Cancer Research

Reprogramming of the ERRα and ERα Target Gene Landscape Triggers Tamoxifen Resistance in Breast Cancer

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Abstract

Endocrine treatment regimens for breast cancer that target the estrogen receptor-α (ERα) are effective, but acquired resistance remains a limiting drawback. One mechanism of acquired resistance that has been hypothesized is functional substitution of the orphan receptor estrogen-related receptor-α (ERRα) for ERα. To examine this hypothesis, we analyzed ERRα and ERα in recurrent tamoxifen-resistant breast tumors and conducted a genome-wide target gene profiling analysis of MCF-7 breast cancer cell populations that were sensitive or resistant to tamoxifen treatment. This analysis uncovered a global redirection in the target genes controlled by ERα, ERRα, and their coactivator AIB1, defining a novel set of target genes in tamoxifen-resistant cells. Beyond differences in the ERα and ERRα target gene repertoires, both factors were engaged in similar pathobiologic processes relevant to acquired resistance. Functional analyses confirmed a requirement for ERRα in tamoxifen- and fulvestrant-resistant MCF-7 cells, with pharmacologic inhibition of ERRα sufficient to partly restore sensitivity to antiestrogens. In clinical specimens ($n = 1041$), increased expression of ERRα was associated with enhanced proliferation and aggressive disease parameters, including increased levels of p53 in ERα-positive cases. In addition, increased ERRα expression was linked to reduced overall survival in independent tamoxifen-treated patient cohorts. Taken together, our results suggest that ERα and ERRα cooperate to promote endocrine resistance, and they provide a rationale for the exploration of ERRα as a candidate drug target to treat endocrine-resistant breast cancer. *Cancer Res; 75(4); 720–31. ©2015 AACR.*

Introduction

The ligand-activated transcription factor ERα is a key driver of the breast cancer phenotype in around 70% of patients (1). Accordingly, endocrine treatment modalities targeting ERα, such as the selective estrogen receptor modulator (SERM) tamoxifen or downregulator (SERD) fulvestrant (Fulv; SERM) constitute the basis for therapeutic intervention in ERα-positive tumors (2). Fulvestrant as a pure ERα antagonist and tamoxifen as partial antagonist counteract the pro–proliferative and antiapoptotic stimuli classically induced by estrogens (3). Beyond the widespread improvements by these treatment regimens, the frequent onset of antiestrogen resistance remains a major limitation (4), underlining the clinical need for alternative drug targets.

A compelling body of evidence suggests involvement of another nuclear hormone receptor, estrogen-related receptor-α (ERRα), in the pathogenesis of breast cancer. Increased ERRα expression was found in mammary tumors and correlated with an impaired disease-free and overall patient survival (5, 6). The pathophysiologic relevance of ERRα has been further demonstrated *in vivo*, as ERRα inhibition in xenograft systems reduces breast tumor growth, and in HER-2/neu–driven breast cancer mouse models, ERRα knockout delays tumor formation (7, 8). Despite the structural relationship, ERα and ERRα share only 33% homology in their ligand-binding domains, resulting in the insensitivity of ERRα to classical ERα ligands such as estrogen and tamoxifen (9–11). Because of the lack of known natural ligands, ERRα is classified as orphan receptor, whereas its transcriptional activity can be abrogated by small-molecule inhibitors (i.e., XCT790; ref. 12).

ERRα and ERα possess a high sequence homology in their central DNA-binding domains (68%), and therefore each recognize the others cognate-binding motif (9, 13). Identification of a subset of common target genes (e.g., *pS2*; refs. 14, 15), raised the hypothesis that ERRα bypasses the requirement for ERα in endocrine-resistant breast cancers and fuels resistance. This concept has been corroborated by the interplay of ERRα and known determinants of antiestrogen resistance such as HER-2/neu and the coactivator AIB1 (6, 8, 16). As a versatile nuclear hormone

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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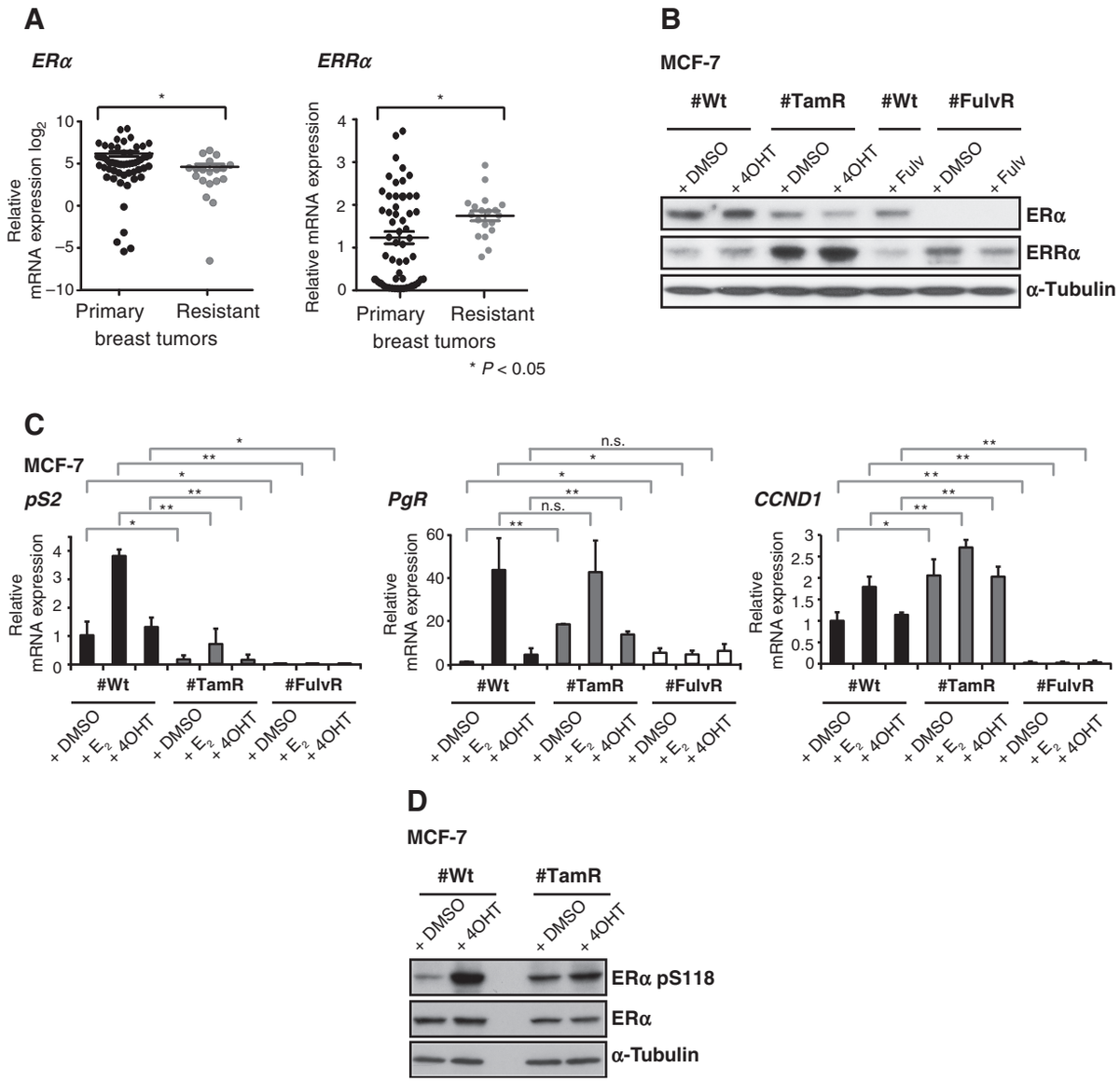

