Orphan nuclear receptors in breast cancer pathogenesis and therapeutic response

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Abstract

Nuclear receptors comprise a large family of highly conserved transcription factors that regulate many key processes in normal and neoplastic tissues. Most nuclear receptors share a common, highly conserved domain structure that includes a carboxy-terminal ligand-binding domain. However, a subgroup of this gene family is known as the orphan nuclear receptors because to date there are no known natural ligands that regulate their activity. Many of the 25 nuclear receptors classified as orphan play critical roles in embryonic development, metabolism, and the regulation of circadian rhythm. Here, we review the emerging role(s) of orphan nuclear receptors in breast cancer, with a particular focus on two of the estrogen-related receptors (ERRα and ERRγ) and several others implicated in clinical outcome and response or resistance to cytotoxic or endocrine therapies, including the chicken ovalbumin upstream promoter transcription factors, nerve growth factor-induced B, DAX-1, liver receptor homolog-1, and retinoic acid-related orphan receptor α. We also propose that a clearer understanding of the function of orphan nuclear receptors in mammary gland development and normal mammary tissues could significantly improve our ability to diagnose, treat, and prevent breast cancer.

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What is an orphan nuclear receptor?

Members of the nuclear receptor superfamily are some of the most abundant regulators of gene expression in higher eukaryotes. These DNA-binding transcription factors play essential roles in key biological processes from embryonic development to differentiation, and their dysregulation has been widely studied in many different pathologies including cancer (Mangelsdorf et al. 1995, Robinson-Rechavi et al. 2003, Novac & Heinzel 2004, Jeong & Mangelsdorf 2009).

Of the 48 members of the human nuclear receptor superfamily, 25 are currently considered to be orphan nuclear receptors (Benoit et al. 2006; Table 1) because they have no known ligand. Most of these 25 receptors adhere to the classical domain structure that typifies ligand-regulated nuclear receptors (discussed in more detail below), with two notable exceptions. Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1 (DAX-1, NR0B1) and small heterodimerization partner (SHP, NR0B2) lack a classical DNA-binding domain (DBD), and cannot act alone to bind DNA (Burris et al. 1996, Seol et al. 1996). The number of nuclear receptors classified as orphans has decreased over the years as new ligands have been discovered. Two such ‘adopted orphans’ are the retinoid X receptors (RXRs) and the peroxisome proliferator-activated receptors (PPARs), which were initially considered to be orphans but have been firmly in the category of liganded receptors for some time. RXRs and PPARs, along with several other formerly orphaned nuclear receptors, form a group now referred to as natural or nutrient sensors that bind 9-cis retinoic acid, oleic, and linoleic acids (Francis et al. 2003, Benoit et al. 2004).

What constitutes an orphan receptor is still the subject of some debate. An example of the lingering
controversy is that orphan nuclear receptors like steroidalogenic factor-1 (SF1, NR5A1) and hepatocyte nuclear factor-4 (HNF4, NR2A1 and NR2A2) have been shown to bind phospholipids and fatty acids respectively (Wisely et al. 2002, Li et al. 2005). However, because the physiological and/or functional relevance of these interactions remain unclear, SF1 and HNF4 are still considered to be orphan nuclear receptors.

### Nuclear receptor structure and function

As a group, most nuclear receptors share a common, highly conserved domain structure (Fig. 1). At the amino-terminus, the activation function-1 (AF1) domain is a highly divergent region that assists in regulating the transcriptional activity of nuclear receptors independent from ligand binding (Kumar & Litwack 2009). The AF1 domain is one of the two major sites for the binding of nuclear receptor

### Table 1 Orphan nuclear receptors in breast cancer

| Gene symbols | Common name | Select ligand(s) | mRNA overexpression in human breast cancer
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DES, diethylstilbestrol; 4HT, 4-hydroxytamoxifen.

*aGSE identification numbers denote publicly available gene expression studies from gene expression omnibus (GEO). For each study, expression of the relevant nuclear receptor is significantly increased in breast cancer versus normal mammary tissue (P ≤ 0.05, pair-wise t-test).*
coregulators, which include coactivator and corepressor proteins that can positively or negatively impact transcriptional activity respectively; it is also an important site of posttranslational modification, including phosphorylation and the addition of small ubiquitin-like modifier proteins (SUMOylation; Takimoto et al. 2003, Cheng et al. 2007, Zhang et al. 2007, Tamasi et al. 2008, Garza et al. 2010). Much less is known about the AF1 domain as compared with other regions of nuclear receptors. One key reason is that the AF1 domain has a high level of intrinsic disorder (ID; Kumar & Litwack 2009), although this is not the only region of these receptors that is disordered (Krasowski et al. 2008). ID regions are characterized by amino acid sequences that are low in hydrophobicity and highly charged, leading to flexible, highly variable tertiary and quaternary protein structures. In general, all transcription factors are enriched in ID regions (Minezaki et al. 2006), and these appear to be critical for the regulation of protein–protein interactions (Dunker et al. 2005). In addition, the distribution of nuclear receptor coactivator proteins that can bind to the AF1 domain and regulate receptor function is tissue- and cell-type specific. It is now apparent that the differential expression and function of the entire group of nuclear receptor coregulators (coactivators and corepressors) in normal versus cancer tissue is a fundamental component of nuclear receptor regulation (Hall & McDonnell 2005, O’Malley & Kumar 2009).

The DBD of the nuclear receptor superfamily is defined by two cysteine-rich zinc finger motifs that permit binding of the receptor to DNA (Freedman et al. 1988). This region is also important in mediating the homo- and heterodimerization of nuclear receptors (Claessens & Gewirth 2004). Proximal to the DBD is the flexible hinge region of the nuclear receptor, which typically contains the nuclear localization sequence (Claessens et al. 2001, Aschrafi et al. 2006, Carrigan et al. 2007). The hinge region is also a key site for posttranslational modifications (Sentis et al. 2005, Chen et al. 2006, Hwang et al. 2009).

