SKBR3 cells were transfected as in (B) and seeded in 96-well plates. Cells were harvested 1, 3, 5, 7, 9, and 11 days after transfection and cell numbers were determined by staining with the DNA dye Hoechst 33258. The data shown are representative of three independent experiments.

See also Figure S4 and Table S5.

Although it is impossible to achieve complete or equivalent knockdown of *ERRα* and *PGC-1β* in every cell line, the data showed that cell lines with high ERRα cluster 3 gene expression tend to be more sensitive to *PGC-1β* knockdown. We further verified that PGC-1β-activated ERRα induced cluster 3 gene expression similar to that activated by PGC-1α in breast cancer cells (Figures S4A–S4C). Together, these data imply that PGC-1β is a major regulator of ERRα activity in breast cancer cells.

Definition of the Signaling Pathways that Regulate the PGC-1β/ERRα Axis

The next step in this study was to define the pathways that were responsible for regulating the expression and/or activity of PGC-1β/ERRα axis. To this end, we undertook a chemical genomic approach to identify signaling modulators that impacted ERRα activity in breast cancer. Specifically, using the cluster 3 ERRα-regulated genes we queried the Connectivity Map (cmap) (Lamb et al., 2006). Developed by the Broad Institute, this resource is essentially a repository of microarray data from sentinel cell lines treated with over 1300 bioactive compounds including those currently used in the clinic. Our query of the cmap identified several classes of compounds that had gene expression profiles that negatively correlated with the expression of the ERRα signature. Among the most interesting compounds implicated in this manner were the phosphatidylinositol 3-kinase (PI3K) inhibitors wortmannin and quinostatin (Table S6). Wortmannin was used to verify the cmap results in SKBR3 cells. It was determined that wortmannin was as effective as XCT790 at inhibiting ERRα target gene expression (Figure 5A). Given that PCG-1β is important for ERRα activity, we tested the possibility that PI3K inhibitors like wortmannin may affect ERRα activity indirectly by regulating the expression of this coactivator. Indeed, treatment with wortmannin dramatically reduced the expression of *PGC-1β* (Figure 5B). Similar results were obtained in cells treated with the more selective PI3K inhibitor LY294002 or the Akt inhibitor GSK716166B (compound 4 described in (Vlahos et al., 1994; Woods et al., 2006)). In contrast, the mitogen-activated protein kinase (MAPK) inhibitor U0126 was without effect (Figure 5C). These results suggest that PI3K and its downstream target Akt are important for the expression of *PGC-1β*. This finding is of particular interest as it has been suggested previously that effector(s) downstream of Her2, including PI3K and MAPK, can phosphorylate and regulate ERRα activity (Ariazi et al., 2007). However, we have mapped and mutated the putative phosphorylation sites within ERRα and have found that the resultant proteins are indistinguishable from the wild-type receptor with respect to their ability to activate target gene transcription (unpublished data). Therefore, it is likely that the major impact of PI3K signaling on ERRα activity results from its ability to upregulate the expression of *PGC-1β*. Consequently, it was important to define (1) the processes upstream of PI3K that impacted *PGC-1β* expression and (2) how PI3K/AKT activation resulted in an upregulation of *PGC-1β* mRNA and protein.

To examine the role of Her2 in PGC-1α/ERRα signaling, the expression of the cluster 3 genes was assessed in cell lines where Her2 is amplified (AU565, SKBR3 and BT474 cells) and a control cell line (BT483), following treatment with the Her2/EGFR dual inhibitor GW2974 (Rusnak et al., 2001). Not surprisingly, we observed quantitative downregulation of *IDH3A* and several ERRα target genes by GW2974 (Figure 6 and data not shown). Importantly, the expression of *PGC-1β* was also decreased following GW2974 treatment (Figure 6). In contrast, *PGC-1α* expression was undetectable in SKBR3 and AU565 cells, and although it is expressed in BT474 and BT483 cells, its expression was not altered by treatment with GW2974. Of note, the expression level of an unrelated cofactor *SRC-2* was not significantly changed upon treatment. The involvement of Her2 signaling pathway in the regulation of *PGC-1β* expression was further confirmed using an additional Her2 inhibitor (Figure S5).