Figure 1.
Characterization of ERα and ERRα in endocrine-resistant breast tumors and MCF-7 cells. A, relative *ERα* and *ERRα* mRNA expression. qRT-PCR revealed decreased *ERα* (*, $P = 0.0169$), but maintenance of *ERRα* expression (*, $P = 0.0477$) in tamoxifen-resistant breast carcinomas. RNA was available from 55 primary untreated (tumor set I) and 19 of 20 recurrent tamoxifen-resistant carcinomas (tumor set II). B, steroid-depleted MCF-7 #Wt, #TamR, and #FulvR cells were exposed to DMSO, 4OHT (1 μmol/L), or fulvestrant (100 nmol/L) for 1 hour and subjected to immunoblotting. C, canonical ERα signaling. Hormone-deprived MCF-7 cells were exposed to DMSO, E_2 (10 nmol/L), or 4OHT (1 μmol/L) for 24 hours, followed by *pS2*, *PgR*, and *CCND1* measurement by qRT-PCR. *, $P < 0.05$; **, $P < 0.01$. n.s., nonsignificant. D, ERα S118 phosphorylation in MCF-7 #Wt and #TamR cells. Steroid-depleted cells were treated for 20 minutes with DMSO or 4OHT (1 μmol/L), respectively, and subjected to immunoblotting.

receptor regulator, AIB1 not only regulates ERα's transcriptional activity (17), but likely dictates ERRα activity by functioning as substitute "protein ligand" (6). However, its influence on tamoxifen resistance is still controversial.

The substantial cross-talk between ERα and ERRα emphasizes the need to elucidate their concerted action in endocrine-resistant breast carcinomas. Beyond recent reports that focused on tamoxifen-induced ERα cistromes (18–20), no comprehensive map of ERα-, ERRα-, and AIB1-binding events in relation to gene expression had so far been described in the tamoxifen-resistant setting.

Table 1. ERα and PR in recurrent tamoxifen-treated breast tumors

ERα/PR expression	Number of tumors ($n = 20$)	ERα$^+$ tumors (%)	Total (%)
ERα$^+$/PR$^+$	13	76.5	65
ERα$^+$/PR$^-$	4	23.5	20
ERα$^-$/PR$^-$	3	NA	15

NOTE: ERα and PR receptor status was evaluated by routine pathologic IHC (tumor set II). Depicted are absolute numbers, percentages of ERα-positive breast tumors, and the total sum of ERα-positive and -negative tumors.
Abbreviation: NA, not available.

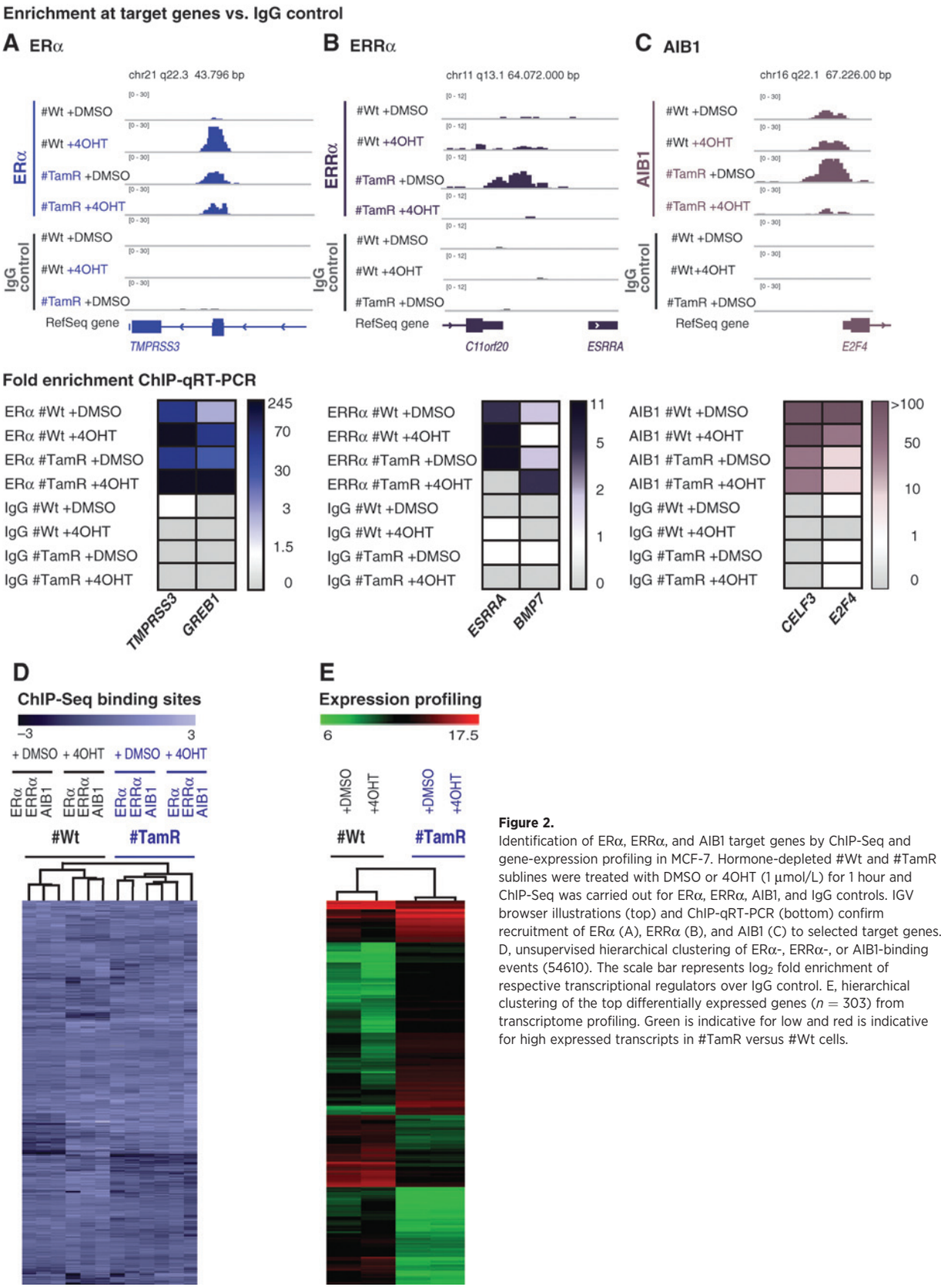

Figure 2.
Identification of ERα, ERRα, and AIB1 target genes by ChIP-Seq and gene-expression profiling in MCF-7. Hormone-depleted #Wt and #TamR sublines were treated with DMSO or 4OHT (1 μmol/L) for 1 hour and ChIP-Seq was carried out for ERα, ERRα, AIB1, and IgG controls. IGV browser illustrations (top) and ChIP-qRT-PCR (bottom) confirm recruitment of ERα (A), ERRα (B), and AIB1 (C) to selected target genes. D, unsupervised hierarchical clustering of ERα-, ERRα-, or AIB1-binding events (54610). The scale bar represents $\log_2$ fold enrichment of respective transcriptional regulators over IgG control. E, hierarchical clustering of the top differentially expressed genes ($n = 303$) from transcriptome profiling. Green is indicative for low and red is indicative for high expressed transcripts in #TamR versus #Wt cells.

