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Review

The role of ERα-36 in regulation of cell function

Yong-Qing Hua[#] and Lena Sahlin[*]

Division for Reproductive Endocrinology and the Pediatric Endocrinology Unit,
Department of Women's and Children's Health, Karolinska Institutet,
Karolinska University Hospital - Solna, Q2:08, 171 76 Stockholm, Sweden

ABSTRACT

ERα-36, a newly described variant of the estrogen receptor alpha (ERα), is widely expressed in normal and tumor tissues. However, the functions of ERα-36 expression have not been fully revealed. ERα-36 is primarily localized to the plasma membrane and cytoplasm and has been shown to play a role in mediating non-genomic estrogen signaling. It has been demonstrated that ERα-36 is involved in cell differentiation, which could imply a key role in e.g. cancer development, nerve differentiation and immune cell maturation. Here we present a review of the recent findings in the field, especially in relation to breast cancer and the immune system, as well as the other ERs.

KEYWORDS: estrogen receptor, ERα-36, non-genomic action, breast cancer, leukocytes, cell differentiation

ABBREVIATIONS

AF, activation function; AKT, protein kinase B; AP-1, activator protein 1; cAMP, cyclic adenosine monophosphate; CREB, cAMP response element-binding protein; E2, 17β-estradiol; EGFR, epidermal growth factor receptor; ER, estrogen receptor; ERE, estrogen response element; ERK, extracellular signal-regulated kinase; GFR, growth factor receptor; HER2, human epidermal growth factor receptor 2; HL-60, human promyelocytic leukemia cells; JUNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; PI3K, phosphoinositide 3-kinase; PIP3, phosphate-dylinositol 3,4,5-triphosphate; PKA, protein kinase A; PKC, protein kinase C; SP1, specificity protein 1; Src, proto-oncogene tyrosine-protein kinase; wt1, Wilms' tumor suppressor gene

INTRODUCTION

Estrogens play many roles in human physiology as well as pathophysiology [1]. They exert numerous effects in the development and maintenance of normal sexual and reproductive functions, and also in the cardiovascular, musculoskeletal, immune, and central nervous systems [2]. Estrogens express their effects after binding to nuclear receptors, historically designated as the estrogen receptor α (ERα) and the estrogen receptor β (ERβ). Estrogen signaling pathways can be divided into four different types [2-4]. I. The classical ligand-dependent genomic action. The hormone binds to the ER and the steroid-receptor complex binds to a hormone-responsive element on the chromatin, denoted ERE (estrogen response element) for estrogens. The steroid-receptor complex interacts as dimers with EREs in targets genes. The complex then recruits transcriptional co-modulators and regulates transcription of the target genes. II. The ERE-independent genomic action. Many estrogen-responsive genes lack the ERE and interacts with

*Corresponding author:
lena.sahlin@ki.se
[#]Home university: Department of Pharmacology on Traditional Chinese Medicine, School of Pharmacy, Nanjing University of Chinese Medicine,
210029 Nanjing, China.

ERs via a tethering mechanism with a combination of ER and a protein, e.g. SP1 or AP1 transcription factors. III. Non-genomic signaling. Very fast estrogen responses, e.g. rapid steroid effects on the electrical activity of nerve cells, activation of NO synthase, calcium mobilization etc., indicate that the steroid hormone interacts with and activates signaling cascades at the cell membrane. There are several candidates for this membrane bound ER, e.g. GPER (G-protein-coupled estrogen receptor 1, also known as GPR30), ERα-46 and ERα-36 [5-8]. IV. Ligand-independent non-genomic action. ER-mediated transcription can occur in a ligand-independent manner in response to signaling via growth factor receptors, subsequently activating intracellular kinase pathways which lead to phosphorylation and activation of the ER.

Discovery of ERα-36

The identification and subsequent cloning of the full length human ERα (66 kDa), the splice variant ERα-46, and the closely related ERβ have had an immense impact on the understanding of estrogen-mediated functions. However, a remaining issue is that not all previously reported findings, especially the rapid non-genomic estrogen effects, can be explained by the known properties of the nuclear ERs already described. In some cases, antagonists of these receptors could not block certain rapid signaling events, which led to the prediction that alternative non-genomic estrogen signaling pathways also exist [9].

