ERK/MAPK regulates ERRγ expression, transcriptional activity, and receptor-mediated Tamoxifen resistance in ER+ breast cancer

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Abstract

Background—Selective estrogen receptor modulators (SERMs) such as Tamoxifen (TAM) can significantly improve breast cancer-specific survival for women with ER-positive (ER+) disease. However, resistance to TAM remains a major clinical problem. The resistant phenotype is usually not driven by loss or mutation of ER; instead, changes in multiple proliferative and/or survival pathways override the inhibitory effects of TAM. Estrogen-related receptor gamma (ERRγ) is an orphan member of the nuclear receptor superfamily that promotes TAM resistance in ER+ breast cancer cells. In this study, we sought to clarify the mechanism(s) by which this orphan nuclear receptor is regulated and, in turn, affects TAM resistance.

Methods—mRNA and protein expression/phosphorylation were monitored by RT-PCR and Western blotting, respectively. Site-directed mutagenesis was used to disrupt consensus ERK target sites. Cell proliferation and cell cycle progression were measured by flow cytometric methods. ERRγ transcriptional activity was assessed by dual-luciferase promoter-reporter assays.

Results—We show that ERRγ protein levels are affected by the activation state of ERK/MAPK, and mutation of consensus ERK target sites impairs ERRγ-driven transcriptional activity and TAM resistance.

Conclusions—These findings shed new light on the functional significance of ERRγ in ER+ breast cancer, and are the first to demonstrate a role for kinase regulation of this orphan nuclear receptor.

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Introduction

Worldwide, breast cancer is the most common cancer in women, with an estimated 1.38 million new cases diagnosed per year [1], and ~70% of breast cancers are estrogen receptor alpha-positive (ER+). ER+ breast cancer can be successfully treated with selective estrogen receptor modulators (SERMs) such as Tamoxifen (TAM) [2], and ER is one of only two robust, reproducible biomarkers that are routinely used to make breast cancer treatment decisions in the clinic [3]. However, the development of TAM resistance is a pervasive problem that affects nearly half of all women with ER+ breast cancer who are treated with TAM [4–6]. Typically, it is not loss or mutation of ER that causes resistance, but changes in proliferative and/or survival pathways in an ER+ breast tumor cell that override the inhibitory effects of TAM. These frequently include alterations in receptor tyrosine kinases, cell cycle regulatory proteins, and mediators of apoptosis.

Distinct from hormone-regulated nuclear receptors such as ER, 25 members of this protein superfamily lack an identified ligand and are thus designated orphan nuclear receptors [7]. Orphan nuclear receptors display constitutive transcriptional activity and have been implicated in numerous developmental and disease processes, including breast cancer [8]. A trio of estrogen-related receptors (ERRα, β, and γ) are well established transcriptional regulators of mitochondrial biogenesis and function, including fatty acid oxidation, oxidative phosphorylation, and the tricarboxylic acid cycle [9, 10] in organs and tissues with high energy requirements, such as the heart and liver. Multiple studies have now shown that the ERRs alter metabolism and oncogene expression in breast and other cancer cells a way that promotes growth and proliferation [11, 12]. In non-transformed mammary epithelial cells, upregulation of endogenous ERRγ after detachment from the extracellular matrix contributes to metabolic reprogramming and, ultimately, the development of resistance to anoikis [13].

As their name implies, ERRs have broad structural similarity to classical ER, but being orphan nuclear receptors they have no (known) endogenous ligand and do not bind estrogen. The third member of this family, ERRγ (ESRRG, NR3B3), is preferentially expressed in ER + breast cancer [14]. Endogenous ERRγ is upregulated during the acquisition of TAM resistance by ER+ invasive lobular breast cancer cells, and exogenous expression of ERRγ in this breast cancer type is sufficient to induce TAM resistance [15]. ERRγ mRNA is also significantly increased in pre-treatment tumor samples from women with ER+ breast cancer who ultimately relapsed following TAM treatment [8]. More recently, nuclear expression of ERRγ protein has been shown to correlate with lymph node-positive status in a small cohort of breast cancer patients [16], and gene-level amplification of ERRγ is significantly enriched in lymph node metastases vs. the primary breast tumor [17].

The goal of the current study is to better understand how ERRγ expression and activity are regulated, and how this regulation contributes to the TAM resistant phenotype in ER+ breast cancer. We show herein that i) modulation of ERK activity directly affects ERRγ protein levels, ii) Serines 57, 81, and 219 are required for ERK-mediated enhancement of ERRγ protein, and iii) mutation of these sites abrogates receptor-mediated TAM resistance and reduces transcriptional activity.

Results

ERRγ mRNA (ESRRG) is increased in pre-treatment tumor samples from women with ER+ breast cancer who relapse within 5 years of TAM treatment [8, 18]. Using the KM plotter tool [19] to test whether there is an association between ERRγ and other clinical parameters in additional patient populations with longer follow-up time, we found that high expression of ESRRG (upper vs. lower tertile) is significantly associated with worse overall survival in ER+ breast cancer patients who received TAM as their only endocrine therapy (Fig 1A, hazard ratio 2.44, logrank p = 0.035). MCF7/RR cells are a TAM-resistant variant of MCF7 [20] that depend on heightened signal transduction through networks regulated by nuclear factor kappa B (NFκB) [21] and glucose-regulated protein 78 (GRP78) [22] for maintenance of the resistance phenotype. By quantitative RT-PCR, expression of ERRγ (Fig. 1B) is increased in resistant MCF7/RR cells vs. sensitive, parental MCF7s. However, MCF7 cells have a mean cycle threshold (C_T) greater than 35, indicative of very low expression outside the optimal range of TaqMan gene expression assays; the mean C_T for MCF7/RR cells is 33. We subsequently performed non-quantitative RT-PCR for ESRRG in independent samples of MCF7 and MCF7/RR cells alongside a human ERRγ ORF cDNA clone (Fig. 1C). While ESRRG mRNA is detectable in both cell lines, the signal intensity observed in ~400 ng cDNA is 40–50% less than that obtained from 800 pg of plasmid. By Western blot, MCF7 and MCF7/RR cells have undetectable ERRγ protein in 67 μg of whole cell lysate, while 25 ng of purified ERRγ protein is observed (Fig. 1D). These data show that MCF7 and MCF7/RR cells express very low levels of receptor mRNA, and that endogenous ERRγ protein is not readily detected in these cells by the available commercial antibodies.