Thus, we analyzed ERRα, ERα, and AIB1 in recurrent tamoxifen-resistant breast cancers and conducted a genome-wide target gene profiling in tamoxifen-resistant and -sensitive MCF-7 cell culture models. Furthermore, we performed functional analyses of ERRα's role in treatment sensitivity and assessed its predictive impact on patient's response toward endocrine therapy.

Materials and Methods

Fresh-frozen, formalin-fixed paraffin-embedded breast cancer specimens and patient characteristics

Fresh-frozen primary pretreatment (tumor set I) and secondary locoregional tamoxifen–resistant (tumor set II) breast cancer specimens were collected between 2001 and 2012 at the University Woman's Hospital Heidelberg (Germany) or obtained from the PATH tumor bank (Munich, Germany; ref. 21). All patients from tumor set II received tamoxifen therapy, and the relapse occurred within the time of or upon completion of treatment. All patients signed informed consents, and the study was approved by the Ethical Committee of the Universities of Heidelberg or Bonn, respectively. Tumor set III, defined as ERα$^+$ (IRS-score $\geq$3) has been described before (22) and comprises like tumor set IV and the publicly available dataset GSE9893, used for survival analyses, pretreatment primary tumors. These patients received adjuvant tamoxifen therapy (22, 23) with a standard duration of 5 years. The median follow-up time was 58 (tumor set IV) and 65.9 (GSE9893) months. Additional patient characteristics are described in Supplementary Table S1.

Cell lines

Antiestrogen-sensitive (#Wt) and -resistant (#TamR and #FulvR) MCF-7 cells were from R.I. Nicholson (Cardiff University, Cardiff, UK) and derived as previously described (24, 25). Cell line authentication was assessed using short tandem repeat profiling. #Wt cells were routinely grown under "maintenance" culture conditions in RPMI containing 5% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL fungizone, and 4 mmol/L glutamine. #TamR and #FulvR cells were cultured in "experimental" medium [phenol-red–free RPMI, 5% charcoal-stripped, steroid-depleted fetal calf serum, antibiotics, fungizone, and glutamine as indicated above] and supplemented with 100 nmol/L 4-hydroxytamoxifen (4OHT) or fulvestrant. If not other specified, #Wt cells were cultured under "experimental" conditions and 4OHT and fulvestrant were withdrawn from #TamR and #FulvR cells 72 hours before experiments.

Western blot analysis, RNA isolation, quantitative real-time PCR, and microarray gene-expression profiling

Western blot analysis, RNA isolation, quantitative real-time PCR (qRT-PCR), and microarray gene-expression profiling (Agilent 4 × 44 k) were performed as described previously (6), and outlined in the supplements with antibodies and primers depicted in Supplementary Tables S2 and S3.

Chromatin immunoprecipitation and ChIP-Seq

Hormone-depleted MCF-7 cells were treated with 4OHT (1 μmol/L) or DMSO for 1 hour. Chromatin immunoprecipitation (ChIP) was performed using the Transcription-Factor-ChIP-Kit (Diagenode) with minor modifications and library preparation according to the NEBNext—ChIP-Seq Sample-Prep Master-Mix Set 1.

Enrichment of biologic processes

Network discovery of enriched target genes in #TamR (vs. #Wt) was computed with Ingenuity Systems IPA (www.ingenuity.com).

Cell viability assays

Cells were treated every 2 days with 4OHT (100 nmol/L), fulvestrant (100 nmol/L), and XCT790 (1 μmol/L) and viability was monitored with the CellTiter-96 Aqueous-Non-Radioactive Cell Proliferation Assay (Promega).

Lentiviral knockdown and Click-iT-Edu cell proliferation assays

Production of lentiviral particles targeting *ERRα* (TRCN-0000022179 and TRCN0000022181; Sigma-Aldrich) and a non-targeting control shRNA (SHC002) was conducted as described previously (26). Proliferation was assessed by the Click-iT-EdU Cell Proliferation Kit (Life Technologies).

Immunohistochemistry on tissue microarrays

The tissue microarray (TMA) was comprised of primary formalin-fixed paraffin-embedded (FFPE) breast tumor samples (tumor set III and IV). Upon ERRα staining (Abcam), data were analyzed as described before (22).

Kaplan–Meier survival analyses

ERRα mRNA expression values were retrieved from a tamoxifen-treated primary breast cancer gene-expression profiling meta-set deposited at NCBI-GEO database (GSE9893; ref. 23). ERRα protein expression was assessed by IHC in tumor set IV.

Statistical analyses and bioinformatic computation

Statistical analyses and bioinformatic computation were performed as outlined in the supplements. *P* values are listed in Supplementary Table S4.

Additional information is provided in the Supplementary Materials and Methods.

Results

ERα and ERRα in recurrent tamoxifen-resistant breast carcinomas and MCF-7–based breast cancer models of endocrine resistance

ERα and *ERRα* mRNA expression was analyzed in a cohort of 19 relapsed breast tumors, which emerged during or after completion of long-term adjuvant tamoxifen treatment (tumor set II, Supplementary Table S1). Comparison with primary treatment-naïve breast carcinomas (tumor set I) revealed a minor downregulation of *ERα* (*, $P = 0.0169$), although most tumors remained *ERα* positive, and slight upregulation of *ERRα* (*, $P = 0.0477$) in tamoxifen-resistant cases (Fig. 1A).

To assess the suitability of *in vitro* model systems for functional analyses, ERα and ERRα expression was studied in a tamoxifen- (#TamR) and a fulvestrant (#FulvR)-resistant MCF-7 subline. In comparison with the corresponding antiestrogen-sensitive parental strain (#Wt), #TamR and #FulvR cells were characterized by a slight and complete reduction in ERα expression, respectively, which is in good agreement with published data (Fig. 1B; refs. 25, 27), regardless of short-term treatment with the respective antiestrogens. Furthermore, an increase in ERRα was detected in the #TamR subline. Hence, the cell lines can be considered as eligible models to investigate the impact of ERα and ERRα on endocrine resistance.