Nuclear receptor DBDs contain a short stretch of amino acids downstream of the two zinc fingers known as the carboxy-terminal extension (CTE; Claessens & Gewirth 2004). The CTE is present in ligand-regulated nuclear receptors like the estrogen receptors (ERs; Schultz et al. 2002), androgen receptor (AR; Schoenmakers et al. 1999), and the vitamin D receptor (Hsieh et al. 1999). However, orphan nuclear receptors such as estrogen-related receptor β (ERRβ, ESRRB, NR3B1) that bind a single half-site rely heavily on the A box of the CTE (which contains a conserved glycine–arginine motif) to permit DNA binding in the minor groove (Gearhart et al. 2003). In addition, residues C-terminal to the A box form intramolecular interactions with the rest of the DBD; together, these interactions serve to stabilize the binding of ERRβ and several other orphan nuclear receptors to DNA.

The carboxy-terminal ligand-binding domain (LBD) and the AF2 domain are essential for the regulation of nuclear receptor transcriptional activity by mediating ligand and receptor interactions and coregulator binding; in some cases, these regions also participate in receptor dimerization (Chandra et al. 2008). Upon the engagement of natural or synthetic ligand, nuclear receptor LBDs undergo a significant conformational change that alters the orientation of several α-helices and β-sheets, most notably the repositioning of helix 12 (H12) that comprises the AF2 domain (Wurtz et al. 1996). H12 repositioning uncovers a hydrophobic binding groove or charge clamp that recruits coregulator proteins containing an LXXLL motif (Westin et al. 1998), and the sum of these changes serves to significantly enhance nuclear receptor transcriptional activity.

In contrast to ligand-regulated nuclear receptors, the orphan nuclear receptors typically display constitutive transcriptional activity. While crystal structures for many orphan LBDs appear to be ligand-filled, some are still modulated by synthetic agonists and antagonists (Table 1). Thus, it remains possible that some of these receptors have natural ligands that have not yet been discovered. Even in the absence of ligand, H12 is often prepositioned for maximal activation, promoting interactions between the orphan nuclear receptor and its coactivators (Greschik et al. 2002, Flaig et al. 2005). It has also become increasingly clear that orphan nuclear receptors are particularly sensitive to the binding of coactivator proteins. Differential coregulator binding can directly affect, which DNA response elements are bound and activated (Gaillard et al. 2007), and modified coactivators that are selective for particular orphans can be designed to further explore the biology of these receptors (Gaillard et al. 2006, Stein et al. 2008).
Benoit et al. (2006) have recently published a comprehensive review of all nuclear receptors currently classified as orphaned, summarizing what is known about their expression patterns, coregulatory molecules, validated transcriptional target genes, and any phenotypes associated with their perturbation in mice. Therefore, we will specifically focus on a review of the evidence supporting a role for orphan nuclear receptors in breast cancer pathogenesis and therapeutic response.

**Introduction to breast cancer**

The American Cancer Society estimates that in 2009, 178,000 women were diagnosed with breast cancer, and over 40,000 women died of breast cancer (Jemal et al. 2009), making it the second-most common cause of cancer-related death in women. Breast cancer is not a single disease; it is classified into multiple histological and molecular subtypes with diverse clinicopathological features and survival outcomes (Olopade et al. 2008, Orlando & Brown 2009). The heterogeneity of breast cancer presents a major challenge for basic, translational, and clinical research. This is most often addressed by studying a combination of model systems, including tumor-derived cell culture models in vitro, xenograft models in immune-deficient mice, and genetically modified rodent models of mammary gland development and cancer. All these approaches are required because no single model is a perfect reflection of the diversity of human breast cancer (Clarke 1996). Recently, expression profiling of breast cancer clinical specimens has sought to uncover gene signatures that can classify tumors into various subgroups. The most widely utilized of these classification systems was developed by Sorlie et al. (2001, 2003) in which tumors are grouped into several classes, including Luminal A, Luminal B, ERBB2, basal-like, and normal-like. While signatures like these may ultimately prove useful in predicting outcome or response to specific therapies, they will require further refinement as additional subdivisions of these breast cancer subtypes (such as claudin low) are identified.

**Orphan nuclear receptors in rodent mammary gland biology and development**

Understanding mammary gland development and tumorigenesis in rats and mice has been instrumental in advancing the study of human breast cancer (Cardiff et al. 2000, Allred & Medina 2008, Marcotte & Muller 2008). However, little is known about the role(s) of specific orphan nuclear receptors in the developmental biology of the rodent mammary gland. This may be due to the fact that orphan nuclear receptor deletion in mice often has serious detrimental effects in multiple tissues that are manifested during embryogenesis or early in life, long before mammary glands develop beyond the rudimentary epithelial tree (Chen et al. 1994, Pereira et al. 1999, Collins et al. 2004, Moral et al. 2008). The creation of mammary gland-specific knockout or transgenic mice for key orphans will be required to explore their function in these tissues. Two orphan nuclear receptors have specifically been studied in the context of mouse mammary epithelial cell differentiation.

**DAX-1**

DAX-1 is an atypical member of the nuclear receptor superfamily because it lacks a DBD and relies on heterodimerization with other transcription factors that can bind DNA. These binding partners include a second orphan nuclear receptor, SF1, and it has been shown that DAX-1 can repress SF1-mediated stimulation of genes regulating steroid synthesis (Wang et al. 2001); DAX-1 can also repress the activity of ERs (Zhang et al. 2000). DAX-1 is strongly up-regulated in HC11 mouse mammary epithelial cells that have been induced to differentiate in vitro by withdrawing serum and growth factors prior to treatment with the lactogenic hormones insulin, dexamethasone, and prolactin (Faulds et al. 2004). It is the withdrawal of epidermal growth factor (EGF), and specifically, the resulting decrease in mitogen-activated protein kinase (MAPK) activity, which leads to DAX-1 induction (Helguero et al. 2006). Once induced, DAX-1 is enriched in the nucleus, and inhibits the transcriptional activity of ERα and ERβ, as well as the proliferation of HC11 cells treated with ERα- and ERβ-specific agonists. Finally, the expression of DAX-1 is significantly induced in the mammary glands of pseudopregnant and lactating 3-month-old C57BL/6 female mice as compared with virgin animals. Together, these data suggest that the lack of sensitivity of pregnant and lactating mammary glands to estrogen stimulation may be due to increased expression of the ER corepressor DAX-1 (Helguero et al. 2006).