Previous studies have shown that with anti-ERα antibodies, raised against the ligand-binding domain of human ERα-66 and used for immuno-precipitation, an additional hitherto unknown 35-39 kDa protein was present in Western blots [10, 11]. Some other studies indicate that an alternative pathway, involving a membrane receptor, may also be involved in cell response to estrogens [12, 13]. Estradiol (E2) was found to directly affect the fluidity of chondrocyte membranes derived from female, but not male, rats. In addition, E2 activates protein kinase C (PKC) in a non-genomic manner in chondrocytes from female rats, and chelerythrine, a specific inhibitor of PKC, inhibits E2-dependent alkaline phosphatase activity and proteoglycan sulfation in these cells, indicating PKC to be involved in the signal transduction mechanism [12, 13].

As the consequence of a search for alternative mechanisms to account for the different findings as described above, a novel, previously unrecognized, 36 kDa variant of full length ERα was identified, cloned, and expressed in HEK 293 cells [8].

Structure of ERα-36

Cellular signaling of estrogens is mediated via ERα and ERβ, both belonging to the nuclear receptor superfamily of transcription factors. Like many other members of this family, ERs contain structurally and functionally well conserved domains (Figure 1). The middle and most conserved domain, the DNA-binding domain (DBD), is involved in DNA recognition and binding, whereas ligand binding occurs in the C-terminal part where the ligand-binding domain (LBD) is located. The N-terminal end is less well conserved and constitutes the most variable domain [2].

There are multiple isoforms of ERα, the full length receptor of 66 kDa and a lower molecular weight variant, ERα-46. In analogy with this ERα-36 has a molecular mass of 36 kDa [8, 14, 15]. ERα-66 is predominantly localized to the nucleus, but ERα-46 has been localized both to the nucleus and the cell membrane [11]. ERα-36 differs from full length ERα by lacking both trans-activation domains (AF-1 and AF-2). It does, however, retain the DNA-binding domain, and partial dimerization and ligand-binding domains of ERα-66 (Figure 1). It holds a unique 27 amino acid domain that replaces the last 138 amino acids encoded by exons 7 and 8 of the human ERα-66 gene [8].There are three myristoylation sites found in ERα-36 which are anticipated to direct the receptor to the plasma membrane. ERα-36 is a unique ERα variant which is predicted to function as a dominant negative effector in ERα-66 mediated estrogen-responsive signaling pathways [14]. ERα-36 is also suggested to have the potential to trigger membrane-initiated mitogenic estrogen signaling [14].

Function of ERα-36 in breast cancer

Growth of ER-positive breast cancer is typically enhanced by estrogens, but ER interactions with DNA are not necessary for this growth to occur, suggesting that non-genomic actions of ERs may

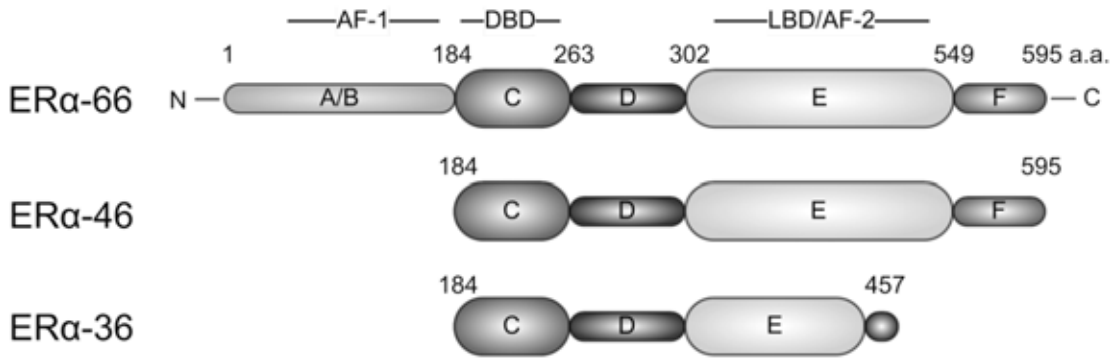

Figure 1. The common structure of the ERα variants, showing the different domains, labeled A-F. There are three main domains in the receptor structure:
The amino terminal (A/B) contains a strong independent trans-activation domain (AF-1).
The DNA-binding domain (C/D), including the hinge region, carries sequences necessary for receptor dimerization, nuclear trans-location and binding to the ERE at DNA.
The carboxy terminal (E/F) the ligand-binding domain contains sequences important for nuclear translocation, receptor dimerization, and a second, hormone dependent, trans-activation domain (AF-2).
The ERα-36 variant holds a unique 27 amino acid domain that replaces the last 138 amino acids encoded by exons 7 and 8 of the human ERα-66 gene [8].