We therefore adapted an exogenous expression model (MCF7 cells transiently transfected with a hemagglutinin (HA)-tagged ERRγ [15, 23]) to determine the mechanism(s) by which this orphan nuclear receptor, when expressed, might modulate the TAM-resistant phenotype. Post-translational modifications such as phosphorylation play essential roles in the regulation of many proteins, including nuclear receptors. At least 8 different phosphorylation sites have been shown to regulate expression or activity of classical (ligand-regulated) ER [24], and a number of these have clinical significance in women with breast cancer who are treated with TAM [4, 25]. In the absence of identified ligand(s), the activity of orphan receptors is thought to be particularly sensitive to regulation by phosphorylation [26–30]. ERK hyperactivation has been associated with TAM resistance *in vivo* and *in vitro* [31, 32], and inhibition of its upstream regulator MEK improves the anti-tumor activity of the steroidal antiestrogen Fulvestrant in ER-positive ovarian cancer [33]. Therefore, we tested whether the activity of ERK or the two other major members of this kinase family (JNK and p38) directly affect exogenous ERRγ in MCF7 cells (Fig. 2A, left panels). The minimal consensus sequence required for phosphorylation of a substrate by any member of the MAPK family is the dipeptide motif S/T-P [34], and ERRγ contains 4 serines (no threonines) that meet these criteria: amino acids 45, 57, 81, and 219. Pharmacological inhibition of pERK by U0126 strongly reduces exogenous ERRγ (HA) levels, but inhibitors of p38 (SB203580) or JNK (SP600125) do not. Furthermore, co-transfection with a mutant, constitutively active form of MEK (MEKDD, [35]) increases pERK and enhances ERRγ (HA) levels (Fig. 2B), as does co-transfection with wild type ERK2 (Fig 2C). Stimulating

MCF7 cells with EGF also increases pERK and enhances exogenous ERRγ (HA), and these effects are blocked by co-treatment with U0126 (Fig 2D). Finally, pharmacological inhibition of pERK by U0126 inhibits exogenous ERRγ (HA) expression in a second ER+ breast cancer cell line, SUM44 (Fig 2E). These data strongly suggest that ERRγ can be positively regulated by ERK.

The putative ERK phosphorylation sites in ERRγ are either located in the N-terminal activation function 1 (AF1) region of the protein (amino acids 45, 57, 81), or in the hinge region downstream of the DNA binding domain (amino acid 219). Tremblay *et al*. [36] have shown that ERRγ and its family member ERRα are regulated by a phosphorylation-dependent SUMOylation motif (PDSM). Phosphorylation at ERRγ S_{45} directs SUMOylation at K40, leading to repression of ERRγ transcriptional activity, and when this serine is mutated to alanine ($S_{45}A$), ERRγ expression and transcriptional activity is enhanced. Therefore, we generated two different variants of ERRγ by site-directed mutagenesis: $S_{45}A$ (part of the PDSM), or $S_{57,81,219}A$ (unknown function). In contrast to wild type and $S_{45}A$ ERRγ, levels of the $S_{57,81,219}A$ variant are decreased by 70% compared to that of wild type ERRγ (Fig. 3A). To determine whether these 3 Serine residues are required for the MEK/ERK-mediated increase in ERRγ levels, wild type or $S_{57,81,219}A$ ERRγ was co-transfected with MEKDD (Fig. 3B). Consistent with data presented in Fig. 2B, activated MEK increases wild type ERRγ by ~3-fold. However, MEKDD is unable to enhance levels of the triple serine mutant. Similarly, treatment with U0126 reduces wild type ERRγ (HA) levels by 70% (consistent with Fig. 2A), but has no further effect on $S_{57,81,219}A$ ERRγ (Fig. 3C). Serines 57, 81, and 219 therefore appear to be required for regulation of ERRγ protein levels by ERK, and their mutation to alanine reduces basal receptor expression.

We next compared $S_{57,81,219}A$ ERRγ to the wild type receptor for its ability to induce TAM resistance. We first used 5-bromo-2′-deoxyuridine (BrdU) incorporation analyzed by fluorescence activated cell sorting (FACS) to measure changes in DNA synthesis (S phase) following 4HT treatment in MCF7 cells transiently transfected with empty vector (control), wild type, or mutant ERRγ (Fig. 4A). As expected, 4HT reduces DNA synthesis by 50% in control (pSG5-transfected) cells. Wild type ERRγ confers significant resistance to 4HT (*p<0.05), but $S_{57,81,219}A$ ERRγ does not. We then tested whether 4HT-mediated induction of the cyclin-dependent kinase (CDK) inhibitors p21 and p27, markers of G0/G1 arrest that are essential for TAM-mediated growth inhibition [37, 38], are altered by exogenous ERRγ. Similar to its effect on ER [39], 4HT increases the expression of both wild type and $S_{57,81,219}A$ ERRγ (Fig. 4B). However, the ~1.5-fold and 1.3-fold induction of p21 and p27, respectively, by 4HT in empty vector transfected cells is reduced or blocked by exogenous expression of wild type, but not mutant, ERRγ. We also measured total and phosphorylated levels of the retinoblastoma tumor suppressor (Rb), a target of active cyclin D1/CDK complexes and another indicator of G1 cell cycle progression. The role of Rb in TAM response and resistance is somewhat contradictory. Some studies report a reduction in pRb in <u>responsive</u> cells following TAM treatment, while others show that loss or downregulation of total Rb is associated with TAM <u>resistance</u> in cell culture models, xenografts, and premenopausal women with ER+ breast cancer [40, 41]. In vehicle-treated conditions, we

observe a strong induction of total and pRb by wild type, but not $S_{57,81,219}$A, ERRγ. When treated with 4HT, the ratio of pRb to total Rb in wild type ERRγ-expressing cells is increased ~2-fold vs. vehicle treatment, and this is driven by a robust decrease in total Rb. In the presence of $S_{57,81,219}$A, ERRγ, pRb remains essentially constant but total Rb is increased in the presence of 4HT. Together, these data show that $S_{57,81,219}$A ERRγ is impaired in its ability to promote TAM resistance, and suggest that this may be due (at least in part) to altered regulation of cell cycle progression by mutant vs. wild type receptor.