**Small heterodimerization partner**

SHP, the other orphan nuclear receptor lacking a DBD, is also strongly induced during HC11 cell differentiation (Faulds et al. 2004). While SHP can serve as an ER corepressor (Johansson et al. 1999), its role in mammary epithelial cell differentiation remains to be elucidated.
Orphan nuclear receptors in breast cancer

While mRNA for all 25 orphan nuclear receptors is significantly over-represented in breast cancer versus normal breast tissue in multiple gene expression microarray experiments in ONCOMINE and the Gene Expression Omnibus (Table 1; Rhodes et al. 2004, Barrett et al. 2009), the significance of only a handful of these receptors has been studied in breast cancer.

Chicken ovalbumin upstream promoter transcription factors, EAR2

Chicken ovalbumin upstream promoter transcription factor-1 (COUP-TFI, NR2F1), -2 (COUP-TFII, NR2F2), and V-erbA-related protein 2 (EAR2, NR2F6) are type III nuclear receptors that preferentially bind to direct DNA repeats as homodimers, although COUP-TFII and EAR2 can also heterodimerize (Avram et al. 1999). COUP-TFs can interact with DNA-responsive elements that are also shared with ERα and ERβ; the consensus estrogen response element (ERE) is a perfect inverted repeat (GGTCA-nnn-TGACC), but there are many genes with imperfect EREs or multiple ERE half-sites upstream of their transcriptional start sites. Both COUP-TFs have a high affinity for these ERE half-sites, while ER preferentially binds consensus EREs (Klinge et al. 1997), and COUP-TFs can inhibit 17β-estradiol (E2)-induced, ER-mediated transcriptional activity by physically interacting with ER and disrupting ER/DNA binding. The COUP-TF/ER physical interaction is mediated by amino acid residues within the DBD and LBD of ER, and conformational changes in these domains induced by ER/DNA-binding reduce COUP-TF/ER interactions (Klinge 1999). COUP-TFI has a higher affinity for ERE half-sites in the presence of E2 and ER, and COUP-TFI inhibits E2-induced ERE activity. These data suggest that specific ligands and the specific sequence(s) of EREs influence the degree to which COUP-TFs activate gene transcription.

Nakshatri et al. (2000) showed that COUP-TFII mRNA expression is increased in ER-positive (ER+) MCF7, T47D, and ZR75-1 and in ER-negative (ER−) MDA-MB-231 breast cancer cells, but reduced in ER− MDA-MB-468 and SkBr3 breast cancer cells, as compared with the nontumorigenic mammary epithelial cell line MCF10A. Overexpression of COUP-TFII inhibits cell proliferation in MDA-MB-435 cells by delaying progression through the G2/M cell cycle transition, which results from its induction of p21 and the subsequent inhibition of cdk2 activity. However, the more recent classification of MDA-MB-435 cells as melanoma (Rae et al. 2004) raises the issue of whether this mechanism of growth inhibition is maintained in breast cancer cell lines. This is further called into question by Moreé et al. (2003) who, in contrast, show that COUP-TFII mRNA and protein expression are highest in SkBr3 and lowest in MCF7 cells. Furthermore, mitogenic signals such as EGF significantly induce COUP-TFII expression in MCF7 cells in a MAPK-dependent manner. Antiproliferative agents such as oncostatin M (OSM) dramatically reduce COUP-TFII expression. However, neither EGF nor OSM have any effect on COUP-TFI expression or function.

DAX-1

DAX-1 is expressed in benign breast disease, carcinoma in situ, and invasive breast cancer (Conde et al. 2004), and is significantly more highly expressed in invasive lobular carcinoma than in benign tissues. In this study, expression and nuclear localization of DAX-1 is positively correlated with lymph node-positive status, while cytoplasmic and nuclear DAX-1 are both positively associated with AR expression.

Estrogen-related receptor α

ERRα (ESRRA, NR3B1) is the most-studied orphan nuclear receptor in the context of breast cancer (Horard & Vanacker 2003, Ariazi & Jordan 2006, Stein & McDonnell 2006, Tremblay & Giguere 2007, ESRRA, in Atlas of Genetics and Cytogenetics in Oncology and Haematology, available: http://atlasgeneticsoncology.org/Genes/ESRRARD44408ch11q13.html). Members of the NR3B or ERR family are classified as type IV nuclear receptors that can bind to the ERE, which it shares with ERα and ERβ, and the SF1 response element (SF1RE), which it shares with SF1 and liver receptor homolog-1 (LRH-1); this latter element is also referred to as the estrogen-related response element (ERRE). The role of ERRα in energy homeostasis and metabolism is well documented (Giguere 2008), and more recently, studies suggest that this orphan nuclear receptor is also involved in the regulation of bone mineral density (Delhon et al. 2009). In breast cancer, ERRα is the most abundantly expressed member of the ERR family. ERRα can induce expression of the estrogen-regulated gene TFF1 (pS2; Surowiak et al. 2006, Markic´ evic´ et al. 2008), through its binding to an ERRE in the TFF1 promoter (Lu et al. 2001). Moreover, ER binds to multiple steroid hormone response element half sites (MHREs) in the ERRα promoter, inducing ERRα expression, and this activity is enhanced by the addition of estrogen (Liu et al. 2003).