play a role [16, 17]. ERα-36 was found expressed in ER-positive and -negative human breast carcinomas [18], and the expression is significantly higher in ER-negative tumors than in ER-positive tumors [19]. Immunohistochemistry analysis was used to evaluate ERα-36 expression in tumors from 896 women with operable primary breast cancer. Results showed that women with ERα-66-positive tumors that also express high levels of ERα-36 are less likely to benefit from tamoxifen treatment [20]. Furthermore, ERα-66 was found to suppress ERα-36 promoter activity in an estrogen-independent manner, and this suppression could be overcome by co-expression with ERα-36 itself or ERα-46 [21]. Many signaling pathways, including cyclic adenosine monophosphate/protein kinase A (cAMP/PKA), the phosphoinositide 3-kinase (PI3K) and the mitogen-activated protein kinase (MAPK), have been described as downstream of ERα-36 in various cell types [15, 22-25]. The MAPK/extracellular signal-regulated kinase (ERK) signaling pathway plays a key role in regulating cell differentiation and proliferation and provides protection against apoptosis under estrogen exposure [26, 27]. It has been demonstrated that ERα-36 inhibits the estrogen-dependent and estrogen-independent transactivation activities of ERα-66 and ERβ. ERα-36, being associated with the plasma membrane, transduces both estrogen- and antiestrogen-dependent activation of the MAPK/ERK signaling pathway and stimulates cell growth [14].

ERα-36 is rapidly activating PKC in response to E2 leading to effects such as stimulation of proliferation, protection against apoptosis and enhancement of metastatic factors. ERα-36 is therefore a candidate for targeting these deleterious effects in order to inhibit them to occur [23]. ERα-36 knockdown cells were more sensitive to the anti-mitotic cancer chemotherapy drug paclitaxel, therefore the c-Jun N-terminal kinase (JUNK) pathway appears to be involved in this mechanism. The ERα-36 knockdown cells also exhibited decreased migration and invasion. These changes were estrogen independent, therefore the findings may indicate that targeting ERα-36 could be a strategy for treating ER-negative breast cancers [19].

Phosphorylation of ERK1/2 by MAPK kinase is involved in the motility and invasiveness of inflammatory breast cancer (IBC) cells [28]. Treatment with E2, anti-estrogenic agents like 4-hydroxytamoxifen and ICI182780, the ERβ specific ligand DPN and the GPER agonist G1 led to a rapid activation of phosphorylated ERK1/2, suggesting involvement of ERα-36, ERβ and GPER in the non-genomic signaling pathway in IBC cells [28].

Wilms' tumor suppressor gene, wt1, encodes the zinc-finger protein WT1 that functions as a dual transcription regulator to activate or suppress gene transcription. High levels of WT1 expression are associated with breast cancer malignancy [29]. WT1 was found to regulate the expression of ERα-66 and ERα-36 oppositely [30]. Furthermore, co-transfection assays showed that all isoforms of WT1 directly activated the promoter activity of the ERα-66 gene while suppressing ERα-36 promoter activity [30].

Function of ER-α36 in immune cells

Estrogens have been suggested to be responsible for the difference between men and women in incidence and prevalence of some immunologic disorders [31, 32]. These conditions are associated with leukocyte infiltration and immune dysfunction. In rats, it has been shown that peripheral blood leukocytes are responsive to estrogens. E2 and selective ER agonists regulate a number of genes related to inflammation and extracellular matrix remodeling [33]. Estrogens are generally considered as enhancers of cell proliferation and the humoral immune response [34]. The exact mechanism(s) by which the hormone modulates the immune cells is not fully understood, since different effects depending on estrogenic substance, receptor and dose are found [34]. Although it is clear that estrogens regulate the immune system, still the outcome of estrogen-induced immune responses is variable [35].