To define the mechanism(s) by which the Her2 signaling impacts *PGC-1β* expression in breast cancer cells, we used

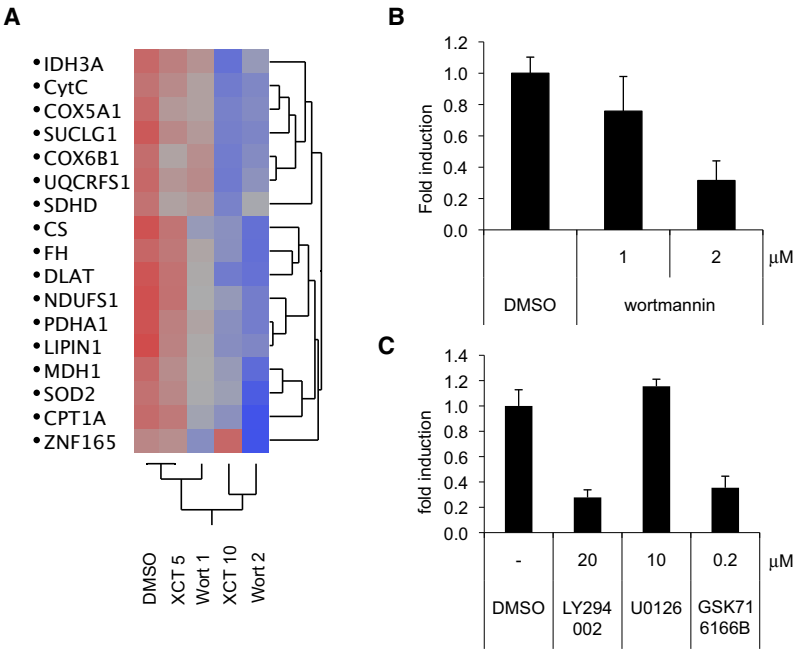

Figure 5. PI3K Inhibitors Attenuates ERRα Activity in Breast Cancer Cells

(A) SKBR3 breast cancer cells were treated with vehicle, 5 and 10 μM XCT790, or 1 and 2 μM wortmannin for 24 hr. RNA was harvested and the expression of ERRα target genes was analyzed by qPCR. Ward hierarchical analysis and heat maps were generated using the JMP software.

(B) SKBR3 cells were treated as in (A) and the expression of *PGC-1β* was analyzed by qPCR.

(C) SKBR3 cells were treated with 20 μM LY294002, 10 μM U0126, or 0.2 μM of the Akt inhibitor GSK716166B for 24 hr. RNA was harvested and the expression of *PGC-1β* was analyzed by qPCR. 36B4 was used as an internal normalization control for each sample and the relative expression of each gene in compound- versus DMSO-treated samples was expressed as fold induction ± SD. The data shown are representative of at least three independent experiments.

See also Table S6.

MCF7 cells, as Her2 is not overexpressed in these cells, but its signaling pathway can be activated by treatment with heregulin (HRG). In addition, since our data indicate that PI3K is important for *PGC-1β* expression, it was of interest to examine whether activation of the insulin-like growth factor 1 (IGF-1) receptor signaling axis, a mechanism by which some breast cancer cells circumvent the inhibition by Her2 inhibitors (Lu et al., 2001), leads to *PGC-1β* upregulation. As shown in Figures 7A and 7B, the expression of PGC-1β at both the mRNA and protein levels was induced in MCF7 cells following treatment with either HRG or IGF-1. HRG binds ErbB3 or ErbB4, which then dimerizes with other members of the ErbB/EGFR family, including Her2, and activates both the MAPK and PI3K signaling cascades. On the other hand, IGF-1 binding to the IGF-1 receptor preferentially activates the PI3K pathway. To further confirm that the PI3K pathway is responsible for the induction of *PGC-1β* by these two growth factors, inhibitors of MAPK and PI3K/Akt pathways as well as the inhibitors of the EGFR/ErbB2 and IGF-1R were used. Not surprisingly, we found that the dual EGFR/ErbB2 inhibitor GW2974 blocked the induction of *PGC-1β* by HRG but not that induced by IGF-1 (Figure 7C). Conversely, the IGF-1R receptor inhibitor ADW742 (Martins et al., 2006) selectively blocked the induction of *PGC-1β* by IGF-1 but not that induced by HRG. As predicted, only the inhibitors of the PI3K pathways (LY294002 and the Akt inhibitor GSK716166B), but not those of the MAPK pathway (U0126, PD98059) blocked the induction of *PGC-1β* by both HRG and IGF-1 (Figure 7C and data not shown). Additional inhibitors for each of the kinases implicated were used to confirm these results (Figures S5 and S6).