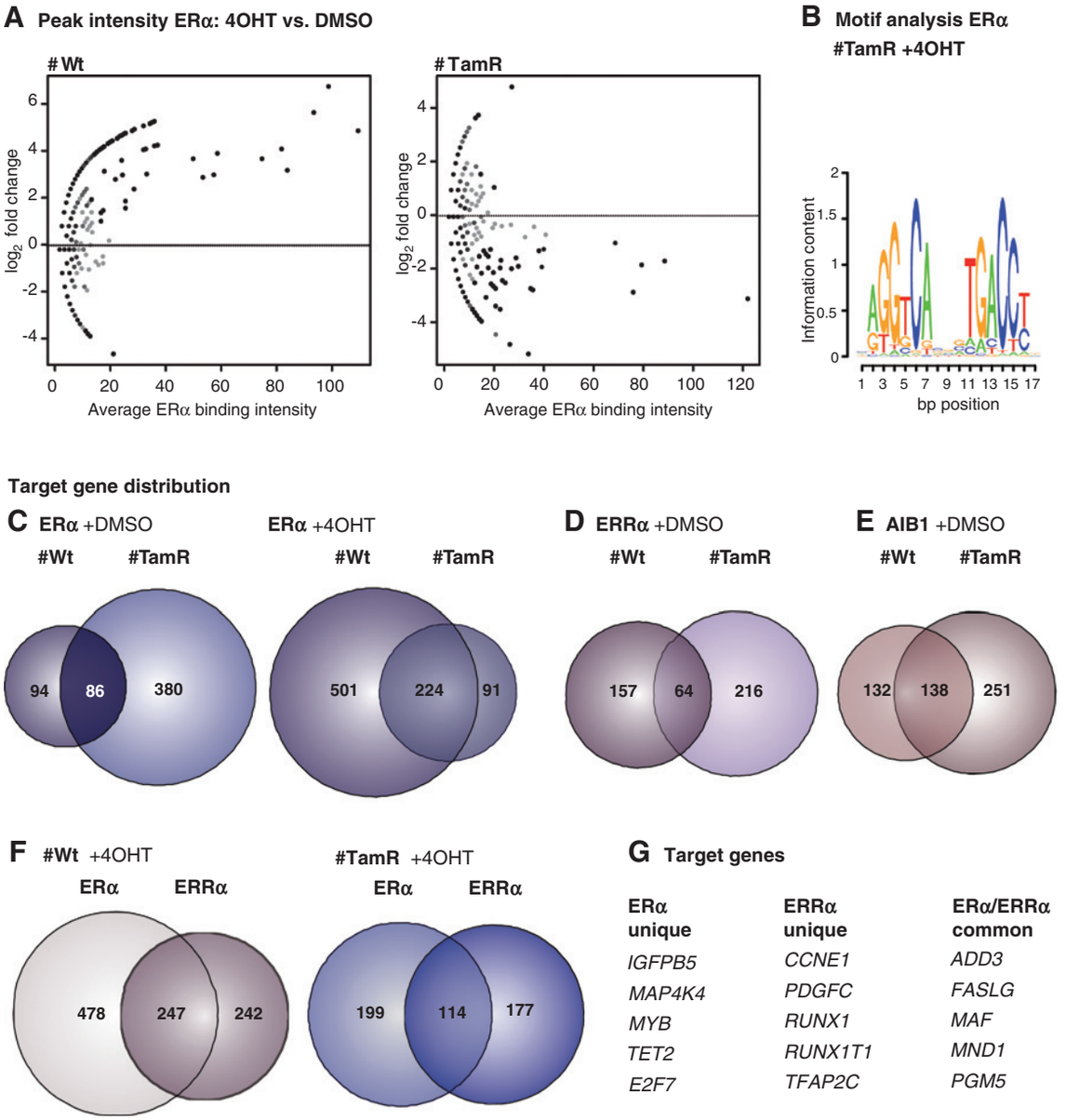

Figure 3.
ChIP-Seq and integrated gene-expression profiling unravels discrete target gene landscapes for ERα, ERRα, and AIB1 between tamoxifen-sensitive and -resistant MCF-7 cells. A, mean average plots (MA-plots) of ERα-binding events in MCF-7 #Wt (left) and #TamR (right) cells upon tamoxifen exposure. The average peak intensity is depicted on the *x*-axis and the $\log_2$ fold ratio of peak intensities between 4OHT and DMSO administration on the *y*-axis. B, weight matrix of the overrepresented ERα consensus-binding motif (ERE) in 4OHT-treated #TamR cells. C–E, Venn diagrams of ERα (DMSO: left, 4OHT right), ERRα- and AIB1-binding sites (intensity cutoff ≥10) associated with gene-expression changes (≥ ±1 $\log_2$ fold change). F, crossover between ERα and ERRα target genes identified by ChIP-Seq and transcriptome profiling in #Wt and #TamR MCF-7 sublines upon tamoxifen administration. G, selected target genes for ERα and ERRα exclusively found in #TamR.

In addition, cell proliferation assays confirmed the treatment sensitivity of #Wt cells toward 4OHT and fulvestrant (Supplementary Fig. S1). Whereas #TamR cells even displayed a slightly increased proliferation rate in the presence of tamoxifen, but a decrease upon fulvestrant treatment, #FulvR cells displayed cross-resistance to both antiestrogens.

In the following, we elucidated whether resistant cells retain canonical ERα signaling. For this purpose, MCF-7 #Wt, #TamR, and #FulvR cells were treated with estradiol (E_2) or 4OHT, and the relative mRNA expression of the established ERα target genes *pS2*, *PgR*, and *CCND1* (28) was measured using qRT-PCR (Fig. 1C). As expected, supplementation of the cell culture media with estradiol resulted in a marked increase in target gene expression in #Wt cells, a feature not observed upon administration of the partial antagonist tamoxifen. In contrast, #TamR cells displayed a highly variable response pattern. Whereas the estradiol-dependent *pS2*

levels (left) were lower in #TamR cells, estradiol-induced *PgR* (middle) was expressed to a similar extent as in the #Wt subline. Irrespective of the presence of estradiol or tamoxifen, *CCND1* (right) exhibited high baseline expression levels in #TamR, but in accordance with the other target genes was lost in ERα-negative fulvestrant-resistant MCF-7 cells. These findings indicate a disengaged target gene regulation in tamoxifen-resistant cells at selected promoters. Moreover, we delineated the phosphorylation status of ERα S118 as a classical activation marker in ERα-positive #Wt and #TamR cells (Fig. 1D). In #Wt cells, phosphorylation was induced in a ligand-dependent manner, whereas high baseline levels of phosphorylated ERα S118 were present even in untreated #TamR cells.

Furthermore, we investigated ERα's ability to induce target gene expression in refractory tamoxifen-resistant breast tumors (tumor set II). The progesterone receptor (PR) serves as a reliable surrogate marker to assess ERα functionality in FFPE patient tumor material (29). Analyses of the complete set ($n = 20$) of immunohistochemically stained specimens revealed that 76.5% of ERα-positive tumors coexpressed PR, whereas the remaining 23.5% lacked notable PR expression (Table 1). Correspondingly, ERα retains its capability for canonical downstream signaling in the majority of tamoxifen-resistant tumors. In combination with the sensitivity of tamoxifen-resistant cells toward ERα loss (as assessed by the SERM fulvestrant; Supplementary Fig. S1B), the modified ERα activity on selected target genes *in vitro* and the slightly increased ERRα expression in tamoxifen-resistant cells suggest that global adaptions in the target gene regulation of the nuclear receptors might contribute to endocrine resistance.