ERRγ directly regulates transcription by binding to EREs or ERREs. Deblois *et al.* identified a hybrid ERRE/ERE element as the major binding site for the family member ERRα in breast cancer [42]. Because $S_{57,81,219}$A ERRγ does not induce TAM resistance, we tested whether this mutant has impaired transcriptional activity at all 3 response elements. In MCF7 cells, activity of mutant $S_{57,81,219}$A ERRγ is significantly reduced by ~30% vs. wild type ERRγ on the ERRE (Fig. 5A) and ERE (Fig. 5B). For the first time, we show that ERRγ can also stimulate transcription from the ERRE/ERE (Fig. 5C). However, activity of the $S_{57,81,219}$A mutant ERRγ at this hybrid element is decreased vs. wild type receptor by <10%. In contrast, the $S_{57,81,219}$A mutant ERRγ shows a 30–40% reduction in transcriptional activity at all 3 response elements in a different ER+ breast cancer cell line (SUM44) (Fig. 5D–F). These data demonstrate that ERK-mediated stabilization of ERRγ positively regulates receptor transcriptional function, and suggest that this is most relevant to ERRE- and ERE-driven activity.

Discussion

In this study, we have shown that ERRγ protein levels are enhanced or stabilized by active ERK, mapped this activity to 3 Serine residues, and demonstrated that impairment of ERRγ phosphorylation at these sites reduces receptor-mediated TAM resistance and transcriptional activity in ER+ breast cancer cells. We propose that ERK-mediated phosphorylation of ERRγ is a key determinant of TAM resistance in ER+ breast cancer cells where this receptor is expressed and drives the resistant phenotype.

To our knowledge this is the first demonstration of direct, functional consequences of phospho-regulation of a member of the ERR family. Ariazi *et al.* initially showed that ERRα transcriptional activity in ER+ breast cancer cells is enhanced by HER2 endogenous amplification (BT474) or exogenous expression (MCF7), and that pharmacological inhibition of AKT or MAPK reduces this activity [26]. They also provide evidence, via *in vitro* kinase assays using GST-tagged ERRα constructs, that multiple receptor sites (particularly in the carboxy-terminus) can be phosphorylated by AKT and MAPK. However, Chang *et al.* reported that in SKBR3 (a HER2-amplified, ER− breast cancer cell line), expression of endogenous ERRα target genes is repressed by AKT, but not MAPK, inhibitors through regulation of the co-activator PGC1β [43]. Moreover, they state that mapping and mutation of the proposed phosphorylation sites in ERRα has no effect on receptor transcriptional activity, which is in direct contrast to our finding that mutation of 3 ERK consensus sites in ERRγ significantly impairs transcriptional activity and receptor-mediated TAM resistance. That ERRα and ERRγ, despite their high sequence similarity and overlapping target genes, have differential functions in breast cancer is an idea that has

gained considerable traction recently [11, 44], and one that our future studies will address, particularly with respect to ERE- and ERRE-containing endogenous target gene selection (see below).

We were surprised by the apparent specificity of ERK for positive regulation of ERRγ in ER + breast cancer cells. All three members of the MAPK family (ERK, JNK, p38) can phosphorylate the same S-P core motif, but our data show that only pharmacological inhibition of ERK reduces ERRγ protein. It should be noted that under these experimental conditions, p38 and JNK are expressed but their activation (phosphorylation) is minimal (Fig 2A, right panels). We therefore cannot rule out the possibility that in other contexts, ERRγ may have the capacity to be regulated by these other members of the MAPK family.

It is not yet clear how inhibition of ERK, or the $S_{57,81,219}A$ ERRγ mutation, ultimately leads to a decrease in receptor levels. One reasonable explanation is a change in proteasomal-mediated degradation of the receptor such that phosphorylation of serines 57, 81, and/or 219 by ERK slows or prevents ubiquitination and degradation of ERRγ. Our data showing that a brief, 2 hour stimulation with EGF is sufficient to enhance ERRγ (HA) expression would be consistent with this. Similar to what we observe here, MEK/ERK-mediated stabilization of the GLI2 oncoprotein results in reduced ubiquitination of GLI2 that requires intact GSK3β phosphorylation sites [45]. Parkin is the only E3 ubiquitin ligase that has so far been shown to ubiquitinate ERRγ (and other members of the ERR family) [46], but knowledge of whether/how parkin is impacted by ERK signaling in breast cancer is limited. In neurons parkin and MAPKs do act in opposition to regulate microtubule depolymerization [47], and in several breast cancer cell lines parkin has been reported to bind microtubules and stabilize their interaction with paclitaxel, leading to enhanced sensitivity to this chemotherapeutic drug [48]. In MCF7 cells, exogenous parkin expression also independently attenuates cell proliferation by causing a G1 arrest [49]. Future studies will determine whether ERK-dependent regulation of ERRγ requires the Parkin and ubiquitin/proteasome pathway.