C-MYC Regulates the Expression of *PGC1-β* in Breast Cancer Cells in Response to Her2/IGF-1 Activation

It has been shown previously that hypoxia downregulates *PGC-1β* mRNA in renal clear cell carcinoma cells by inducing MXI1, a repressor of C-MYC activity (Zhang et al., 2007). Because both Her2 and IGF-1 signaling have been shown to regulate C-MYC mRNA and/or protein stability, we hypothesized that the increase in *PGC-1β* mRNA by these growth factors could be mediated by C-MYC. A time course study revealed a rapid induction of *PGC-1β* mRNA following treatment of cells with either HRG or IGF-1 (Figure 8A). However, under the same conditions, treatment with HRG but not IGF-1 resulted in an induction of *C-MYC* mRNA. In contrast, it was observed that there is a significant increase in C-MYC protein expression following both HRG and IGF-1 treatment, an effect that precedes the induction of *PGC-1β* mRNA (Figures 8A and 8B). To definitively demonstrate the involvement of C-MYC in the regulation of *PGC-1β* by HRG and IGF-1, we pretreated cells with an inhibitor of C-MYC, 10058-F4, which blocks the C-MYC/Max heterodimerization (Lin et al., 2007; Wang et al., 2007). As shown in Figure 8C, 10058-F4 efficiently inhibited the induction of *PGC-1β* mRNA by both HRG and IGF-1 (Figure 8C). Importantly, 10058-F4 also abolished the induction of PGC-1β protein levels by HRG and IGF-1, confirming that the induction of PGC-1β protein by these growth factors is a transcriptional event requiring C-MYC activity. Additionally, overexpression of C-MYC led to increased *PGC-1β* expression whereas depletion of C-MYC, using siRNA, resulted in the expected decrease in the expression of this coregulator (Figure S7). Further, using a chromatin immunoprecipitation assay (ChIP), we demonstrated that C-MYC is prebound to the two previously described C-MYC binding sites within intron 1 of *PGC-1β* (Zhang et al., 2007). Importantly, treatment with both HRG and IGF-1 further enhanced the recruitment of C-MYC to this region (Figure 8D). Taken together, these data indicate that C-MYC serves as a conduit for signaling events initiated at the membrane, facilitating *PGC-1β* expression and the induction of ERRα transcriptional activity.

DISCUSSION

We believe that this work will have a near term clinical impact as it provides a mechanism to stratify tumors based on their ERRα activity. The finding that the ability of an ERRα antagonist to