ERRα may play distinct roles in ER+ versus ER− breast cancer. ERRα activity at ERE sites is highly dependent on cell context, acting as a transcriptional
activator in ER− cells but a repressor in ER+ cells (Kraus et al. 2002), and ERRγ expression is only positively associated with TFF1 expression in ER− breast tumors (Heck et al. 2009). Posttranslational modification of ERRγ provides one potential explanation for these context-dependent activities. Phosphorylation of ERRγ by the HER2/MAPK/AKT pathway is a critical determinant in the receptor’s ability to recruit coactivator proteins and activate transcription from ERE sites in ER+ cells (Ariazi et al. 2007). In breast tumors, ERRγ mRNA expression is negatively correlated with ER+ status but shows a strong positive correlation with HER2 expression (Ariazi et al. 2002). Phosphorylation can subsequently lead to SUMOylation of ERRγ, which also modulates its activity (Vu et al. 2007, Tremblay et al. 2008). Most recently, Wilson et al. (2010) have shown that ERRγ acetylation by p300 coactivator-associated factor inhibits ERRγ transcriptional activity, while histone deacetylase 8 (HDAC8) and a homolog of sirtuin 1 (Sirt1) can reverse acetylation and increase receptor binding to DNA.

Discerning the relevant estrogen signaling pathways controlled by ERRγ versus ER is potentially challenging. However, two recent studies have convincingly shown that genes induced by ERRγ in breast cancer cells have very little overlap with those transcribed in response to ER activation. First, using a variant of PPAR γ coactivator-1α (PGC-1α) engineered to selectively activate ERRs but not ER or other nuclear receptors, Stein et al. (2008) found that very few ERRγ-regulated genes are related to estrogen signaling. Instead, ERRγ appears primarily to control oxidative stress signaling and aerobic metabolism (particularly key components of electron transport and the tricarboxylic acid cycle) in ER+ MCF7 breast cancer cells; ERRγ also induces the expression of vascular endothelial growth factor (Stein et al. 2009).

More recently, Deblois et al. (2009) have uncovered why the gene targets of ERRγ and ER in breast cancer cells are so distinct. Using comparative genome-wide chromatin immunoprecipitation, they determined that the binding sites utilized by ERRγ and ER, in the vast majority of cases, do not overlap. They did identify a group of genes (n=212, 18% of the total) that are targets of both receptors. However, while the promoter regions of some of these dual targets have distinct EREs and ERREs in close proximity, the majority of these coregulated genes are driven by an entirely novel DNA response element termed an ERRE/ERE, in which an ERRE (underlined) merges with a classical ERE (bold) (TCAAGGTCAANNTGACCT). These ERRE/ERE sites are only occupied by a single receptor (ERRγ or ER) at a time.

In ER− MDA-MB-231 breast cancer cells, siRNA targeted to ERRγ does not inhibit proliferation in vitro but does significantly reduce these cells’ migratory ability and delays xenograft tumor growth (Stein et al. 2008). In contrast, Chisamore et al. (2009b) have shown that inhibiting ERRγ signaling using a novel synthetic antagonist of the receptor (compound A; N-[(2Z)-3-(4,5-dihydro-1,3-thiazol-2-yl)-1,3-thiazolid-2-ylidene]-5Hdibenzo[a,d][7] annulen-5-amine), blocks cell proliferation in ER+ (MCF7, T47D) and ER− (BT-20, MDA-MB-231) breast cancer cell lines in vitro. Compound A has no effect on ERRγ mRNA expression, but abrogates ERRγ-mediated transcriptional activity in MCF7 cells and accelerates degradation of the receptor by the ubiquitin-proteasome pathway (Chisamore et al. 2009a). A different ERRγ antagonist, XCT790, also inhibits MCF7 and MDA-MB-231 proliferation in vitro and tumor formation in nude mice (Bianco et al. 2009). Like compound A, XCT790 does not inhibit ERRγ mRNA expression but instead enhances ERRγ protein degradation; XCT790 also blocks interaction of ERRγ with its coactivator PGC-1α (Lanvin et al. 2007). These findings suggest that pharmacological inhibition of ERRγ may represent a promising therapeutic approach, particularly in ER− breast cancer.

ERRγ appears to integrate well with known breast tumor molecular subtypes (Sorlie et al. 2001). ERRγ target genes are specifically enriched in the ERBB2 or HER2 cluster (Deblois et al. 2009). Greater than 80 ERRγ targets have prognostic value in several independent datasets, and a subset of these has independent prognostic value beyond ER and ERBB2 status in multivariate analyses. Other ERRγ target genes identified by Deblois et al., including GRB7 and ERBB2, are already incorporated into the OncotypeDX diagnostic test (Paik et al. 2004). Finally, gene expression analysis of distinct murine brain and bone metastases of a breast tumor line established from a patient with advanced breast cancer found that ERRγ, its coactivators PGC-1α and PGC-1β, and several known ERR target genes that control oxidative phosphorylation and the tricarboxylic acid cycle were selectively enriched in brain metastases.

**Estrogen-related receptor γ**

ERRγ (ESRRG, NR3B3) shares 90% sequence identity with the DBD, and ~60% sequence identity with the LBD, of ERRγ (Horard & Vanacker 2003). Like ERRγ, ERRγ is implicated in metabolism and cancer (Ariazi & Jordan 2006, Giguere 2008, ESRRG, in Atlas of Genetics and Cyto genetics in Oncology and
Haematology, available: http://atlasgeneticsoncology.org/Genes/ESRRGID45840ch1q41.html). However, the role of ERRγ in the etiology of breast cancer remains unclear.