A reduction in $S_{57,81,219}A$ mutant ERRγ protein levels, and its attendant failure to induce TAM resistance or promote cell cycle progression in MCF7 cells, is not perfectly correlated with impaired transcriptional activity. $S_{57,81,219}A$ mutant ERRγ is significantly less active at ERRE and ERE sites. However, Figure 5C shows that activity of the $S_{57,81,219}A$ mutant at the hybrid ERRE/ERE element is surprisingly near wild type in MCF7 cells, but reduced by 30% in SUM44 cells (Fig. 5F). Because these divergent results were obtained using identical, plasmid-borne heterologous promoter constructs (3 tandem ERRE/ERE sequences functioning as enhancers of the SV40 core promoter) under similar experimental conditions, we hypothesize that this context-dependent difference in mutant ERRγ activity could be due to a difference in either the repertoire of co-regulatory proteins, or the expression of ERα, in MCF7 vs. SUM44 cells. The latter possibility is interesting in light of what is known about the interplay between family member ERRα and ERα at these hybrid response elements. Using serial ChIP assays Deblois *et al*. showed that in MCF7 cells, ERRα and ERα cannot simultaneously occupy these hybrid sites, and reduction of ERα by siRNA enriched ERRα binding to these sequences in the promoter regions of *FAM100A* and *ENO1* [42]. We previously reported that SUM44 cells have high basal expression of ERα [15], which represents 3-fold enrichment in mRNA and protein levels vs. MCF7 cells (p<0.001, data not

shown). This might mean that where competition with ERα is limited (*i.e.* in MCF7 cells), $S_{57,81,219}$A mutant ERRγ is more readily recruited to ERRE/ERE sites. However, $S_{57,81,219}$A mutant ERRγ is still unable to fully induce TAM resistance in MCF7 cells and shows compromised activity at ERE inverted repeats and the ERRE half site in these cells. This implies that phosphorylated, wild type ERRγ may preferentially activate ERE- and ERRE-regulated target genes to promote the TAM-resistant phenotype.

Materials and Methods

Cell Lines, Culturing Conditions, and Reagents

ER-positive, Tamoxifen-responsive MCF7 cells were originally obtained from Dr. Marvin Rich (Karmanos Cancer Institute, Detroit, MI). The ER-positive, Tamoxifen-resistant variant of MCF7 (MCF7/RR cells) was a kind gift of Dr. W. B. Butler (Indiana University of Pennsylvania, Indiana, PA) [20]. ER-positive, Tamoxifen-responsive SUM44 cells have been described previously [15]. All cells tested negative for *Mycoplasma* spp. contamination, and were maintained in a humidified incubator with 95% air: 5% carbon dioxide. MCF7 and MCF7/RR cells were cultured in modified improved minimal essential medium (IMEM; Life Technologies, Grand Island, NY) with phenol red (10 mg/L) supplemented with 5% fetal bovine serum (FBS). SUM44 cells were cultured in serum-free Ham's F12 medium (1.25 mg/L phenol red) with insulin, hydrocortisone, and other supplements (SFIH) as described previously [15, 50].

4-hydroxytamoxifen (4HT; Sigma, St. Louis, MO) was dissolved in 200-proof ethanol, stored as a 10 mM stock at −20°C, and used at the concentrations indicated. The MEK inhibitor U0126, JNK inhibitor SP600125 and p38 inhibitor SB203580 (Tocris Bioscience, Ellisville, MO) were dissolved in dimethyl sulfoxide (DMSO), stored as 10 and 50mM stocks (respectively) at −20°C, and used at the concentrations indicated. Poly-L-lysine was purchased from Sigma. Recombinant human epidermal growth factor (EGF) was purchased from PeproTech (Rocky Hill, NJ) and used at the concentration indicated.

Expression Constructs and Reporter Plasmids

An ORF cDNA clone for human ERRγ (AB020639.1) was purchased from GeneCopoeia (Rockville, MD). Wild type, HA-tagged murine ERRγ (pSG5-HA-ERR3, 100% protein sequence identity to human ERRγ transcript variant 1) has been described previously [15, 23]. The serine-to-alanine variants (S_{45}A and $S_{57,81,219}$A) were generated using the QuikChange Lightning site-directed mutagenesis kit (Stratagene, La Jolla, CA), confirmed by automated DNA sequencing (GENEWIZ, South Plainfield, NJ), and have been deposited at Addgene (Cambridge, MA; plasmid #s 37849 and 37850, respectively). Amino acid numbers correspond to transcript variant 1. Plasmids encoding constitutively active MEK (pBabe-puro-MEK-DD, [51]) and wild type, HA-tagged ERK2 (pCDNA-HA-ERK2 WT, [52]) were obtained from Addgene (plasmid #s 15268 and 8974, respectively).

The estrogen response element (ERE)-containing promoter reporter construct (3xERE-luciferase) has been described previously [15, 53]. To generate the estrogen-related response element (ERRE)-containing reporter (3xERRE-luciferase, [54]) and the hybrid ERRE/ERE-

responsive reporter (3xERRE/ERE-luciferase, [42]), oligonucleotides were synthesized (IDT, Coralville, IA), annealed, and cloned into KpnI/BglII-digested pGL3-Promoter vector (Promega, Madison, WI) using standard techniques. Oligonucleotide sequences are as follows:

ERRE forward: 5′…
CCGGACC**TCAAGGTCA**CGTTCGGACC**TCAAGGTCA**CGTTCGGACC**TCAAGGTCA**GGATCCA…3′

ERRE reverse: 5′…
gatctGGATCC**TGACCTTGA**GGTCCGAACG**TGACCTTGA**GAACG**TGACCTTGA**GGTCCGggtac…3′

ERRE/ERE forward: 5′…
CCGGACC**TCA***AGGTCACCTTGACCT*CGTTCGGACC**TCA***AGGTCACCTTGACCT*CGTTCGGACC**TCA***AGGTCACCTTGACCT*GGATCCA…3′

ERRE/ERE reverse: 5′…
gatctGGATCC*AGGTCAAGGTGACCT***TGA**GGTCCGAACG*AGGTCAAGGTGACCT***TGA**GAACG*AGGTCAAGGTGACCT***TGA**GGTCCGggtac…3′

Bold indicates consensus ERRE sequences, *underlined italics* indicate consensus ERE sequences, and small letter sequences highlight KpnI and BglII sites. Proper annealing and insertion were confirmed by automated DNA sequencing (GENEWIZ), and plasmids have been deposited at Addgene (plasmid #s 37851 and 37852, respectively).

Clinical Data

The KM Plotter tool (http://kmplot.com/analysis/) [19] was used to evaluate ERRγ mRNA expression (Affymetrix ProbeID 207981_s_at) in publicly available breast cancer gene expression data from 65 patients selected by the following parameters: overall survival (OS), upper vs. lower tertile of ESRRG expression, ER-positive tumors (including those for which ER+ status is extrapolated from gene expression data), Tamoxifen as only form of endocrine therapy, and any chemotherapy.