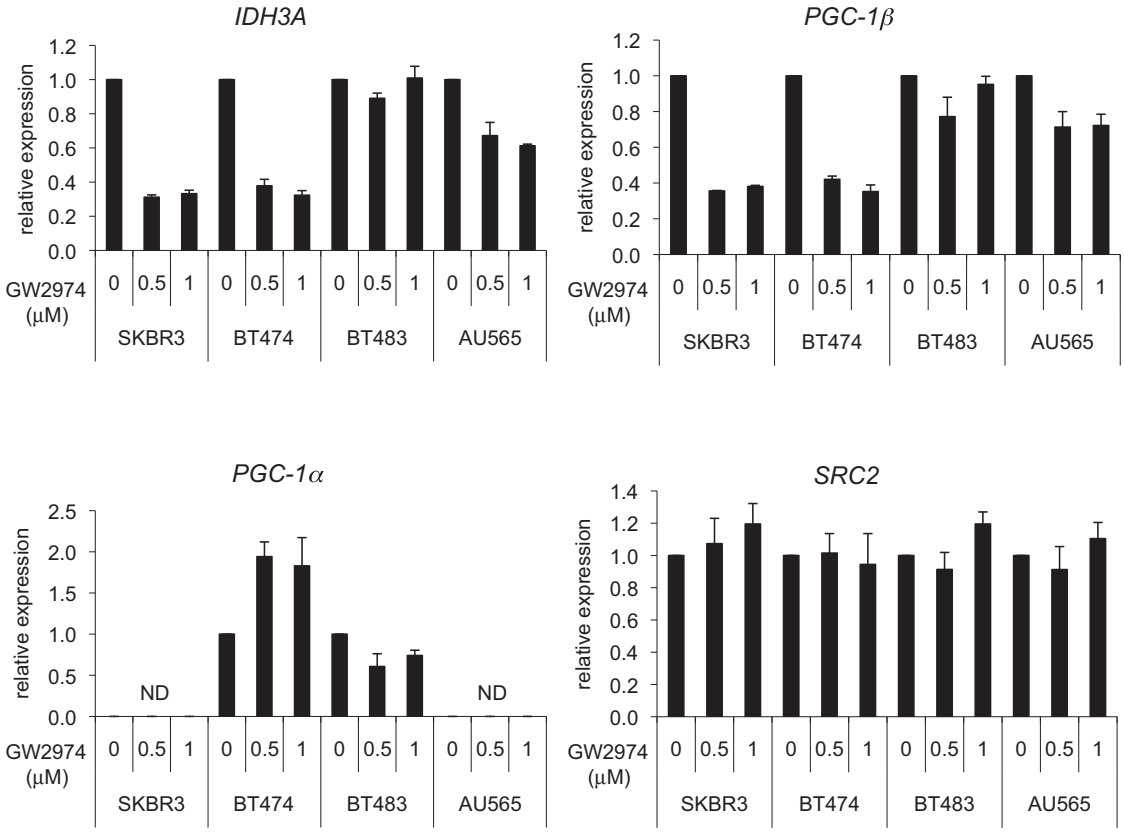

Figure 6. Her2 Signaling Pathway Regulates ERRα Activity Partially through Modulating the Expression of PGC-1β

SKBR3, BT474, and AU565 cells (Her2-amplified) and BT483 breast cancer cells (not Her2-amplified) were treated with vehicle or 0.5 and 1 μM of the dual EGFR/Her2 inhibitor GW2974 for 24 hr. RNA was harvested and the expression of *IDH3A* (ERRα target gene), *PGC-1β*, *PGC-1α*, and an unrelated coactivator *SRC2* were analyzed by qPCR. 36B4 was used as an internal normalization control for each sample and the ratio of each gene in compound- versus DMSO-treated samples was expressed as relative expression (the expression values of DMSO treated samples of each cell line was set to 1). The data shown are average ±SEM of three independent experiments. "ND" stands for not detectable. See also Figure S5.

inhibit cell growth tracks with the activity of ERRα in cancer cells provides a strong rationale for targeted therapy of tumors in which this receptor is transcriptionally active. Additionally, the finding that the ERRα signaling axis is downstream of Her2 and IGF-1R suggests that interventions that include the combined administration of an ERRα antagonist and inhibitors of Her2/IGF-1R signaling may have clinical utility.

Clearly, the cluster 3 gene signature does not represent the full and unbiased spectrum of ERRα activity. Rather, it represents a facet of ERRα activity that is relevant to patient prognosis. Indeed, we have determined that cluster 3, enriched in metabolic genes, is manifest in tumors that also express genes associated with highly proliferative phenotypes (Table S2). By leave-one-out strategy, we cannot pinpoint an individual gene that would explain the full predictive capacity of this cluster (data not shown), suggesting this activity is the sum of contributions of multiple genes within this cluster. However, our data lend credibility to the hypothesis that cluster 3 reflects a sum of genes involved in oxidative phosphorylation and TCA pathways, whose expression is coordinately elevated by ERRα signaling to satisfy the elevated energy demands of a rapidly dividing tumor cell. However, we cannot definitively conclude that cluster 3 genes are responsible for all the phenotypes observed. It is also possible that ERRα-regulated genes contained within other clusters may also contribute significantly to the pathogenesis of breast cancer.