Genome-wide landscape of ERα-, ERRα-, and AIB1-binding sites in tamoxifen-resistant MCF-7 cells

To map ERα- and ERRα-binding events in the endocrine-resistant setting across the genome, we selected the ERα- and ERRα-expressing MCF-7 #Wt and #TamR cells and subjected them to ChIP sequencing (ChIP-Seq) after tamoxifen or vehicle (DMSO) treatment. In addition, AIB1 was included into the experimental setup to evaluate its binding pattern in resistant cells. The ChIP reaction was monitored by the detection of precipitated protein–DNA complexes using Western blot analysis (Supplementary Fig. S2A). The ChIP-Seq approach identified a vast enrichment of ERα upon tamoxifen treatment on classical target genes such as *TMPRSS3* and *GREB1* (18) in #Wt cells, underlining the robustness of the method (Fig. 2A top, for the complete panel Supplementary Fig. S2B). Compared with the ERα frequency distribution, ERRα and AIB1 predominantly showed lower but broader peaks (Fig. 2B and C top; Supplementary Fig. S2C–S2D). The ChIP-Seq data were further validated by ChIP–qRT-PCR confirming the recruitment of all factors to known and newly identified cognate-binding elements (ERα: *TMPRSS3*, *GREB1*; ERRα: *ESRRA*, *BMP7*; AIB1: *E2F4*, *CELF3*; Fig. 2A–C, bottom; Supplementary Fig. S3).

Different ERα, ERRα, and AIB1 target gene repertoires between tamoxifen-sensitive and -resistant breast cancer cells

Hierarchical clustering of all ChIP-Seq peaks, occupied by either ERα, ERRα, or AIB1 under any treatment condition (Fig. 2D), demonstrated clearly binding clusters specific for MCF-7 #Wt or #TamR cells. Because binding of a transcription factor not necessarily implies a biologic outcome on transcriptional level, gene-expression profiling was conducted under the same experimental conditions. Hierarchical clustering of the top differentially regulated transcripts again revealed distinct transcriptional networks between #Wt and #TamR cells (Fig. 2E; Supplementary Table S5). These findings are in accordance with the SAM analyses (FDR < 0.01), which unraveled a large proportion of significantly upregulated (1,060) and downregulated (1,429) transcripts in #TamR compared with #Wt cells.

Subsequently, we interrogated the target gene repertoires of ERα, ERRα, and AIB1 in detail (Fig. 3; Supplementary Table S6) and dissected the global ERα chromatin occupancy in response to tamoxifen. Tamoxifen facilitated a rapid recruitment of ERα to DNA interaction sites in #Wt cells (Fig. 3A, left). Hitherto unexpected was the inverse recruitment pattern in the #TamR subline, as we observed reduced chromatin binding of the ERα/tamoxifen complex in comparison with the nonligand-bound receptor (Fig. 3A, right). We further applied consensus-binding motif analyses to assess whether ERα still executes genomic signaling in tamoxifen-treated #TamR cells. The classical estrogen-response element (ERE; ref. 18) emerged as the most prevalent ERα DNA interaction site, approving maintenance of canonical genomic signaling in tamoxifen-resistant cells (Fig. 3B).

To identify functionally relevant target genes, high-affinity–binding events were combined and set in relation to expression changes. Beyond a minor proportion of common ERα target genes in untreated cells [DMSO: $n = 86$ (15.4%); Fig. 3C, left] and a slight upregulation upon tamoxifen administration [4OHT: $n = 224$ (27.5%); Fig. 3C, right], the majority remained unique in #Wt and #TamR cells. Closer examination of the total number of ERα target genes in #Wt (DMSO, $n = 180$; 4OHT, $n = 725$) and #TamR (DMSO, 466; 4OHT, 315) sublines further uncovered ligand-independent binding of ERα in tamoxifen-resistant cells in line with previous data (19).

Substantial differences were also observed for the ERRα and AIB1 target gene repertoires in tamoxifen-sensitive compared with -resistant sublines. Only 14.7% of ERRα target genes were commonly regulated in #Wt and #TamR cells (Fig. 3D), whereas a higher degree of overlap was observed for AIB1 target genes (26.5%, Fig. 3E). Correspondingly, the majority of targets were differentially controlled between #Wt (total target genes: ERRα, $n = 221$; AIB1, $n = 270$) and #TamR cells (total target genes: ERRα, $n = 280$; AIB1, $n = 389$). This lack of cross-binding reactivity was maintained independent of the applied tamoxifen treatment (data not shown). Thus, we uncovered a global reprogramming of ERα, ERRα, and AIB1 target regulation in tamoxifen-resistant breast cancer cells.

We further investigated whether AIB1 specifically colocalizes to ERα or ERRα target genes in tamoxifen-resistant cells. However, we did not observe marked quantitative alterations with functional relevance in the overlapping target gene spectra (Supplementary Fig. S4A–S4B). Although we did not notice pronounced AIB1 expression changes in tamoxifen-resistant MCF-7 cells, we surprisingly detected a significant downregulation of *AIB1* mRNA in relapsed tamoxifen-resistant breast cancer specimens (Supplementary Fig. S4C–S4D). As our data did not provide an indication for an involvement of AIB1 in the development of resistance, we focused on the impact of ERα and ERRα in subsequent analyses.

ERα and ERRα functionally cooperate to promote endocrine resistance beyond their distinct target cistromes

To examine, whether ERα and ERRα regulate similar or different target spectra in tamoxifen-resistant cells, the overlap of these