Neutrophils form an essential part of the immune system. Women have a higher neutrophil count than men, with the numbers changing during the menstrual cycle [36, 37]. Both ERα and ERβ are present in neutrophils and E2 up-regulates both ERs in women, but only ERα in men [38]. In HL-60 cells, a human promyelocytic leukemia cell-line capable of undergoing terminal differentiation into neutrophils [39], an ER localized to the plasma membrane was found [40]. A recent study by our group showed that ERs are functionally active in differentiated neutrophil like HL-60 cells, regulating genes involved in important physiological functions. A receptor variant of ERα, corresponding to a molecular weight of 36 kDa, was observed in differentiated neutrophil like HL-60 cells, but not in the undifferentiated cells [41]. In addition, also GPER expression was found in both undifferentiated and differentiated HL60 cells, as was nuclear ERβ, whereas ERα-66 and GPER was localized mainly to the membrane fraction and, to a lesser degree, the cytosol [41].

The expression of the membrane bound receptor occurs throughout the cell cycle, progressively increasing as cells mature [40]. The effects of ER ligands, including a non-internalizable estradiol-BSA conjugate, were found to cause activation of the MAPK/ERK pathway. Thus, there is a membrane localized ER in HL-60 cells that can cause ERK activation [40], and thereby mediate non-genomic actions by estrogen stimulation.

ERα-36 and GPER

GPER is still a controversial issue, it has been presented as a membrane bound non-classical ER (reviewed in [42-44]), or just a modulator of the classical or truncated ERs [45, 46]. It is also claimed that no reliable GPER deficient mouse models exists [47]. GPER has though been shown to mediate non-nuclear responses of estrogens and is suggested to be an alternative membrane receptor for estrogens [5, 48, 49]. A study from 2000 shows that E2 activates ERK-1/-2 not only in MCF-7 cells, which express both ERα and ERβ, but also in SKBR3 breast cancer cells, which express neither of these receptors. MDA-MB-231 breast cancer cells (ERα-, ERβ+) are GPER deficient and insensitive to ERK-1/-2 activation by E2. Transfection of these MDA-MB-231 cells with a GPER complementary DNA resulted in conversion to an estrogen-responsive phenotype. This data provide evidence that estrogen-induced ERK-1/-2 activation occurs independently of known ERs, but requires GPER expression [50]. Confocal fluorescence microscopy revealed that E2-Alexa 546 staining co-localized almost completely with GPER-GFP expression in the endoplasmic reticulum and Golgi, and that the binding could be counteracted by excess E2. The intensity and pattern of staining of the fluorescent E2 was consistent with the level and pattern of GPER protein expression. Activation of GPER by E2 resulted in intracellular calcium mobilization and synthesis of phosphatidylinositol 3,4,5-trisphosphate (PIP3) in the nucleus [49]. Sanden *et al.* demonstrated that the GPER agonist G1 and E2,

both stimulated GPER-dependent cAMP production, a defined plasma membrane event, as well as recruitment of β-arrestin-2 to the plasma membrane. Then, using FLAG- and hemagglutinin-tagged GPER, they demonstrated that these events occur in the plasma membrane [51]. Knockdown studies in the seminoma cell-line TCam-2 indicate that ERα-36 is a downstream target of E2-activated GPER/PKA/CREB (cAMP response element-binding protein) pathway and necessary for cell proliferation [24]. Similar results were found in breast cancer cells [46]. GPER signaling activates ERα-36 promoter activity and induces ERα-36 expression; however, ERα-36 functions were independent of GPER. Activities of GPER in response to estrogen were in this model via its ability to induce ERα-36 expression [46].

DISCUSSION

There are several signaling cascades, such as the IP3/PKC, PI3K/AKT, MAPK/ERK and cAMP/PKA pathways, implicated in estrogen induced non-genomic action. ERα-36 has been demonstrated to be involved in all these pathways, which we have tried to summarize in Figure 2. ERα-36 and ERα-46 have both been found in membranes, cytosol and, to a minor degree, the nuclei [11, 14, 49, 52, 53]. ERα-36 is mostly associated with the plasma membrane where it transduces both estrogen- and antiestrogen-dependent activation of the MAPK/ERK signaling pathway and stimulates cell growth [14, 30], but was also found in cytoskeleton microfilaments [24], suggesting a role of estrogens in cell motility. GPER has also been described as present