In addition to validating a role for ERRα in the pathogenesis of breast tumors, the results of this study highlight additional pathways that can be targeted for the treatment of tumors in which this receptor is active. For instance, the set of ERRα regulated genes generated from cluster 3 is most predictive of unfavorable outcome, and this cluster is enriched in genes coding enzymes of TCA cycle and oxidative phosphorylation pathway (6 and 20 genes, respectively, by GO annotations). Although a strong link between cellular metabolism and cancer has been established, most efforts in this area were directed toward an understanding of the processes that regulate aerobic glycolysis and how these could be targeted for therapy. More recently, however, it has become clear that metabolic pathways that fulfill the high demand for biosynthetic intermediates by rapidly dividing cancer cells are also likely to be useful points for therapeutic intervention. Of note in this regard is the observation that inhibition of fatty acid synthase (FAS), acetyl CoA carboxylase (ACC), ATP citrate lyase, or pyruvate dehydrogenase kinase (PDK) can negatively inhibit the growth of tumors (Bauer et al., 2005; Beckers et al., 2007; Hatzivassiliou et al., 2005; Kuhajda et al., 2000; Pathania et al., 2009; Pizer et al., 1996). Indeed, several drugs

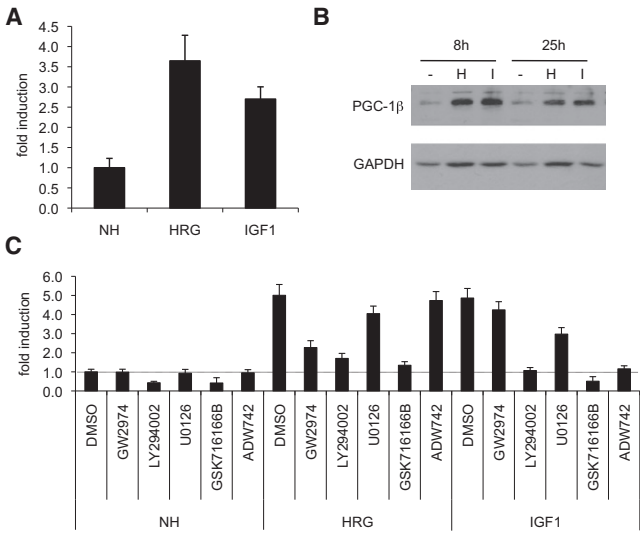

Figure 7. Activation of Her2 or IGF-1R Signaling Pathways Enhances the Expression of PGC-1β

(A) MCF7 breast cancer cells were serum starved for 24 hr and then treated with vehicle or 100 ng/ml of the indicated growth factors for 8 hr. RNA was harvested and the expression of *PGC-1β* was analyzed by qPCR.

(B) MCF7 cells were treated as in (A) and whole-cell extracts were collected 8 and 25 hr after growth factor stimulation. Western immunoblot was used to determine the expression of PGC-1β. GAPDH was used as a loading control.

(C) MCF7 cells were serum starved and pretreated for 1 hr with various kinase inhibitors followed by 8 hr treatment with vehicle, heregulin, or IGF-1. RNA was harvested and the expression of *PGC-1β* was analyzed by qPCR. For all qPCR analysis, 36B4 was used as an internal normalization control for each sample and the relative expression of each gene in treatment versus vehicle-treated samples was expressed as average fold induction ±SD. The data shown are representative of at least three independent experiments.

See also Figure S6.

targeting the TCA cycle and OXPHOS are currently being evaluated as treatments for various cancers (Pathania et al., 2009). Thus, in addition to ERRα antagonists, agents that inhibit these pathways are likely to have therapeutic efficacy in tumors exhibiting high ERRα activity. Furthermore, we observed both similarities and differences in the way the expression of each of the four signatures correlates with patient survival. Based on these observations we hypothesize that ERRα may have distinct activities, some good and some bad for the tumor growth. It will be interesting to see if pharmaceuticals targeting one aspect (cluster 3 genes) of ERRα activity will be more beneficial than targeting the overall ERRα activity in tumors manifesting high ERRα activity. This result provided us with an impetus to explore approaches that may enable the dissection of the distinct pathways regulated by this multifaceted receptor and to identify tools with which to develop "pathway-selective modulators" of this receptor.