Reverse Transcription PCR (RT-PCR)

RNA was extracted from subconfluent monolayers of exponentially growing cultures using the RNEasy Mini kit (Qiagen, Valencia, CA). One microgram of total RNA was DNase treated and reverse transcribed using Super Script II and other reagents from Life Technologies. Quantitative RT-PCR was performed for individual cDNA samples (1:5 dilution) using TaqMan Gene Expression Assays for ESRRG and RPLP0 as described previously [15]. Standard (non-quantitative) RT-PCR was performed on 400 ng of cDNA or 800 pg of the human ERRγ ORF cDNA clone with primers designed to amplify ESRRG or RPLP0 using TaqSelect DNA polymerase from Lucigen (Middleton, WI) under the following PCR conditions: 94°C for 2 min; 35 cycles of 94°C for 30 sec, 54°C for 30 sec, and 72°C for 1 min 24 sec; final extension of 72°C for 10 min; 4°C hold.

| ESRRG | Forward: GGAGGTCGGCAGAAGTACAA
Reverse: GCTTCGCCCATCCAATGATAAC | 241 bp |
| RPLP0 | Forward: ACCATTGAAATCCTGAGTGA
Reverse: AATGCAGAGTTTCCTCTGTG | 187 bp |

Transient Transfection and Immunoblotting

Cells were seeded on 6-well, 12-well, or 100 mm plastic tissue culture dishes one day prior to transfection with the indicated expression constructs using Lipofectamine 2000 or Lipofectamine LTX (Life Technologies), or JetPrime (VWR, Radnor, PA) according to the manufacturer's instructions. For transfections using Lipofectamine 2000, wells were pre-coated with poly-L-lysine. Transfection complexes were removed (and, where indicated, 4HT or kinase inhibitors were added) at 4–6 hours post-transfection. For the growth factor stimulation experiment, 4–6 hours post-transfection the cells were washed twice in sterile PBS and cultured in low-serum (0.5% FBS) conditions overnight (~20 hours) before treatment with EGF in the presence or absence of U0126 for 2 hours. For both transfected and non-transfected cells, wells and dishes were lysed in modified radioimmunoprecipitation assay (RIPA) buffer [55] supplemented with CompleteMini protease inhibitor and PhosSTOP phosphatase inhibitor tablets (Roche Applied Science, Penzburg, Germany). Polyacrylamide gel electrophoresis and protein transfer were performed as described previously [15, 55]. Nitrocellulose membranes blocked in either 5% nonfat dry milk or 7.5% bovine serum albumin (BSA) in Tris-buffered saline plus Tween (TBST) for 1 hour were incubated overnight at 4°C with primary antibodies for: phosphorylated Erk1/2 (1:1000), total Erk1/2 (1:1000), total MEK (1:1000), phosphorylated JNK (1:5000), total JNK (1:500), phosphorylated p38 (1:1000), total p38 (1:1000), phosphorylated Rb Ser780 (1:1000), total Rb (1:1000) (all from Cell Signaling, Beverly, MA); ERRγ (1:100, ab82319 from Abcam, Cambridge, MA); p21 (1:300, sc-756), p27 (1:500, sc-528) from Santa Cruz Biotechnology, Dallas, TX; or the HA epitope tag (1:500, HA.11 clone 16B12, Covance, Princeton, NJ). For ERRγ detection, 25 ng of purified protein corresponding to human ERRγ transcript variant 2 (Origene, Rockville, MD) was run alongside 67 µg whole cell lysates. As a loading control, all membranes were re-probed with β–actin primary antibody (1:5000–1:10,000, Sigma) for 1 hour at room temperature [15]. Horseradish peroxidase-conjugated secondary antibodies (1:5000) and enhanced chemiluminescent detection were performed as described previously [15].

FACS Analysis of Bromodeoxyuridine (BrdU) Incorporation

MCF7 cells were seeded in poly-L-lysine-coated 6-well plastic tissue culture plates at a density of 2.5×10^5 cells per well, respectively, one day prior to transfection with 4 µg HA-ERR3, the $S_{57,81,219}A$ variant, or empty vector (pSG5) using Lipofectamine 2000. Four to 6 hours post-transfection, transfection complexes were removed and cells were treated with 1 µM 4HT or ethanol vehicle. 48 hours later, BrdU was added to a final concentration of 10 µM for an additional 18–20 hours. Cells were fixed and stained using the APC (allophycocyanin) BrdU Flow Kit with 7-AAD (7-amino-actinomycin D; BD Pharmingen, San Jose, CA) according to the manufacturer's instructions with one modification: during

incubation with the APC-conjugated anti-BrdU antibody, cells were co-stained with AlexaFluor488-conjugated anti-HA antibody (Covance) at 1:50–1:100. Fluorescence-activated cell sorting (FACS) was performed on a BD FACSAria instrument. For wild type- and mutant-transfected cells, data are presented for only HA-positive (*i.e.* AlexaFluor488-stained) cells; for empty vector-transfected cells, data are presented for all sorted cells.

Promoter-Reporter Luciferase Assays

MCF7 and SUM44 cells were seeded in poly-L-lysine-coated 24- and 12-well plastic tissue culture plates at 7.5×10^4 and 2.0×10^5 cells per well, respectively. The following day, cells were co-transfected with 500 or 1000 ng HA-ERR3, the $S_{57,81,219}A$ variant, or empty vector (pSG5), 290 or 580 ng 3xERE-, 3xERRE-, or 3xERRE/ERE-luciferase, and 10 or 20 ng pRL-SV40-Renilla (internal control), respectively. Transfection complexes were removed and media were replaced 4–6 hours post-transfection. Twenty-four (MCF7) and 48 (SUM44) hours post-transfection, cells were lysed and analyzed for dual-luciferase activity as described previously [15].