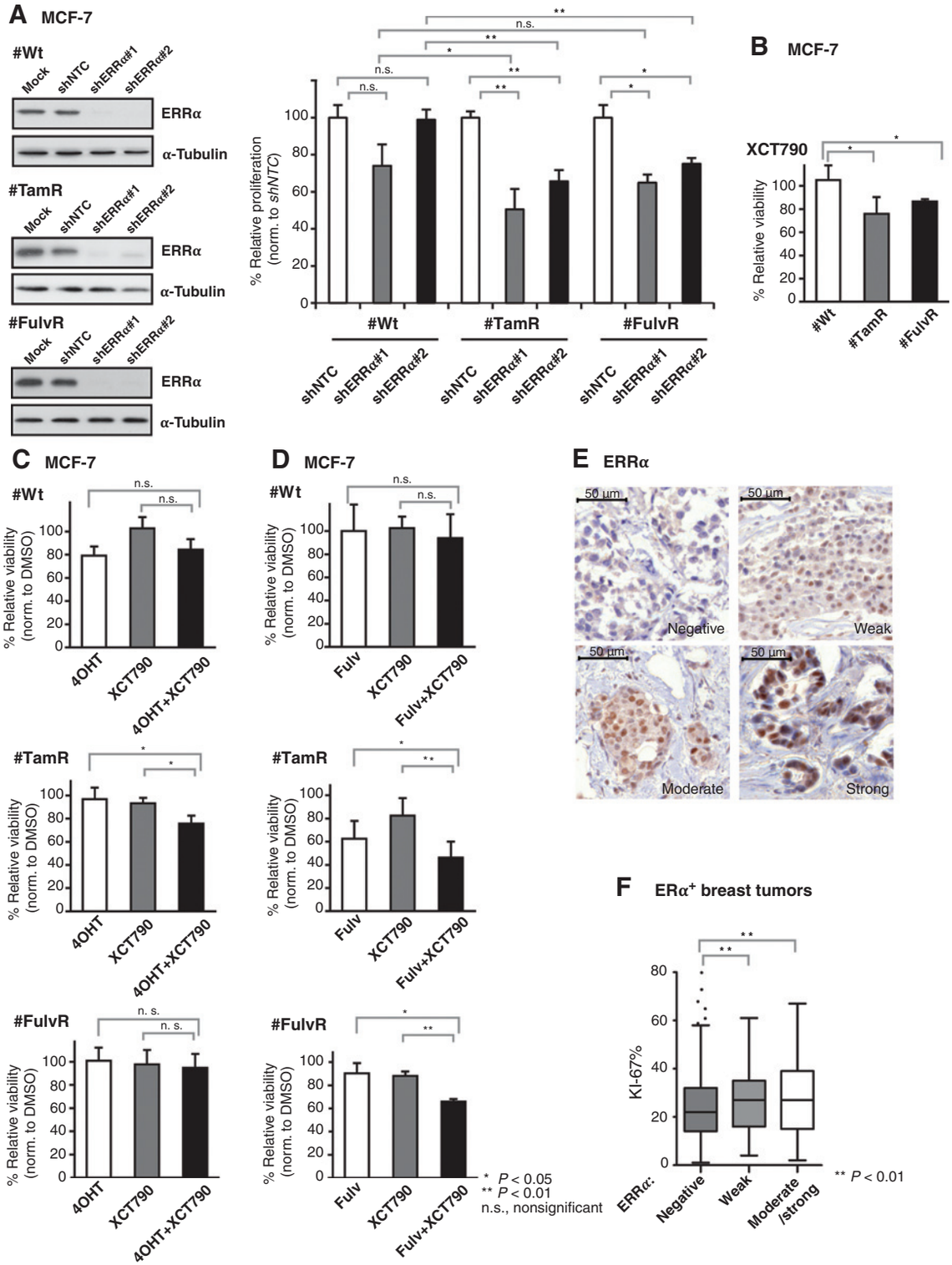

Figure 4.
ERRα inhibition reduces proliferation in endocrine-resistant MCF-7 cells. A, lentiviral knockdown of ERRα in MCF-7 #Wt in "maintenance medium" and #TamR and #FulvR cells in steroid-depleted medium with two independent *shRNAs* (*shERRα#1* and *shERRα#2*). Western blotting was done to ascertain ERRα protein knockdown (left). Cell proliferation measured by Click-iT–EdU was adjusted to nontarget *shRNA* control (*shNTC*, right). (*Continued on the following page.*)

spectra was determined. Surprisingly, despite a small subset of commonly regulated genes, striking differences were observed for their target cistromes in both tamoxifen-sensitive and -resistant MCF-7 cells (Fig. 3F; Supplementary Fig. S4E and Supplementary Table S7). Comparison of the relative percentage of shared target genes in #Wt (DMSO, 26.5%; 4OHT, 25.5%) and #TamR cells (DMSO, 15%; 4OHT, 23.3%) unraveled an even smaller proportion of common target genes in resistant cells.

To elucidate the functional relevance of deregulated target genes, which were specifically enriched in resistant cells, deregulated networks were inferred using Ingenuity (Supplementary Table S8). ERα target genes were associated with cellular processes such as proliferation and cell death (e.g., *MAP4K4*, *MYB*, and *TET2*, Fig. 3G). Although ERRα induced a different set of downstream effectors (e.g., *CCNE1*, *PDGFC*, and *RUNX1)*, these were associated with similar pathophysiologic processes (e.g., proliferation), suggesting a functional redundancy between ERα and ERRα. Furthermore, we identified common ERα and ERRα target genes, which have been already linked with different forms of resistance in other tumor types (e.g., *ADD3*; ref. 30).

The potential of ERα to promote endocrine-resistant breast cancer proliferation and to predict patient outcome has been demonstrated earlier (31, 32). Correspondingly, we set out to assess the impact of ERRα on the proliferation behavior in MCF-7 #Wt, #TamR, and #FulvR cells, as another antiestrogen-resistant model, using lentiviral-mediated *shRNA* knockdown. Western blot analyses confirmed effective silencing of ERRα expression and cell proliferation assays detected a significant reduction in proliferation of #TamR and #FulvR cells upon ERRα knockdown with two independent shRNAs that was not evident in #Wt cells (Fig. 4A). Remarkably, #TamR cells were stronger affected from ERRα loss than #Wt cells, suggestive for a higher requirement of resistant cells on functional ERRα signaling. Comparable effects of the ERRα knockdown on proliferation were achieved in the presence of the respective antiestrogens (Supplementary Fig. S5), indicating that the contribution of ERRα to overcome the tamoxifen- and fulvestrant-induced growth inhibition is not tightly linked to the ERα function.

The transactivation properties of ERRα can be abolished with the small-molecule inhibitor XCT790 (12). Administration of XCT790 impaired the viability of MCF-7 #TamR and #FulvR cells to a stronger extent than #Wt cells as measured by MTS assays (Fig. 4B).

Next, we interfered simultaneously with the function of ERRα and ERα (Fig. 4C). Dual targeting of ERα and ERRα resulted in a significant reduction of cell viability in #TamR cells. This finding was confirmed in cells cultured in the presence of fulvestrant instead of tamoxifen (Fig. 4D). Similar effects were further observed in #FulvR cells (Fig. 4C and D, bottom). In accordance with the established cross-resistance of fulvestrant-resistant breast cancers toward tamoxifen (33), #FulvR cells were unresponsive toward tamoxifen, but were partly resensitized toward fulvestrant upon simultaneous inhibition of ERRα.

As ERRα sustained proliferation *in vitro*, we investigated its impact on cell proliferation in breast cancer specimens. ERRα and KI-67 protein expression was determined by immunohistochemical staining of a TMA covering 1,041 informative ERα-positive primary breast carcinomas (Fig. 4E and for extended panel Supplementary Fig. S6, Supplementary Table S1, tumor set III). ERRα was primarily located in the nucleus, although we also observed weak cytoplasmic staining. We here considered solely nuclear staining in line with our focus on the transcriptional activities of ERRα. In good agreement with the *in vitro* findings, strong nuclear ERRα expression, found in approximately 13% to 20% of tumors, associated with a higher proliferation index (**, $P = 0.0033$, Fig. 4F). Within the same cohort, we observed a positive dependency between ERRα expression and increased p53 expression (**, $P = 0.0042$) and the association with AIB1 (**, $P < 0.0001$, Supplementary Table S9), which has been previously reported by our group in another patient cohort (6).