Our ERRα signature was built based on the activity of ERRα when it was activated by PGC-1α. Our subsequent analysis has led us to conclude that the closely related coactivator PGC-1β may be a more relevant coactivator for ERRα in breast cancer cells. The expression of *ERRα* and *PGC-1β* correlate very well in most commonly used breast cancer cell models tested, and knockdown of *PGC-1β* interferes with ERRα target gene expression. Underscoring the importance of this finding is the fact that the expression of *PGC-1β* is controlled by growth factor signaling pathways, i.e., Her2, IGF-1R, and PI3K, the significance of which in breast cancer and other tumors is well established. We have observed statistically significant higher Cluster 3 ERR signature scores in ErbB2-overexpressing subtype of tumors in one data set containing such information, but not in the other (data not shown). Extremely small sample size may have contributed to the latter observation. Furthermore, we have demonstrated in this report that the ERRα coactivator PGC-1β is a transcriptional downstream target of both Her2 and IGF-1R pathways, which converge on the transcription factor C-MYC. Thus, it is likely that any manipulation that impacts C-MYC expression and/or stability will effect *PGC-1β* expression and, ultimately, ERRα transcriptional activity. Therefore, we expect that the PGC-1β/ERRα signaling axis will be activated in more than just Her2-positive tumors and therefore we do not expect Her2 status to always correlate with ERRα cluster 3 gene expression. For instance, the Wnt signaling pathway, also frequently upregulated in tumors, has been shown to regulate the transcription of C-MYC by inducing β-catenin/TCF/LEF (He et al., 1998). Further, because of its ability to inhibit GSK3β, the Wnt pathway may also regulate the stability of C-MYC protein posttranslationally (Cook et al., 1996; Sears et al., 2000). Indeed, we found that inhibition of GSK3β in MCF7 cells also led to increased expression of *C-MYC* and *PGC-1β* (data not shown).

C-MYC is a proto-oncogene whose expression is dysregulated in approximately 70% of human tumors. It is involved in a diverse range of biological processes, including proliferation, apoptosis, and differentiation. It also regulates ribosomal biogenesis to affect protein synthesis and influence glycolysis and mitochondrial biogenesis to control cellular metabolism (Li et al., 2005). The fact that *PGC-1β* is a direct target of C-MYC raises the possibility that some of these well-established functions of C-MYC may be mediated by PGC-1β and ERRα. It has been proposed that the ability of C-MYC to regulate both glycolysis and mitochondrial activity confers upon cancer cells the ability to both generate ATP and synthesize the synthetic intermediates needed to sustain rapid cell proliferation (Gordan et al., 2007). Our observation that the PGC-1/ERR-regulated TCA cycle and OXPHOS genes track with unfavorable clinical outcomes may reflect the need of more aggressive tumors to produce biosynthetic intermediates, a hypothesis that is currently under investigation.

In summary, we have used a genomic approach to demonstrate that high ERRα cluster 3 gene expression correlates with unfavorable clinical outcomes in breast cancer. Furthermore, we have provided evidence that compounds that antagonize ERRα activity may have utility in the treatment of patients whose tumors exhibit heightened ERRα activity. Thus, although an activating ligand for this receptor has not yet been identified, the results of this study provide strong justification for the continued pharmacological exploitation of this receptor.

EXPERIMENTAL PROCEDURES

Chemicals

XCT790, GSK716166B, and ADW742 were gifts from W. Zuercher (GlaxoSmithKline, RTP, NC). LY294002 was purchased from Alexis