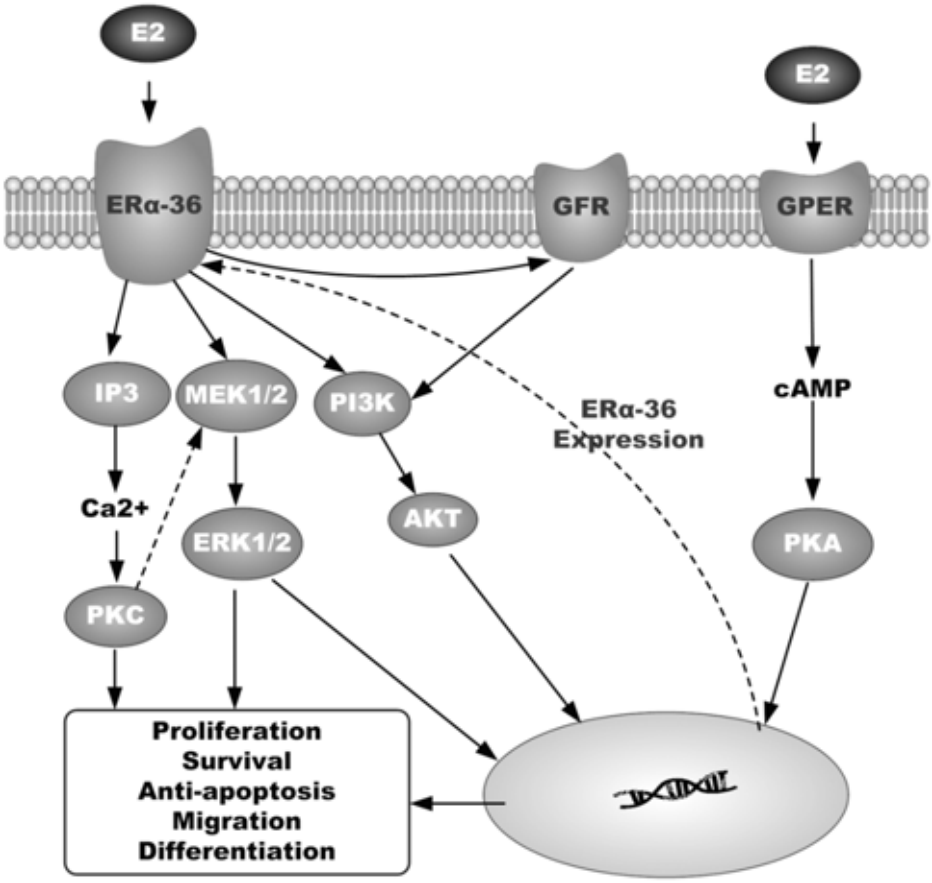

Figure 2. The non-genomic estrogen signaling pathways. E2 binding of ERα-36 may induce activation of PKC via IP3 [23, 46]; ERK activation via MEK1/2 [14, 46] and AKT via PI3K [15, 67]. There are cross talk between growth factor receptors (GFR) and non-genomic estrogen signaling [60, 68]. GPER is activating PKA via cAMP, which also activates ERα-36 promoter activity to induce ERα-36 expression [24, 46].

in the plasma membrane [54], endoplasmatic reticulum and/or Golgi [48]. Thus, all of them have been localized to the plasma membrane, but neither of them solely to the membrane. To simplify the figure, all receptors are localized to the membrane (Figure 2). Growth factor receptors (GFR)s cross talk with ERs, as shown for ERα-66 [55, 56] and GPER [57]. It is possible that growth factor-signaling pathways may also contribute to induction of ERα-36 expression via the Src/MAPK/AP-1 pathway [15]. It is highly probable that all the hitherto identified membrane receptors, suggested to bind estrogens, could add to estrogen mediated activity and function. Further investigations are needed to identify which membrane receptor that is most important for which estrogenic action.