ERRα associates with a shortened overall survival in endocrine-treated patients with breast cancer

As the *in vitro* findings suggested an involvement of ERRα in the pathogenesis of tamoxifen resistance, we examined the potential of ERRα expression to predict patients' outcome upon tamoxifen treatment. *ERRα* mRNA expression data were retrieved from a gene-expression profiling dataset (GSE9893; ref. 23) of adjuvant tamoxifen-treated primary breast tumors. Subsequent Kaplan–Meier estimates revealed a significantly reduced overall survival (OS; *, $P = 0.02905$, Fig. 5A) for patients with high *ERRα*-expressing tumors. Likewise, the predictive power of ERRα on protein level was analyzed by IHC. The above-mentioned TMA contains a subset of patients with a tamoxifen monotherapy treatment history (tumor set IV). Analysis of this patient cohort again uncovered a correlation between ERRα expression and a shortened OS (**, $P = 0.00974$, Fig. 5B).

The impact of standard clinicopathologic parameters on survival was evaluated in multivariate analyses for both patient cohorts (Table 2). Cox analyses revealed the following independent predictors for an early recurrence upon endocrine therapy: ERRα [HR, 3.30; *, $P = 0.0118$ (GSE9893) and HR, 2.72; **, $P = 0.007$ (tumor set IV)], grade [HR, 2.74; **, $P = 0.0047$ (GSE9893)] and PR [HR, 0.39; *, $P = 0.0442$ (GSE9893)]. ERRα was the only factor identified in both patient cohorts and also upon stratification to grade and PR in the GSE9893 dataset, ERRα was still the only significant factor (HR, 3.72; **, $P = 0.0095$, Supplementary Table S10).

Altogether, these data emphasize that high ERRα expression in breast tumors correlates with a diminished response toward endocrine therapy underlining the contribution of ERRα to the acquisition of endocrine resistance.

(*Continued.*) B–D, MCF-7 #Wt, #TamR, and #FulvR cells were exposed to inhibitors against ERα (100 nmol/L 4OHT or fulvestrant) and/or ERRα (1 μmol/L XCT790). Cell viability under steroid-depleted conditions was monitored by MTS assays (d6). B, ERRα inhibition by XCT790. Dual inhibition of ERα and ERRα with 4OHT (C) or fulvestrant (D) and XCT790. Bar charts indicate the mean + SD of at least three independent experiments. E, examples of immunohistochemical ERRα stainings in ERα-positive breast carcinomas on a TMA at a ×400 original magnification. F, immunohistochemical evaluation of ERRα and KI-67 expression in ERα-positive breast carcinomas ($n = 1{,}041$; **, $P = 0.0033$) on the TMA. ERRα expression was stratified according to negative ($n = 728$), weak ($n = 242$), and moderate/strong ($n = 71$) expression. KI-67 is displayed as a percentage of positive cells.

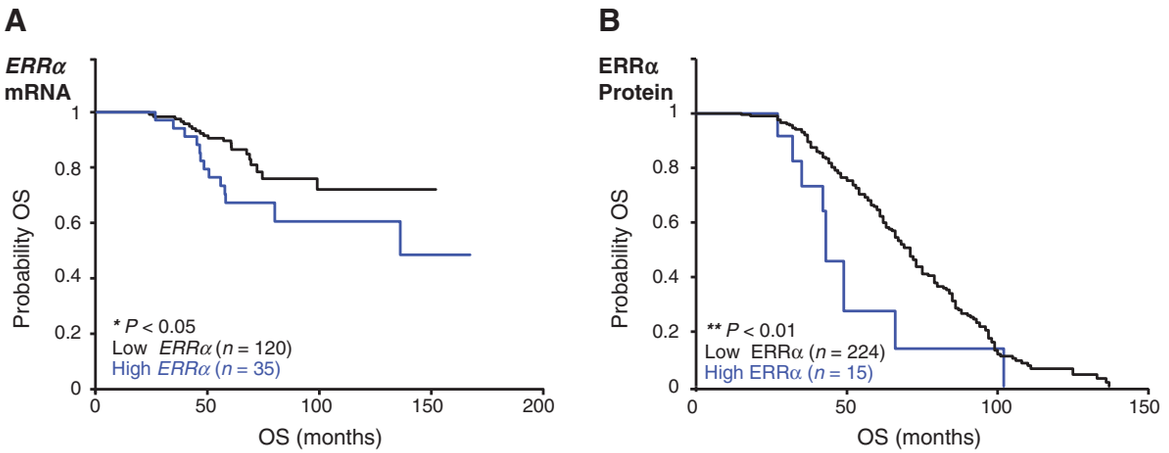

Figure 5.
ERRα correlates with an adverse outcome in tamoxifen-treated patients with breast cancer. Kaplan–Meier survival curves to relate ERRα expression with OS in primary adjuvant tamoxifen-treated primary tumors. A, *ERRα* mRNA expression data were dissected from the microarray dataset GSE9893 (*, $P = 0.02905$; ref. 23). B, ERRα expression based on nuclear immunostaining of ERRα on the TMA (tumor set IV, $n = 239$; **, $P = 0.00974$).

Discussion

Integrated molecular analyses uncovered a multitude of mechanisms underlying antiestrogen resistance, which initially appeared as attractive targets for therapeutic intervention in breast cancer (4). However, in line with the anticipated heterogeneity of drug-tolerant cell populations, the monotherapeutic efficacy of most of them yielded disappointing results in the clinic, highlighting the need for alternative drug targets and combined treatment approaches. ERRα has been identified as a prognostic biomarker for an unfavorable patient outcome in mammary carcinomas (5, 13, 34), underlining a pathophysiologic relevance of ERRα in the etiology of breast cancer.

Table 2. Effects of covariables on survival in tamoxifen-treated primary breast tumors

	Coefficient (95% CI)	HR	*P*[a]
ERRα mRNA[b]			
pT stage	−0.0401 (0.4364)	0.9607	0.9268
pN stage	0.7179 (0.5586)	2.0501	0.1987
Grade	1.0085 (0.3566)	2.7415	**0.0047**
Histologic subtype	−6.4472 (21.6028)	0.0016	0.7654
ERα	0.2072 (1.0837)	1.2302	0.8484
PR	−0.9323 (0.4634)	0.3936	**0.0442**
ERRα	1.1951 (0.4746)	3.3039	**0.0118**[c]
ERRα protein[d]			
pT stage	−0.055835 (0.11429)	0.946	0.630
pN stage	−0.436730 (0.16969)	0.646	**0.010**
Grade	−0.093228 (0.11397)	0.911	0.410
Age	0.002032 (0.00893)	1.002	0.820
HER-2	0.020006 (0.12145)	1.020	0.870
p53	−0.097467 (0.09137)	0.907	0.290
ERRα	0.999811 (0.37229)	2.718	**0.007**
AIB1	−0.000672 (0.23996)	0.999	1.000

Abbreviation: CI, confidence interval.
[a]*P* values <0.05 were considered as significant and are indicated in bold.
[b]Clinical data were retrieved from a publicly available dataset (GSE9893). All cases used for Kaplan–Meier survival analyses were included into multivariate analysis.
[c]ERRα was still a significant ($P = 0.0095$) independent predictor upon stratification to grade and PR status (Supplementary Table S10).
[d]From tumor set IV (total, $n = 239$), 47 cases were not included into multivariate analysis due to missing clinical variables.