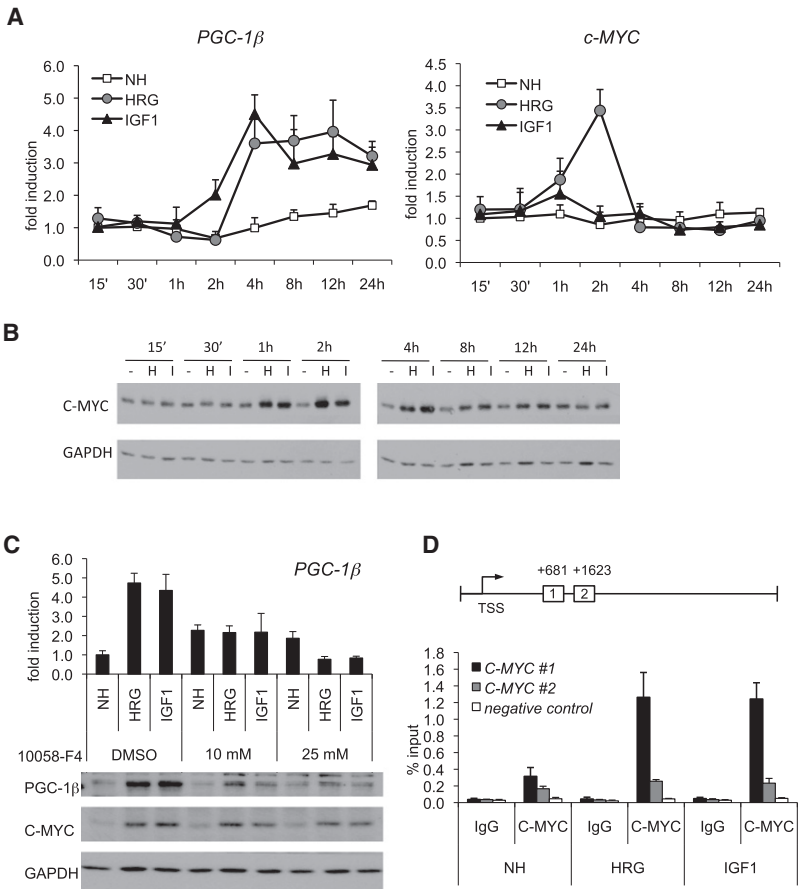

Figure 8. C-MYC Integrates IGF-1 and Her2 Signaling to Activate the Transcription of PGC-1β

(A and B) MCF7 breast cancer cells were serum starved for 24 hr and then treated with vehicle or 100 ng/ml of the indicated growth factors for different time points. RNA and protein were harvested. The expression of *PGC-1β* and *C-MYC* transcripts was analyzed by qPCR (A) and the expression of C-MYC proteins was assessed by western immunoblots (B).

(C) MCF7 cells were serum starved and pretreated with 10 or 25 μM of the C-MYC inhibitor 10058-F4, followed by 8 hr treatment with vehicle, heregulin (HRG), or IGF-1. RNA and whole-cell extracts were collected and the expression of *PGC-1β* RNA and protein, as well as C-MYC protein was analyzed. For all qPCR analysis, 36B4 was used as an internal normalization control for each sample and the relative expression of each gene in treatment versus vehicle-treated samples was expressed as fold induction ±SD. The data shown are representative of at least three independent experiments.

(D) MCF7 cells were serum starved and treated with vehicle, HRG, or IGF-1 for 90 min, and the chromatin was crosslinked with formaldehyde. Chromatin immunoprecipitation was performed using either an IgG control or an antibody that recognizes C-MYC. Precipitated chromatin was reverse crosslinked and quantitated by qPCR using primers spanning the putative C-MYC binding sites in the intron 1 of *PGC-1β*.

See also Figure S7.

Biochemicals (Plymouth Meeting, PA), wortmannin from Cayman Chemical (Ann Arbor, MI), U0126 from Promega (Madison, WI), and GW2974 and 10058-F4 from Sigma (St. Louis, MO). Heregulin, EGF, and IGF-1 were purchased from Peprotech (Rocky Hill, NJ) and Actinomycin D from Calbiochem (La Jolla, CA). Oligonucleotides were synthesized by either IDT DNA (Coralville, IA) or Sigma-Genosys (St. Louis, MO). siRNAs were purchased from Invitrogen (San Diego, CA). Kinase inhibitors were used at the following concentrations unless otherwise specified: 1 μM GW2974, 10 μM LY294002, 10 μM U0126, 0.2 μM GSK716166B, and 0.5 μM ADW742.

Generation of ERRα Signature in hMEC Cells

Primary human mammary epithelial cells were cultured in MEBM (Cambrex, East Rutherford, NJ) with MEGM bullet kit supplemented with 5 mg/ml transferrin and 10^{-5} M isoproterenol. To generate the ERRα signature, hMECs were serum starved for 36 hr followed by infection with MOI = 150 of adenoviruses expressing PGC-1α 2x9 or PGC-1α L2L3M. The generation of variant PGC-1α viruses were described previously (Gaillard et al., 2006). Comparable expression levels of the two PGC-1α variants were verified by western blot analysis (data not shown). RNA was collected 16 hr after infection and purified using QIAGEN RNeasy kit (Valencia, CA). Ten biological replicates of each virus infection were collected. Probe preparation and hybridization to the Hu133A2.0 Affymetrix High Throughput Array system (Affymetrix, Santa Clara, CA) were performed by the Duke microarray facility.