Image Analysis and Statistics

NIH Image J (http://rsbweb.nih.gov/ij/) was used to perform densitometry. All statistical analyses were performed using GraphPad Prism 5.0c for Mac (La Jolla, CA), with the exception of the hazard ratio and logrank p value in Fig. 1A, which were generated by the KM Plotter tool. All data are presented as the mean ± standard deviation (SD), and statistical significance is defined as p 0.05. qRT-PCR, BrdU incorporation, and promoter-reporter luciferase assays were analyzed by t test or one-way analysis of variance (ANOVA) with *post-hoc* Tukey's or Dunnet's multiple comparison tests.

Acknowledgments

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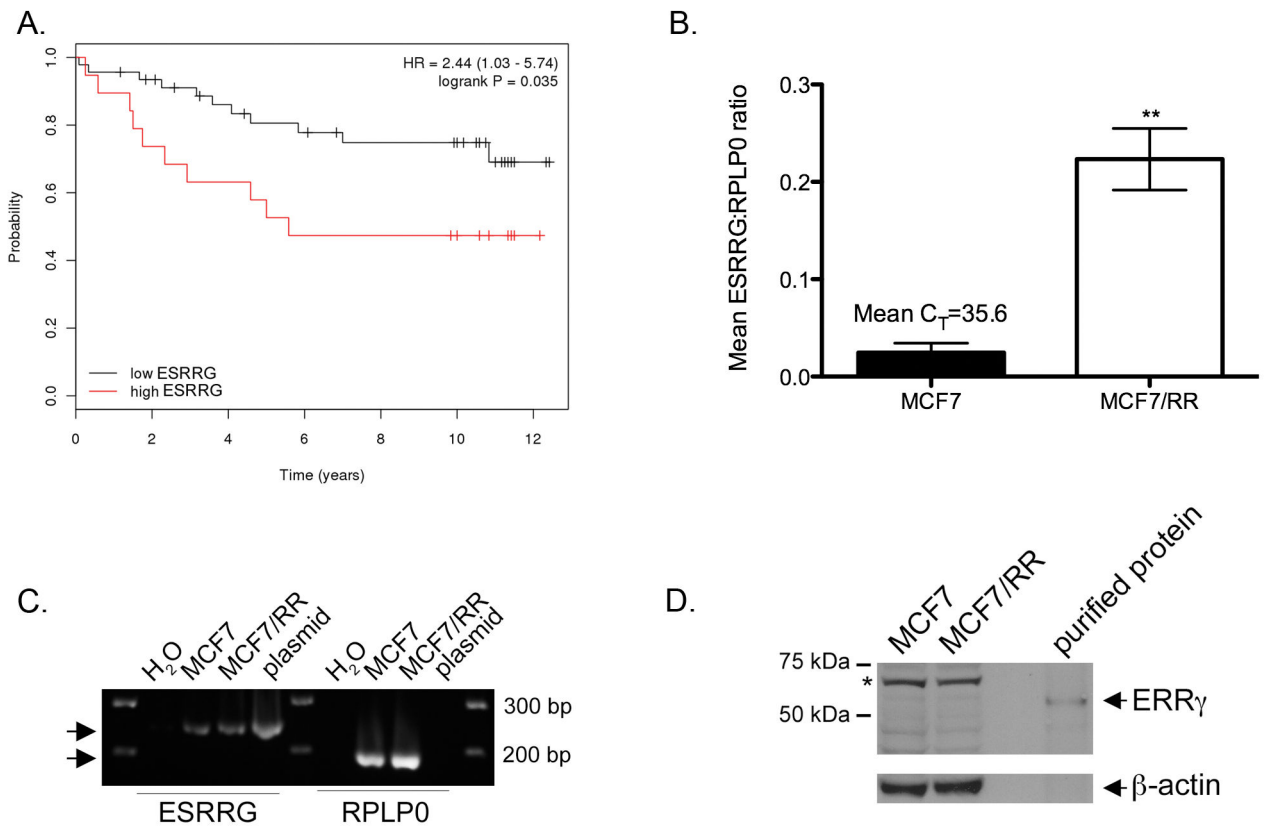
Figure 1. ERRγ expression in ER+ breast tumors and breast cancer cells
A, Expression of ESRRG in ER+, TAM-treated breast tumors is associated with worse overall survival. HR = hazard ratio calculated by [19]. B, Relative expression of ESRRG normalized to RPLP0 in MCF7 and MCF7/RR cells by quantitative RT-PCR. Mean cycle threshold (C_T) values for parental (MCF7) cells are shown. Bars, n=3 replicates from a representative assay performed independently twice. Error, standard deviation (SD).
**p 0.01 for t test. C, Expression of ESRRG and RPLP0 in MCF7 and MCF7/RR cells by non-quantitative RT-PCR. Upper and lower arrowheads identify ESRRG and RPLP0 amplicons, respectively. Plasmid denotes ERRγ ORF cDNA clone. D, Expression of ERRγ protein in MCF7 and MCF7/RR cells by Western blot analysis. *denotes a non-specific band detected by the ERRγ antibody. Purified protein denotes human ERRγ transcript variant 2. β–actin = loading control.
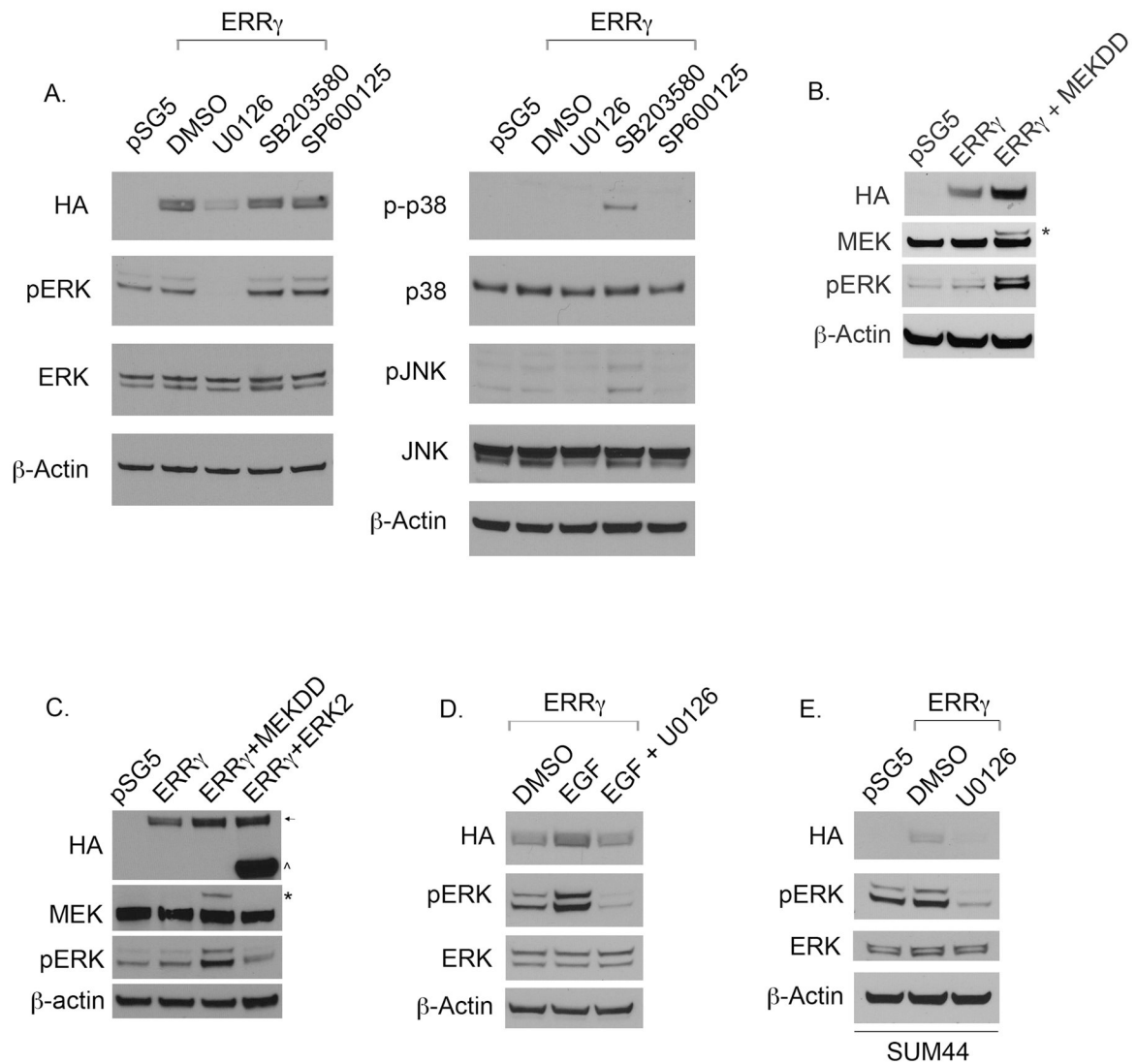
**Figure 2. Effect of MEK and ERK on ERRγ protein levels**

