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Experimental Models for Evaluating Non-Genomic Estrogen Signaling

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Abstract

Non-genomic effects of estrogen receptor α (ERα) signaling have been described for decades. However, the mechanisms and physiological processes resulting solely from non-genomic signaling are poorly understood. Challenges in studying these effects arise from the strongly nucleophilic tendencies of estrogen receptor, and many approaches to excluding ERα from nucleus have been explored over the years. In this review, we discuss past strategies for studying ERα's non-genomic action and current models, specifically H2NES ERα, first described by Burns et al. 2011. In vitro and preliminary in vivo data from H2NES ERα and H2NES mice suggest a promising avenue for pinpointing specific non-genomic ERα action underlying mechanisms.

Keywords

Estrogen Receptor alpha; non-genomic signaling; rapid action

1. Introduction

1.1. Non-Genomic Estrogen Signaling

Estrogen receptors play a crucial role in the maintenance of the female and male reproductive systems. They also bring about a wide range of effects in other tissues and organ systems. Known estrogen receptors include estrogen receptor α (ERα), estrogen receptor β (ERβ), and G protein-coupled estrogen receptor 1 (GPER1/GPR30). Investigators in the 1970's observed rapid estrogenic effects in uterine stimulation and first proposed that these rapid actions could be modulated by estrogen receptors localized to the plasma membrane where they also elicited signal transduction events. Elevation of uterine cAMP levels and eosinophilic infiltration [1] and calcium mobilization in endometrial cells following estrogen exposure [2] were the earliest observations of these rapid effects. In 1977, Pietras and Szego observed substantial binding of estrogen to the plasma membrane of endometrial and liver cells, and concluded the binding site was likely an estrogen receptor

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[3] due to the high affinity of 17β-estradiol (E2) to the binding site,. Since then, non-genomic effects of estrogen receptor have been attributed to the increase of intracellular calcium concentration via activation of PLCβ [4], activation of Gα and Gβ proteins [5], regulation of potassium channels, activation of MAPK cascades, activation of lipid kinases such as phosphatidylinositol 3-kinase (PI3K), and adenylate cyclase [6].

Evidence for plasma membrane-localized estrogen receptor was introduced when Pedram and Levin isolated membrane bound estrogen receptor from a breast cancer cell line and with mass spectrometry confirmed its identity was identical to the *ESR1* gene product ERα [7]. However, attributing non-genomic signaling and action of estrogen to the membrane localized ERα might be considered dubitable because ERα protein lacks known kinase or phosphatase motifs, thus it is unknown how E2 induces ERα-mediated non-genomic signal transduction events.

Non-genomic estrogen signaling is also carried out through GPER1, which was originally identified as an orphan G protein-coupled receptor 30 (GPR30) [8]. Of note is the fact that aldosterone binds GPR30 with higher affinity than estrogen [9], creating contention of whether GPER1/GPR30 should be considered an estrogen-specific receptor. Nonetheless, activation of GPER1/GPR30 elicits a variety of signal transduction pathways that execute estrogen's functions in vitro. Several different GPER1/GPR30 knock-out mouse models have been published however results are variable therefore making general conclusions difficult [10–13]. One of the mutant mouse models used to report reproductive and estrogenic functionality and phenotypes showed no change in body weight, visceral adiposity, glucose tolerance, or fertility, and normal estrogenic responses in the uterus and mammary gland of female mice, in contrast to the ERα knock-out mouse phenotypes [13].

In this review, we focus on previous and current efforts seeking to elucidate how ERα mediates non-genomic estrogen action.

1.2. ERα Structure and Mechanisms of Action

Like other nuclear receptors, the structure of ERα is characterized by several motifs: the amino-terminal domain (A/B-domain), the DNA-binding domain (DBD; C-domain), the hinge region (D-domain), the ligand binding domain (LBD; E-domain) and the carboxy-terminal domain (F-domain) [14].

ERα's classical mechanism of signaling involves its localization to the nucleus where it directly binds to estrogen responsive DNA elements (ERE). This action results in changes in gene expression involving either stimulation or repression [15]. ERα's other mechanism of action in the nucleus involves tether-mediated signaling, in which it binds to other transcription factors such as c-Jun and Sp1, which in turn bind to AP-1 and Sp-1 DNA response elements to elicit gene expression changes [16]. The third mechanism of ERα action is non-nuclear, non-genomic signaling in the cytoplasm of cells [17]. At least, the E-domain is involved in non-genomic signaling [18] but the involvement of other domains of ERα is still unclear.