ERα-66, ERα-46, ERα-36 and GPER are all present in one or more of the breast cancer cell lines T47D (ERα+/ERβ+), MCF7 (ERα+/ERβ+), MDA-MB-231 (ERα-/ERβ+) and SKBR3 (ERα-/ERβ-) [54]. E2 stimulates a strong intracellular increase of calcium mobilization in ERα-positive breast cancer MCF7 cells and a modest increase in ERα-negative breast cancer SK-BR-3 cells [46, 58, 59]. It was suggested that E2 interacts with ERα-36 at the cell membrane to activate PKC. Because PKC can activate ERK1/2, to rapidly enhance phosphorylation of proteins that promote proliferation, this pathway may lead to indirect effects on gene expression [23]. ERα-36 expression is frequently associated with HER2 expression in breast cancer specimens [20]. ERα-36 was found to mediate non-genomic estrogen signaling via the EGFR/Src/ERK signaling pathway in ER-negative breast cancer cells [60]. Effects of mitogenic estrogen signaling exhibit a biphasic, dose-response curve, i.e. estrogens at low concentrations stimulate cell proliferation, while at high concentrations, estrogens inhibit cell growth. ERα-36 is involved in the biphasic estrogen signaling of ER-negative breast cancer cells [25].

Testosterone has been identified as one important factor in the pathogenesis of endometrial cancer [61]. ERα-36 is, as demonstrated by immuno-cytochemistry, localized to the plasma membrane of both the ERα- and androgen receptor-negative endometrial Hec1A cancer cells [15]. Testosterone induced ERK and AKT (protein kinase B) phosphorylation, which could be abrogated by ERα-36 short hairpin RNA knockdown or the kinase inhibitors U0126 and LY294002, or the aromatase inhibitor letrozole. These findings suggest that ERα and ERα-36 might be involved in testosterone induced carcinogenesis [15].

Besides the cancer cell lines mentioned above, ERα-36 expression has also been identified in e.g. human gastric cancer cells where it was expressed mainly on the plasma membrane and in the cytoplasm, and its expression showed high correlation to lymph node metastasis [62]. Results from the TCam-2 study show that ERα-36 expression is rapidly induced after estrogen treatment in a GPER-dependent manner, and ERα-36 is required for E2-dependent epidermal growth factor receptor (EGFR) expression [24]. ERα-36 was also found expressed in airway epithelial and smooth muscle cells, predominately localized on the plasma membranes, and the expression levels were increased after sensitization to allergen [63].

It has been shown that murine macrophages, both *in vivo* and *in vitro*, present both mRNA and protein of GPER [64]. In addition, treatment with E2 *in vitro* resulted in a rapid decrease of toll-like receptor-4 (TLR4) expression on the macrophages. Two different GPER specific agonists that interact with neither ERα nor ERβ could mimic this effect in primary macrophages and a cell line. Specific ERα and ERβ agonists failed to show any effect on TLR4 expression [64]. Silencing RNA to specifically knock-down the GPER expression in the macrophages showed that the effects by E2 or G1 on TLR4 expression were abolished. ERα-46 has been found present in macrophages along with ERα-66 [65]. Macrophages have not, as yet, been shown to express ERα-36 [64, 65], but it has been suggested that G1 might also interact with ERα-36 [46]. Hence, it cannot be excluded that ERα-36, if present, may play a role in the E2 and G1 effects on TLR4 expression. However, the ER antagonist and GPER agonist ICI182780 decreases TLR4 levels, while the selective ERα agonist PPT failed to decrease TLR4 expression in the macrophages [64]. Short time E2 treatment of peritoneal macrophages has been shown to reduce the LPS-induced increase in inflammatory mediator

expression [66], which is in agreement with the fact that G1 reduced LPS binding in macrophage-like cells and inhibited production of key inflammatory cytokines [64]. Thus, this allows for a role of estrogens in reduction of inflammatory responses. Since we found ERα-36 expression in differentiated, but not undifferentiated HL-60 cells, and GPER expression in both types, these membrane associated receptors might be involved in the estrogen regulated inflammatory responses [41].

In conclusion, we believe that ERα-36, as one of the membrane bound ERs, mediates many important functions via estrogen induced non-genomic effects. ERα-36 has been shown to be present in many breast cancers but also in immune cells like leukocytes. To what extent ERα-36 acts differently, or independently of, the other described ERs like full-length ERα and ERβ or the suggested membrane bound variants like ERα-46 and GPER, still remains to be determined. There are many possibilities for estrogen signaling in breast cancer and immune cells, and the hormone and its receptors are important components both in breast cancer development and prognosis as well as in inflammatory and autoimmune disease incidence and prevalence. The increased amount of ER variants adds to the complexity of estrogen regulation.

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