A, Inhibition of ERK, but not p38 or JNK, reduces exogenous ERRγ expression. MCF7 cells were transiently transfected with the pSG5 empty vector or HA-ERRγ, then treated with DMSO vehicle, 5 μM U0126 (MEK inhibitor), 25 μM SB203580 (p38 inhibitor), or 10 μM SP600125 (JNK inhibitor) for 24 hours prior to lysis and Western blot analysis. Left panels show ERRγ (HA) levels, phosphorylated ERK (pERK), and total ERK from a representative experiment repeated at least twice. Right panels show total and phosphorylated p38 and JNK (p-p38 and pJNK, respectively) from the same experiment. β–actin = loading control. B, Constitutively active, mutant MEK enhances ERRγ protein levels. MCF7 cells were transiently co-transfected with HA-ERRγ and either MEKDD or additional pSG5 empty vector. *denotes the transfected MEKDD construct. β–actin = loading control. C, Exogenous, wild type ERK2 enhances ERRγ protein levels. MCF7 cells were transiently co-transfected with HA-ERRγ and either MEKDD, wild type HA-tagged ERK2, or additional pSG5 empty vector. *denotes the transfected MEKDD construct. The arrowhead and ^ denote transfected HA-ERRγ and HA-ERK2, respectively. β–actin = loading control. D,