2. Models of Non-Genomic Estrogen Action

Little is known about the precise physiological effects of non-genomic ERα signaling, and pinpointing these effects has proven to be complicated due to the difficulty in controlling for the strong nucleophilic nature of ERα. Observations of its non-genomic effects have been made by blocking RNA and protein synthesis for ERα-mediated gene expression, leading to the conclusion that non-genomic action can stimulate cAMP levels through adenylate cyclase activity [19]. Earlier pharmacological studies attempted to use E2 covalently conjugated with BSA (E2-BSA) to test for non-genomic E2 action, proposing that the E2-BSA complex could not enter the cells [20]. This approach was brought into question when Stevis et al. reported continuous leaching of free E2 from the E2-BSA conjugates and observation that E2-BSA stimulates sustained MAPK activity where free E2 does not activate under the same conditions. These results warned that biological activity of E2-BSA can lead to erroneous conclusions regarding the effects of E2 at the membrane [21]. Second generation approaches have employed estrogen-dendrimer conjugates (EDCs), where estradiol is confirmed to be covalently linked as another means to explore estrogen receptor signaling outside of the nucleus in both in vitro and in vivo models [22]. EDCs are multiple E2 molecules conjugated with polyamidoamine dendrimer macromolecules that are excluded from the nucleus due to their size and charge [23]. Utilization of EDCs has contributed to the findings that non-genomic ERα activates p44/42 MAPK (ERK1/2), Shc, and Src [23], stimulates vascular endothelial cell migration and proliferation, and protects against vascular injury without creating uterotrophic responses [22]. Additionally, use of EDCs in mice has shown that non-genomic ERα may prevent cortical bone loss post-ovariectomy [24] and reverse hepatic steatosis [25]. However, conclusions drawn from pharmacological studies in vivo to explore non-genomic ERα are limited by the fact that endogenous estrogen is present in non-ovariectomized animals and activates gene transcription.

Another method of studying non-genomic ERα action is the alteration of the receptor to create a mutant ERα that cannot localize to the membrane. Theoretically, any estrogenic effects seen in cells or animals with such mutation(s) are due to nuclear effects only, therefore loss of wild-type-associated phenotypes could be attributed to the loss of non-genomic action. Palmitoylation of cysteine 451 in the E-domain of ERα in mice (cysteine 447 in human ERα) causes the receptor to localize to the plasma membrane [26]. Taking advantage of this necessary modification, the C451A-ERα mutant mouse line was generated, in which C451A-ERα has an alanine instead of a cysteine at position 451 of ERα [27, 28]. Alanine cannot be palmitoylated, thus the C451A-ERα cannot bind to the plasma membrane. This was confirmed in primary hepatocytes [27]. C451A-ERα was used to show E2-dependent carotid artery reendothelialization and endothelial NO synthase activation did not occur when ERα could not associate with the plasma membrane [27]. In C451A-ERα mice, uterine response to a 28-day exposure to E2 was normal as was the endometrial endothelial proliferative response to 24-hour E2 exposure, however the ovaries were abnormal, with hemorrhagic and cystic follicles and no corpora lutea. Additionally, luteinizing hormone levels were significantly higher than normal [27].

The same point mutation in receptor position 451 was used by another group to create nuclear-only ERα mice (NOER), however these mice had differences in phenotype compared to C451A-ERα mice [28]. Pedram et al. observed that these mice had an abnormal uterine response to a 21-day E2 exposure [28]. These authors did not assess the acute response to E2, gene expression, or proliferation like Adlanmerini et al., making comparisons between the studies difficult. The contrasting phenotypes of these two mouse models, despite both models having the same mutation, might call into question the construction of the models. Indeed, hepatocytes in the C451A-ERα mouse showed a 55% reduction of membrane ERα [27], whereas in the NOER mouse, hepatocytes show no membrane ERα [28]. Pedram et al. postulated the incomplete reduction of membrane ERα to be the root of the inconsistent phenotypes of those mice [28]. While these nuclear-only ERα models are useful to study what happens when ERα cannot associate with the membrane, it is impossible to show the physiological function and signaling of membrane associated ERα directly. To complement such question, a membrane only ERα mouse model (MOER) is useful which was generated by Pedram et al. [18]. MOER mouse expresses a transgenic human ERα E-domain, which contains the palmitoylation site for localization to the plasma membrane, in an ERα knockout background. The uterus and vagina of MOER mice are atrophic, the ovaries have hemorrhagic cysts with no corpora luteum, mammary glands are underdeveloped, and there is increased visceral fat accumulation. All these effects are hallmark phenotypes of the ERα knockout mice [29]. E2 could activate ERK and PI3K in the liver cells isolated from MOER mice, in contrast to the liver cells isolated from ERα knockout mice. This mouse model, while effective in modeling effects of ERα at the membrane, is limited by the fact that only the E-domain of the receptor is present. Other domains of ERα that may play significant roles in protein interaction as part of cytoplasmic signaling are no longer present.