One of the more puzzling aspects of the relationship between ERRα and ERRγ is that at first glance, they appear to have opposite prognostic value in breast cancer. The different associations arise from a study, in which 38 unselected breast tumors were compared to nine different populations of mammary epithelial cells (Ariazi et al. 2002). Within the tumor samples, ERRα mRNA expression correlates significantly with ER− and progesterone receptor-negative (PR−) status, and with HER2+ status. ER−/PR−/HER2+ tumors are more aggressive than ER+/PR+ tumors, leading to the conclusion that ERRα is a marker of poor clinical outcome. The ability of ERRα (Suzuki et al. 2004) and its target genes (Deblois et al. 2009) to function independently as poor prognostic factors have been subsequently confirmed. In direct contrast, breast tumors expressing ERRγ are significantly more likely to be ER+ and PR+ (Ariazi et al. 2002). ER+/PR+ status is, overall, a marker of good outcome. However, unlike ERRα, ERRγ has not been shown to have independent prognostic value, positive or negative, in any study. ERRγ's association with good outcome remains linked to its overrepresentation in ER+/PR+ tumors, not all of which have a good prognosis (see section on endocrine therapy below).

Liver receptor homolog-1

LRH-1 (NR5A2) is a type IV nuclear receptor that binds as a monomer to the SF1RE DNA response element (Fayard et al. 2004). LRH-1 is an important regulator of bile acid homeostasis, reverse cholesterol transport from peripheral tissues to the liver, and steriodogenesis; LRH-1 can bind C16 and C18 phospholipids such as phosphatidylycerol and phosphatidylethanolamine (Ortlund et al. 2005). LRH-1 mRNA and protein expression is significantly elevated in breast tumors and their surrounding adipose tissue as compared with normal breast (Zhou et al. 2005, Miki et al. 2006). LRH-1 is positively associated with ER+, PR+, and AR-positive (AR+) status, but negatively correlated with increased tumor stage, grade, and HER2+ status. Interestingly, LRH-1-positive status is associated with improved survival in women with PR+ breast cancer, but worse survival in women with PR− breast cancer. The establishment of LRH-1 as an ER target gene (Annicotte et al. 2005) has additional implications for endocrine therapy responsiveness in ER+ breast cancer.

Nerve growth factor-induced B, nuclear receptor-related 1, and neuron-derived orphan receptor 1

Nerve growth factor-induced B (NGFI-B, NR4A1; also known as Nur77 or testicular receptor 3 (TR3)), nuclear receptor-related 1 (NURR1, NR4A2), and neuron-derived orphan receptor 1 (NOR1, NR4A3) make up a group of closely related type IV nuclear receptors. A key feature of all three receptors is their apparent lack of a true ligand-binding pocket (Wang et al. 2003), although various diindolylmethanes (DIMs) have been shown to have agonist activity toward NGFI-B (Dae Cho et al. 2010). NGFI-B plays an interesting dual role in cell fate, functioning as a prosurvival factor when found in the nucleus but strongly inducing apoptosis when it translocates to the mitochondria, binds Bcl-2, and promotes cytochrome c release (Ferri & Kroemer 2001, Moll et al. 2006). MCF7 cells express NGFI-B, and DIM treatment of this cell line inhibits cell proliferation and induces apoptosis (Chinthalapalli et al. 2005); NOR1 is also strongly induced early in the apoptotic process of MCF7 cells (Ohkubo et al. 2000).

Rev-Erbα

V-erbA-related protein 1 (Rev-Erbα, NR1D1; also known as EAR1) is a type IV nuclear receptor that regulates metabolism and circadian rhythm (Duez & Staels 2009). Despite its orphan status, Rev-Erbα and the related Rev-Erbβ (NR1D2) are known to bind heme (Burris 2008). Rev-Erbα is localized to chromosome 17q21, a region that is frequently amplified in breast cancer; this region also harbors ERBB2, and Rev-Erbα is often coamplified and coexpressed with ERBB2 in breast tumors (Dressman et al. 2003, Chin et al. 2006). More recently, Davis et al. (2007) have reported that amplification of Rev-Erbα together with two other genes (SMARCE1 and BIRC5) functions as an accurate prognostic index independent of patient age or tumor stage in ER−/PR− breast tumors.

Retinoic acid-related orphan receptor α

Retinoic acid-related orphan receptor α (RORα, NR1F1) is a monomeric type IV nuclear receptor that can, under some circumstances, bind to cholesterol or cholesterol sulfate (Kallen et al. 2004). RORα is better known for its ability to regulate cerebellar development, the immune response, circadian rhythm, and resistance to atherosclerosis (Jetten 2004). However, several splice variants of this receptor are expressed in ER+ (MCF-7 and T47D) and ER− (BT-20, MDA-MB-231)
breast cancer cells (Dai et al. 2001). RORα is also localized to a well-known site of genomic instability (15q22.2), its expression is reduced in breast and other hormonally regulated tumors, and overexpression of RORα in MCF12F cells significantly inhibits their proliferation (Zhu et al. 2006).

TR2 and TR4

Human TR2 (NR2C1) and TR4 (NR2C2) are classified as type III nuclear receptors, although they do not exclusively form homodimers (Lee et al. 2002). Both TRs are generally considered to be transcriptional repressors; TR2 is implicated in adipocyte proliferation (Gupta et al. 2007) and erythroid cell differentiation (Tanabe et al. 2007), while TR4 plays an important role in gluconeogenesis (Liu et al. 2007) and promyelocyte proliferation (Koritschoner et al. 2001). Both TR4 (Shyr et al. 2002) and TR2 (Hu et al. 2002) can repress ER transcriptional activity, and radiation-stimulated p53 induction in MCF7 cells down-regulates TR2 expression (Lin & Chang 1996).