EGF-mediated enhancement of ERRγ protein levels is reversed by concomitant ERK inhibition. MCF7 cells were transiently transfected with HA-ERRγ, then cultured in low-serum conditions for 20 hours before treatment with DMSO vehicle, 25 ng/ml EGF, or 25 ng/ml EGF plus 5 μM U0126 for 2 hours. β–actin = loading control. E, Inhibition of ERK reduces exogenous ERRγ expression in a second ER+ breast cancer cell line. SUM44 cells were transiently transfected with the pSG5 empty vector or HA-ERRγ, then treated with DMSO vehicle or 5 μM U0126 for 22 hours. β–actin = loading control.
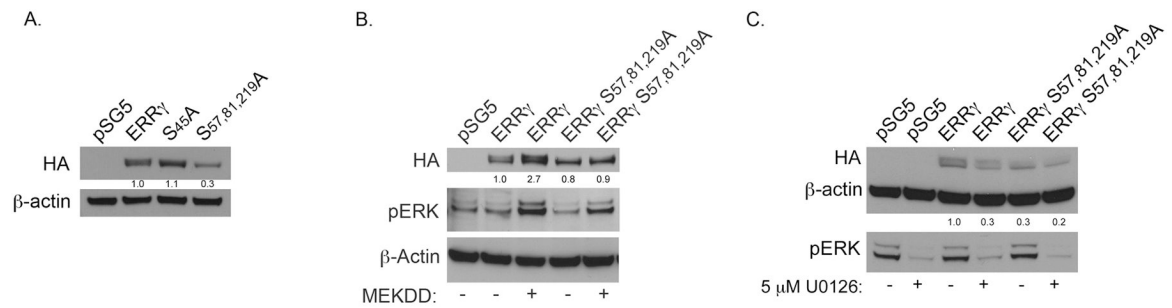
Figure 3. Contribution of serines 57,81, and 219 to ERRγ protein levels
A, Concomitant serine-to-alanine mutation at residues 57, 81, and 219 reduces basal HA-ERRγ levels. B, MEKDD fails to increase protein levels of $S_{57,81,219}A$ HA-ERRγ. C, Erk inhibition does not reduce $S_{57,81,219}A$ HA-ERRγ. MCF7 cells were transiently transfected and treated with 5 μM U0126 or DMSO vehicle for 24 hours where indicated (C) prior to lysis and Western blot analysis. β–actin = loading control. Densitometric values for the ratio of HA:β-actin are normalized to the level of wild-type receptor in the absence of treatment (1.0). Data are from representative experiments that were performed independently at least 3 times.
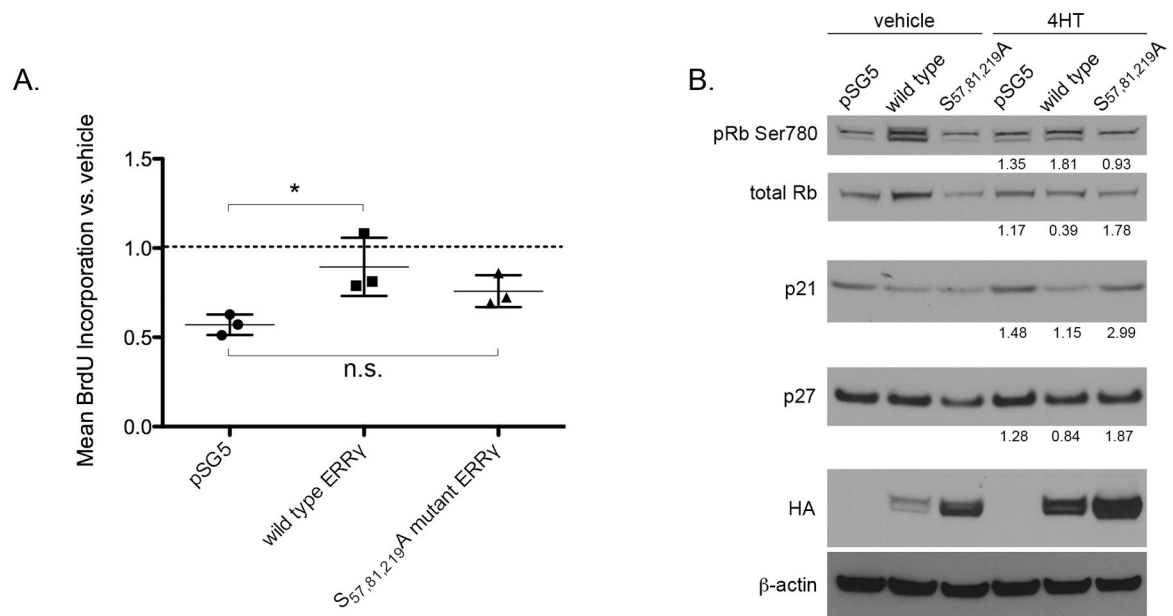
Figure 4. Effect of S$_{57,81,219}$A mutation on Tamoxifen response

A, Inhibition of BrdU incorporation by 4HT is reversed by wild type but not S$_{57,81,219}$A HA-ERRγ. MCF7 cells were transiently transfected as shown, treated with ethanol vehicle or 1 μM 4HT for 48 hours, then incubated with BrdU for an additional 18–20 hours before fixation and staining for HA and BrdU. Dashed line denotes BrdU incorporation in vehicle-treated cells (set to 1.0). Points, n=3 independent assays. Error, SD. *p<0.05 for *post hoc* Dunnet's test following one-way ANOVA for pSG5 vs. wild type HA-ERRγ; n.s. denotes no statistical significance between pSG5 and S$_{57,81,219}$A mutant HA-ERRγ. For transfections with wild type or S$_{57,81,219}$A ERRγ, data are from HA-positive, FACS-sorted cells only. For transfections with the empty vector pSG5 control, data are from all cells in the population. B, 4HT-mediated induction of cell cycle inhibitors p21 and p27 is reversed by wild type but not S$_{57,81,219}$A HA-ERRγ, and the phosphorylation state of Rb is differentially affected by wild type vs. mutant receptor. MCF7 cells were transiently transfected as shown, then treated with ethanol vehicle or 2.5 μM 4HT for 21 hours prior to lysis and Western blot analysis. β–actin = loading control. Densitometric values for the ratio of the indicated proteins to β-actin in 4HT-treated conditions are normalized to the level of their expression in the absence of treatment (1.0) for each transfected construct; for pRb Ser780, the ratio of phosphorylated:total signal (which was then normalized to β-actin) is shown. Data are from a representative experiment that was performed independently 3 times.
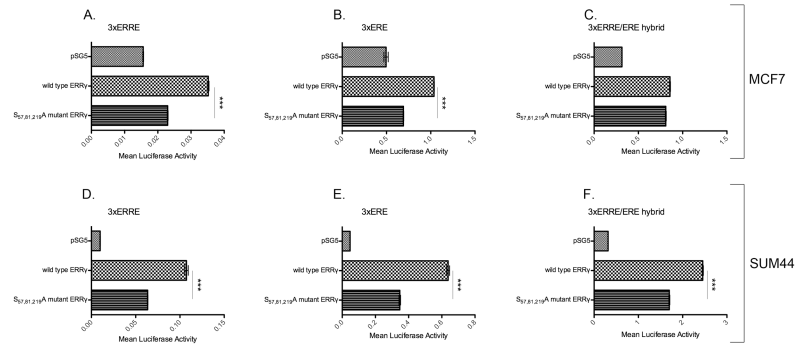
Figure 5. Effect of S$_{57,81,219}$A mutation on ERRγ transcriptional activity
MCF7 and SUM44 cells were transiently co-transfected with pSG5 empty vector, wild type HA-ERRγ, or S$_{57,81,219}$A HA-ERRγ plus the ERRE- (A, D), ERE- (B, E), or ERRE/ERE-driven promoter-reporter luciferase construct (C, F) and the Renilla internal control for 24 (MCF7) or 48 hours (SUM44) prior to lysis and luciferase assay. Bars, luciferase:Renilla ratio of n=3 replicate wells from a representative assay performed 3 times independently. Error, SD. ***p 0.001 for one-way ANOVA with *post hoc* Tukey's tests.