Nevertheless, the role of the interplay between ERRα and ERα in endocrine resistance has been basically undetermined. Our combined *in vitro* and tumor data suggest that ERα and ERRα adopt a novel set of target genes in tamoxifen-resistant cells, and that they functionally cooperate to promote endocrine resistance, despite the apparent existence of specific target gene spectra. We further provide evidence that ERRα is a predictor of reduced patient survival upon tamoxifen therapy, and that its inhibition leads to a reduced proliferation in endocrine-resistant cells.

Because of the limited availability of respective patient material, only few studies so far included tamoxifen-resistant recurrent tumors. Apart from the overall diminished ERα expression, which is in good agreement with earlier reports (35, 36), three quarters of tamoxifen-resistant tumors retained the capacity for canonical ERα signaling as assessed by the surrogate marker PR. The target gene-specific differential ERα-driven transcriptional response found in tamoxifen-resistant MCF-7 cells, and the selective regulation of target genes, which are linked to endocrine resistance (e.g., *CCND1*; ref. 4), denotes that ERα actively primes expression of target genes with relevance to resistance. This hypothesis is supported by the reversal of the tamoxifen-resistant phenotype upon disruption of the DNA-binding ability of ERα (37) and the recent identification of mutations in ERα in endocrine-resistant tumors (38, 39). For ERRα, there is currently no appropriate readout system available to access its activation status in breast tissue, but the slight upregulation in tamoxifen-resistant tumors and MCF-7 cells point at a causative role of ERRα in the acquisition of resistance.

Although AIB1 seems to function as the major coactivator of ERα and ERRα in primary breast cancer (6, 17), conflicting results have been generated with respect to its role in tamoxifen resistance. The cumulating evidence, which argues against a strong contribution (40, 41), is consistent with our data as we detected a downregulation of AIB1 in recurrent tamoxifen-resistant tumors.

The genome-wide reprogramming of ERα and ERRα to a newly acquired set of target genes raises the hypothesis that this redirection drives endocrine resistance. This assumption is in accordance with similar findings by Hurtado and colleagues and Ross-

Innes and colleagues (19, 20), who evaluated ERα-binding sites identified by ChIP-Seq, but did not focus on the global combination with transcriptional changes.

In the past, the structural relationship between ERRα and ERα and the corresponding existence of a few commonly regulated target genes (e.g., *pS2*; refs. 9, 14), led to the hypothesis that ERRα circumvents the requirement for ERα in antiestrogen-resistant breast carcinomas. Our data unambiguously demonstrate the existence of specific target repertoires for ERα and ERRα in tamoxifen-resistant breast cancer cells, implying that both engage in different transcriptional activities. Because ERRα is not directly affected by ERα antagonists such as tamoxifen (11), it can stimulate target gene expression independently of ERα to promote tamoxifen resistance. The resulting relative small proportion of overlapping target genes (15%–26.5%) is compatible with earlier reports that uncovered a distinct binding site preference in endocrine-responsive breast cancer (15, 42).

ERRα and ERα are regulated by oncogenic pathways, such as EGFR- or HER-2/neu, which are often inappropriately activated in antiestrogen resistance (4, 16, 43) and affect posttranslational modifications on nuclear hormone receptors. Irrespective of the presence of a ligand, tamoxifen-resistant MCF-7 cells are characterized by a constitutive phosphorylation of ERα at S118, which stimulates ligand-independent activation and reduces ERα's affinity for DNA and tamoxifen binding (44). If inappropriate growth factor signaling indeed induces ligand-independent ERα binding via S118 phosphorylation and a corresponding target gene adaption on the genome-wide scale, our data provide a plausible biologic explanation for the clinically observed phenomenon that activated growth factor signaling abrogates response toward endocrine therapy (4).

ERRα's mode of action is poorly understood, but it appears to act in a constitutive active cell context-, and promoter-dependent manner (14, 45, 46). It is assumed to possess specific signaling activities in dependency of the intrinsic breast cancer subtypes (15). As it has been hypothesized that *Luminal B* carcinomas, which largely overlap with antiestrogen-resistant tumors (47), evolve from endocrine-responsive *Luminal A* tumors (47–49), a subgroup switch would provide a rational explanation for the changed ERRα target spectrum in tamoxifen-resistant cells.

Despite the existence of entirely different transcriptional networks, we identified ERα and ERRα target genes in tamoxifen-resistant cells that feed into similar resistance-promoting processes such as cell proliferation (i.e., ERα, *CCND1* and ERRα, *CCNE1*; ref. 4), indicative for a functional redundancy. Several lines of evidence from our analyses underscore that the predicted functions of specific deregulated target genes in resistant cells indeed have phenotypic implications regarding proliferation: First, we found a positive relationship between ERRα and the proliferation marker KI-67 in a large panel of ERα-positive breast carcinomas. Second, our functional analyses unraveled that tamoxifen-resistant cells have a higher requirement for ERRα than sensitive cells to sustain proliferation. The comparable phenotype in fulvestrant-resistant cells may point at a broader function of ERRα in the adaption toward endocrine therapy in permanently ERα-depleted cell populations. This idea is in good agreement with earlier *in vitro* and mouse xenograft experiments, which also indicated an indispensable role of ERRα for the proliferation of ERα-negative breast cancer cells (7, 42). Importantly, the preclinical observations were reflected by the clinical course: ERRα expression correlated with a reduced OS in independent endocrine-treated patient cohorts.

Currently, drugs targeting ERRα that possess the desired properties for a clinical application are not yet available, but a principle druggability of the transcription factor has been demonstrated *in vitro* and *in vivo* (7, 42). Moreover, the respective ERRα knockout mouse is characterized by a fairly moderate phenotype (50), which increases the likelihood of tolerable side effects.

In conclusion, we identified ERRα as a potential key mediator of endocrine resistance and underline the prevailing view that ERα preserves a pivotal role in tamoxifen-resistant breast carcinomas. Despite the existence of distinct ERRα and ERα target repertoires, their concerted action presents a potential resistance scenario and reinforces the concept of combinatorial targeting strategies directed against both nuclear receptors. Hence, we provide a rationale to consider ERRα as a potential drug target in endocrine-resistant breast carcinomas that do not respond to first-line antiestrogen therapy.

Disclosure of Potential Conflicts of Interest

R. Büttner has ownership interest (including patents) in Targos Molecular Pathology Inc. and is a consultant/advisory board member for Roche, Novartis, Qiagen, Boehringer, GlaxoSmithKline, MSD, Bristol-Myers Squibb, and Lilly. No potential conflicts of interest were disclosed by the other authors.

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