Orphan nuclear receptors and response to chemotherapy

Several cytotoxic chemotherapies play a key role in the clinical management of locally advanced and/or metastatic breast cancer (Shajahan et al. 2008). Anthracyclines (e.g. doxorubicin) induce DNA damage by intercalating into DNA while also inhibiting the activity of topoisomerase II and inducing reactive oxygen species (ROS). Alkylating agents (e.g. cisplatin) also induce DNA damage, but do so by forming DNA adducts that block DNA synthesis. In contrast, antimetabolites (e.g. 5-fluorouracil, 5-FU) inhibit the enzyme thymidylate synthase, which normally produces thymidine 5'-monophosphate (dTMP) that is used in the synthesis of DNA. 5-FU can also affect mRNA translation or inhibit rRNA processing (Burger et al. 2010, Kudo et al. 2010). Finally, two subclasses of antimicrotubule drugs are key inhibitors of tumor cell growth and inducers of tumor cell death by apoptosis and other mechanisms; the taxanes (docetaxel and paclitaxel) stabilize, while the Vinca alkaloids (vincristine and vinblastine) promote the destruction of the cellular microtubule network.

Estrogen-related receptor α

There is some evidence that supports a role for ERRα in response to 5-FU-based chemotherapy. Uridine phosphorylase (UPase) is one of the essential enzymes required for metabolizing 5-FU (Maring et al. 2005). UPase mRNA expression is positively regulated by ERRα and the nuclear receptor coactivator PGC-1α (Kong et al. 2009b). Specifically, PGC-1α/ERRα complexes bind to the UPase promoter, and the ability of PGC-1α to induce UPase transcription in MCF7 cells is abrogated by the ERRα-specific small molecule inhibitor XCT790. Moreover, PGC-1α overexpression sensitizes SkBr3 breast cancer cells to cell death induced by 5-FU, and this sensitization is also reversed by XCT790. These data suggest that the coexpression of PGC-1α and ERRα in breast tumors may indicate enhanced sensitivity to 5-FU-containing treatment regimens.

In contrast, this same group has shown that in multidrug-resistant HepG2 cells overexpressing the MDR1 drug transporter (R-HepG2), XCT790 alone can inhibit growth, alter mitochondrial membrane potential, and stimulate the production of ROS that ultimately induce caspase-dependent apoptosis (Wu et al. 2009). R-HepG2 cells are resistant to both doxorubicin and paclitaxel; however, while XCT790 can synergistically restore paclitaxel sensitivity, it has neither an additive nor synergistic effect on doxorubicin response. The authors propose that XCT790s ability to induce ROS is better complemented by paclitaxel’s stabilization of the microtubule network (thus leading to synergistic inhibition of cell growth). Since doxorubicin already can induce ROS, additive or synergistic interactions are not seen because XCT790 and doxorubicin affect similar downstream pathways. It is not immediately clear whether XCT790 might have similar effects on doxorubicin and/or paclitaxel response in multidrug-resistant breast cancer cells.

Nuclear receptor-related 1

One of the NR4 orphan nuclear receptors, NURR1, has been implicated in resistance to doxorubicin. Overexpression of NURR1 significantly decreases the expression of a proapoptotic member of the BCL2 gene family (BAX), while protecting cells in culture from apoptosis following doxorubicin treatment (Zhang et al. 2009). The DBD of NURR1 was subsequently found to interact with the C-terminus of the tumor suppressor p53. NURR1/p53 interaction prevents oligomerization of p53, attenuating the induction of p53 target genes (which include BAX). While these studies were not performed in breast cancer cells, BAX is important in doxorubicin sensitivity in MCF7 breast cancer cells (Kong et al. 2009a) and human breast tumors (Parton et al. 2002, Chintamani et al. 2004), suggesting that NURR1 could play an important role in this context.
Orphan nuclear receptors and response to endocrine or hormonal therapy

ER-α-positive (ER+) breast tumors comprise ~70% of annually diagnosed breast cancer cases (Jemal et al. 2009). Adjuvant or neoadjuvant endocrine therapy is among the least toxic, best tolerated, and most effective therapies available to patients with ER+ breast cancer. There are currently three major classes of endocrine therapy used in the clinic: selective ER modulators (SERMs), selective ER downregulators (SERDs), and aromatase inhibitors (AIs; Crago et al. 2010).

The prototypical SERM is the triphenylethylene tamoxifen, which competes with estrogen (E2) for binding to ER and in breast tissue is most often an antagonist; however, in bone, brain, and endometrial tissues, tamoxifen can act as a partial agonist (Clarke et al. 2001). In contrast, Fulvestrant (ICI 182 780, or Faslodex) functions as a SERD, a pure antagonist of ER in all tissues that compete for E2 binding and accelerates degradation of the receptor via the ubiquitin-proteasome pathway. Steroidal and nonsteroidal AIs ( exemestane, letrozole, and anastrozole) are inhibitors of the cytochrome P450 family member CYP19A1 (aromatase) that catalyzes the conversion of testosterone to E2, therefore depriving ER+ breast tumors of growth-stimulatory estrogen signaling (Santen et al. 2009). Tamoxifen is effective in the treatment of premenopausal and postmenopausal breast cancer patients. However, AIs are only indicated for use in postmenopausal women with breast cancer, where the major site of estrogen synthesis has shifted from the ovaries to the adrenal gland and adipose tissue.

As successful as each of these classes of endocrine therapies has been in the treatment of ER+ breast cancer, resistance to tamoxifen, fulvestrant, and/or AIs is a significant and widespread clinical problem (Riggins et al. 2005, 2007, Buzdar 2008, Macedo et al. 2009, Musgrove & Sutherland 2009). The proposed mechanisms of endocrine resistance are varied, and can include ER downregulation, silencing, or mutation, ER posttranslational modifications, changes in the expression profile(s) of nuclear receptor coregulators, and altered expression of key networks of growth factor signaling and/or apoptosis. Importantly, several orphan nuclear receptors have demonstrated potential to play important direct or indirect roles in endocrine therapy response and resistance.