Cell Culture

All cell lines were obtained from ATCC (Manassas, VA) and maintained in a 37°C incubator with 5% CO_2. SKBR3, BT474, AU565 were cultured in RPMI, and MCF7 cells were cultured in DMEM/F12 (Invitrogen). All media were supplemented with 8% fetal bovine serum (Sigma), 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids (Invitrogen). For serum starvation, MCF7 cells were split into regular media for 2 days, washed with serum and phenol-red free DMEM/F12 then placed in the same media for 24 hr before growth factor treatments. For kinase inhibitor experiments, serum starved cells were pretreated with the indicated concentrations of compounds for 1 hr before HRG or IGF-1 (100 ng/ml) stimulation.

Gene Expression Analysis

RNA was purified using the Aurum RNA kit (Bio-Rad Laboratories, Hercules, CA). Detailed protocol and primers used are listed in Supplemental Experimental Procedures. Fold induction was calculated using the $2^{-\Delta\Delta Ct}$ method and 36B4 was used as the normalization control. Data shown are representative of at least three independent experiments. Clustering heatmaps were generated by Ward hierarchical clustering using the JMP software (SAS Institute, Cary, NC).

Proliferation Assay

SKBR3 (5000 cells/well) were transfected using DharmaFECT1 (with 100 nM siRNAs for *ERRα*, *PGC-1β* or controls [LowGC, MedGC, and mock]) and seeded in 96-well plates containing regular RPMI. Cells were harvested 1, 3, 5, 7, 9, or 11 days after transfection and were assayed for DNA content using Hoechst 33258 (Sigma) and the fluorescence was read at excitation 346 nm and emission 460 nm using a Fusion microplate reader (PerkinElmer, Waltham, MA). Data shown are representative of three independent experiments.

Sensitivity to ERRα Antagonist

Breast cancer cells were cultured in RPMI, DMEM, or DMEM/F12 to mimic conditions in which the microarray data sets were generated (Bild et al., 2006; Neve et al., 2006). Detailed culture conditions are described in Supplemental Experimental Procedures. For proliferation assays, cells were seeded between 3000 and 7500 cells/well in triplicates in 96-well plates for 2 days so that cells were about 30%–40% confluent at the time of treatment. Half of the media (50 μl) was removed and 50 μl of fresh media containing 2× concentrations of compounds was added every other day for a total of three treatments. Cells were harvested on day 6 and cell number was determined

by DNA content using Hoechst 33258 DNA dye. Percent inhibition was calculated as 100*[1-(fluorescence of compound-treated cells)/(fluorescence of DMSO treated cells)]. The percent response was plotted against the average medium p values (probability of ERRα activation) determined for each cell line using each ERRα-regulated gene clusters. Correlation and statistical analyses were done using the Prism software (GraphPad, La Jolla, CA). Data shown was the average of four independent experiments.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation assay was performed as previously described (Norris et al., 2009). Detailed protocol and primers used are included in the Supplemental Experimental Procedures.

Analysis of global Gene Expression

Microarray data obtained in hMEC cells were analyzed using dCHIP software (Li and Wong, 2001). Model based expression indices were calculated on $\log_2$-transformed data using PM-MM differences. Clustering was performed by centroid linkage with 1-correlation distance metric; rows were standardized by subtracting the row mean and dividing by standard deviation.

Predictive Modeling

The algorithm for predicting a pathway activity based on the expression data from a set of responsive genes has been described previously (Bild et al., 2006; West et al., 2001). Detailed protocol see Supplemental Experimental Procedures.

Survival Analysis

For determining the correlation between ERRα pathway activation and RFS, clinical samples were ordered according to mean posterior probability of ERRα activation and divided at the average value into two groups, "High ERRα activity" and "Low ERRα activity." Kaplan-Meyer survival analysis was then performed comparing the RFS in the two groups. p values were obtained from the Mantel-Cox test. Microarray data of clinical samples were obtained from previously published patient de-identified data sets. The statements of IRB approval were included in the cited original manuscripts.

ACCESSION NUMBERS

The hMEC microarray data have been deposited in GEO: GSE23061.

Supplemental Material

Supplemental Material includes Experimental Procedures, seven figures, six tables and can be found online at doi:10.1016/j.ccr.2011.08.023.

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