Chicken ovalbumin upstream promoter transcription factors

Like the conflicting effects of the COUP-TFs on cell proliferation (Nakshatri et al. 2000, Moré et al. 2003), results from studies of the role(s) of these receptors in tamoxifen resistance are also contradictory. A significant decrease in COUP-TFII protein expression is observed in three different MCF7-derived resistance models. Inhibition of COUP-TFII by siRNA in sensitive MCF7 cells prevents tamoxifen-induced growth inhibition (Riggs et al. 2006). However, COUP transcription factors have been shown to enhance ER transcriptional activity by forming tight homodimer complexes (Métivier et al. 2002). These physical complexes increase the affinity of ER for MAPK, resulting in increased ER phosphorylation at serine 118 and enhanced ER transcriptional activity. Thus, under certain circumstances, overexpression of COUP-TFs might negate the inhibitory effects of tamoxifen on ER. Moreover, overexpression of COUP-TFI has been shown to stimulate MCF7 cell growth and migration, via an ER-dependent pathway, by selectively up-regulating genes involved in cell proliferation; these effects are seen in the presence and the absence of E2 (Le Dily et al. 2008). Discerning the true role of COUP-TFI and COUP-TFII in ER signaling and endocrine resistance will require further study to clarify their seemingly conflicting activities.

DAX-1

The ability of DAX-1 to function as a corepressor for ER (Zhang et al. 2000) has potentially significant implications for responsiveness to antiestrogens. However, more is known about the role of DAX-1 in estrogen synthesis through its negative regulation of aromatase mRNA expression in ovarian granulosa cells (Gurates et al. 2003) and endometrial cells (Gurates et al. 2002); its family member SHP has also been shown to inhibit aromatase expression (Kovacic et al. 2004). In contrast, deletion of DAX-1 in male mice significantly upregulates aromatase expression (Wang et al. 2001). To our knowledge, there have been no studies of DAX-1 in the peripheral and breast adipose tissues of women with breast cancer. Given that these sites are the major source of estrogen synthesis in postmenopausal women (Macciò et al. 2009), and that DAX-1 expression is significantly higher in preadipocytes than in mature adipose tissue (Kim et al. 2008), studies of DAX-1 in the context of AI-responsive and -resistant breast cancer could be informative.

Estrogen-related receptor α

Although the expression of ERRα in breast tumors is inversely associated with ER+ status (Ariazi et al. 2002), there are a number of ways in which this orphan nuclear receptor might affect endocrine therapy responsiveness. First, a small subset of ER+ breast cancers also expresses HER2. HER2-dependent
phosphorylation allows ERRα to activate transcription from EREs (Ariazi et al. 2007), potentially bypassing the inhibitory effects of SERMs and SERDs. Given that addition of the HER2 inhibitors trastuzumab or gefitinib to endocrine therapy appears to significantly improve disease-free survival in women with ER+/HER2+ breast cancer (Buzdar 2009, Johnston 2009), the contribution of ERRα to endocrine resistance, when it is expressed in ER+/HER2+ tumors, should be explored further. In addition, at least two genes identified as ERRα targets with prognostic value in breast cancer (Deblois et al. 2009) are known effectors of tamoxifen resistance. GRB7 is a breast cancer antiestrogen resistance gene that can confer estrogen independence and antiestrogen resistance to breast cancer patients (van Agthoven et al. 2009b), and has independent prognostic value in tamoxifen-resistant breast cancer patients (van Agthoven et al. 2009a). Cyclin E1 (CCNE1), particularly the low-molecular weight forms of this cell cycle regulator, has also been implicated in tamoxifen resistance (Dhillon & Mudryj 2002, Akli et al. 2004).

Alternatively, the ERRα-specific inhibitor XCT790 has been shown to enhance SERD activity by accelerating fulvestrant-dependent degradation of ER in MCF7 breast cancer cells (Lanvin et al. 2007). The mechanism by which this occurs is not entirely clear; the extent of ERRα degradation in response to XCT790 is greater in ER−/MDA-MB-231 cells than ER+ MCF7 cells, but when ER expression is restored in MDA-MB-231 cells, the ability of XCT790 to induce ERRα degradation is unchanged. Furthermore, transfection of MCF7 cells (which express both ER and ERRα) with siRNA specific for each receptor does not lead to enhanced sensitivity of the other receptor to its antagonist. The authors ultimately proposed that ER and ERRα heterodimerize in breast cancer cells in a way that confers protection to each receptor from protein degradation induced by their specific antagonists, and suggest that XCT790 may therefore be useful in combination with fulvestrant to improve endocrine therapy response in the clinic (Lanvin et al. 2007). However, heterodimerization of ER and ERRα has not been convincingly demonstrated, so this mode of action currently seems somewhat unlikely. Finally, in breast cancer cells, ERRα forms a transcriptional complex on aromatase promoters I.3 and II with the nuclear receptor coactivators proline, glutamate, and leucine-rich protein 1 (PELP1) and proline-rich nuclear receptor coactivator 2 (PNRC2), thereby inducing aromatase expression and promoting localized estrogen synthesis (Rajhans et al. 2008).

**Estrogen-related receptor γ**

As discussed above, the expression of ERRγ in breast tumors is associated with ER + and PR + status (Ariazi et al. 2002). This has led to the notion that ERRγ is a marker of good prognosis and response to endocrine therapy. However, 30% of women have ER+/PR+ breast tumors that are intrinsically (de novo) resistant to tamoxifen, and when initially responsive patients present with recurrent, endocrine-resistant disease, in most cases, these recurrent/resistant tumors retain ER expression (Riggins et al. 2005). Therefore, genes that are primarily expressed in ER + breast tumors are not necessarily good prognostic factors. A clear example is X-box-binding protein 1 (XBP1), a key mediator of the unfolded protein response (Feldman et al. 2005). XBP1 is coexpressed with ER in breast tumors (Lacroix & Leclercq 2004, Tozlu et al. 2006, Wilson & Giguère 2008). However, XBP1 is an ER coactivator that induces ligand-independent activation of the receptor (Ding et al. 2003a,b, Fang et al. 2004), XBP1 upregulation is evident in some antiestrogen resistant cells (Gu et al. 2002), and its ectopic overexpression leads to tamoxifen resistance and estrogen independence in multiple ER + breast cancer cell lines (Gomez et al. 2007). The presence of spliced or active XBP1 in breast tumors is also significantly associated with poor clinical response to tamoxifen (Davies et al. 2008).

We have shown that ERRγ can play an important functional role in the acquisition of tamoxifen resistance by breast cancer cell lines derived from invasive lobular carcinoma (Riggins et al. 2008). The tamoxifen-resistant variant of the SUM44 breast cancer cell line (Ether et al. 1993), LCCTam, expresses significantly more ERRγ mRNA and protein, and knockdown of this orphan nuclear receptor restores tamoxifen-mediated growth inhibition. In contrast, ectopic overexpression of ERRγ cDNA independently induces tamoxifen resistance in SUM44 cells and another model of ILC, MDA-MB-134 VI (Reis-Filho et al. 2006). The mechanism by which ERRγ mediates tamoxifen resistance is under active investigation. As discussed above, ERRγ can induce gene transcription from ERE and ERRE sites, and one of the more active metabolites of tamoxifen (4-hydroxytamoxifen, 4HT) inactivates ERRγ with respect to transcription from both of these response elements (Greschik et al. 2004). However, studies in nonbreast cancer cell lines show that in the presence of 4HT, ERRγ can potently activate transcription from activator protein-1 (AP1) sites (Hupunen et al. 2004). We subsequently demonstrated that the resistant LCCTam cells have significantly higher AP1 activity.
in the presence of tamoxifen, and that a peptide inhibitor of API effectively restores tamoxifen responsiveness (Riggins et al. 2008). Because the full compliment of endogenous ERRγ/API target genes are unknown, current studies are focused on identifying these targets in order to understand how this orphan nuclear receptor contributes to tamoxifen resistance.

Importantly, ERRγs function in tamoxifen resistance may not be restricted to ILC. We have recently found that ERRγ mRNA expression is significantly increased in the MCF7/RR model (Butler & Fontana 1992) of tamoxifen resistance (O Z Maniya, M M Mazzotta, and R B Riggins, unpublished observations). This is supported by in silico reanalysis of gene expression microarray data from a study of 60 women with breast cancer who were treated only with tamoxifen (Ma et al. 2004). Pretreatment mRNA levels of ERRγ are significantly elevated in tumors from breast cancer patients who recurred within 5 years of tamoxifen therapy compared with those from breast cancer patients who did not recur (Fig. 2), and ~80% of this patient population had invasive ductal carcinoma. Further studies are needed to better understand how ERRγ functions in the full spectrum of tamoxifen-resistant, ER+ breast cancer.

Liver receptor homolog-1

LRH-1 strongly induces aromatase expression from promoter II in preadipocytes (Clyne et al. 2002) and breast adipose tissue (Clyne et al. 2004), and its ability to do so can be inhibited by SHP (Kovacic et al. 2004). The presence of higher levels of LRH-1 mRNA in breast tumors and their surrounding adipose tissue, as compared with normal breast, mirrors the expression of aromatase in these tissues (Zhou et al. 2005). More recent studies show that cooperation between LRH-1 and GATA transcription factors (Bouchard et al. 2005) or PGC-1α (Safi et al. 2005) strongly induces aromatase expression.

As stated above, LRH-1 is an ER target gene (Annicotte et al. 2005), and tumoral LRH-1 expression is associated with improved survival in women with PR+ breast cancer but worse survival in women with PR− breast cancer (Miki et al. 2006). Together with its demonstrated ability to induce transcription of aromatase, these data suggest that LRH-1 may be a marker of intact aromatase/estrogen/ER signaling that indicates improved sensitivity to endocrine therapies such as tamoxifen or an AI. Two clinical studies provide some support for this. Using specimens from the P025 trial in which letrozole was compared with tamoxifen, women with PR+ breast tumors that also expressed aromatase trended toward having improved time to progression when treated with letrozole (Lykkesholm et al. 2009). In the P024 trial that also compared letrozole to tamoxifen, increased aromatase expression at baseline was significantly associated with improved relapse-free survival in multivariate analyses (Ellis et al. 2009).

Retinoic acid-related orphan receptor α

RORα has also been shown to induce aromatase expression by enhancing its transcription from promoter I.4, and can significantly increase aromatase activity in ER+T47D and MCF7 breast cancer cells (Odawara et al. 2009). This study also showed that RORα and aromatase expression are positively correlated in breast cancer clinical specimens. RORα deletion in mice suggests an even broader role for this gene in regulating multiple components of steroidogenesis other than aromatase, including several sulfotransferases and hydroxysteroid dehydrogenases (Kang et al. 2007). However, a different study reports that RORα expression is reduced in breast cancer compared with normal tissue (Zhu et al. 2006), so it is still unclear how this orphan nuclear receptor functions in breast tumors or modulates endocrine therapy response.

Summary and perspectives

The field of breast cancer research has much to gain from the continued study of orphan nuclear receptors. Many of these unique transcription factors have the potential to transform our understanding of how tumorigenesis is integrated with fundamental
physiological processes such as metabolism, regulation of circadian rhythm, and obesity (Sahar &
Sassone-Corsi 2009, Froy 2010). We also have a great deal to learn about how orphan nuclear receptors function in normal mammary tissue in mice and humans. Exploring these unresolved areas of orphan nuclear receptor biology could ultimately lead to discoveries that will improve our ability to diagnose, treat, and prevent breast cancer.

Declaration of interest
The authors declare that they have no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
R B Riggins and R Clarke developed the original idea and outline, while all authors made significant contributions to the writing and editing of this manuscript.

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