A more robust model was necessary to study the effects of non-genomic, non-nuclear ERα to account for its action in both the plasma membrane and the cytoplasm. The D-domain of ERα provided a novel opportunity to create a mutation excluding ERα from the nucleus. This domain is most commonly known as the hinge region because it is a flexible linker between the DBD and the LBD [30], but is also involved in tethered-mediated transcriptional regulation [16] and contains putative nuclear localization signals (NLS) [31]. It is also the site of several post-translational modifications including phosphorylation, acetylation, methylation, ubiquitination, and sumoylation [32–37].

Due to its NLS, the D-domain was targeted to prevent ERα localization to the nucleus. Earlier studies deleted this hinge domain and incorporated myristoylation and palmitoylation sequences to drive localization to the membrane [38]. This model demonstrated that nuclear ER genomic responses were lost but some rapid estrogenic effects were induced [38]. However, this approach may be problematic because the deletion of the D-domain loses the part of ERα protein surface. In a different approach, without deleting any functional domains, Burns et al. created the H2NES ERα mutant, which has point mutations of NLS and an incorporated nuclear export signal (NES) sequence in the D-domain [39]. In vitro studies of H2NES ERα demonstrate that it is not localized to the nucleus even in the presence of ligand, or only very transiently localized in the nucleus, allowing observation of

estrogenic effects mediated by membrane associated or cytoplasmic ERα, thus affirming that it is a useful model of non-nuclear ERα actions.

3. H2NES ERα

3.1. In Vitro Studies of H2NES

Burns et al. confirmed the putative nuclear localization sequences of ERα using the computational analysis tools LOCTree and Motif Scan. A bipartite NLS was observed in the D-domain. First, the H1 ERα mutant was created, in which the arginine and lysine residues in 267 to 275 amino acids of mouse ERα were mutated to alanine. Some H1 ERα was localized to the cytoplasm in the absence of E2 but largely visualized in the nucleus. In the presence of E2 all H1 ERα quickly translocated into the nucleus, thus its nucleophilic nature was only very weakly reduced. Of note, it also lacks the ability to bind to c-Jun, a transcription factor involved in tether-mediated ERα interaction with estrogen response elements [39]. H2 ERα mutant contains swapped alanines at the arginine and lysine residues in amino acid positions 260 to 275 of mouse ERα. H2 ERα was primarily localized to the cytoplasm in the absence of E2 and all H2 ERα quickly translocated into the nucleus with E2 in the same manner as H1 ERα. To completely exclude ERα from the nucleus, H2NES ERα was created, in which the nuclear export signal (NES; LXXXLXXLXL) was incorporated into the H2 mutant by mutating the residues at position 273 and 274 to leucines [39]. The H2NES ERα was non-nuclear even in the presence of E2, which was confirmed in both HeLa cells and Ishikawa ERα (-) cells using confocal microscopy imaging [39, 40]. When exposed to the leptomycin B, which is an inhibitor for nuclear export signaling, H2NES ERα was seen in the nucleus [39]. This result suggested that H2NES ERα does move into the nucleus but the NES causes it to be rapidly transported back into the cytoplasm. To determine if H2NES ERα could bind to DNA, an in vitro ERE binding assay was performed, which showed that H2NES ERα does bind to the perfect palindromic ERE DNA fragment similar to wild-type (WT) ERα [39]. Given that H2NES ERα appeared to still move into the nucleus and maintained DNA-binding ability, reporter assays were conducted to determine if H2NES ERα could activate ERE mediated gene expression. H2NES ERα activated the artificial 3X ERE fused reporter but not the reporter which fused with endogenous pS2 ERE sequence in H2NES ERα stably transfected Ishikawa cells [40]. Additionally, H2NES ERα failed to activate AP-1 reporter in the HeLa cells, indicating a reduction of genomic activity and substantiating that the D-domain possesses residues necessary for tethered ERα activities [39].

Microarray analysis was performed to evaluate endogenous gene expression in the H2NES ERα Ishikawa cells. Microarray data revealed no differences in gene expression in H2NES ERα Ishikawa cells at 4 or 24 hours post 10 nM E2 treatment compared to the ER negative parental Ishikawa cells. In contrast, WT and H1 ERα stably transfected Ishikawa cells showed changes in gene expression comparable to each other at the 4-hour time point suggesting they are possibly regulated by direct binding to an ERE because both WT and H1 ERα maintain nuclear localization and ERE-binding activity. The genes that were upregulated in WT and H1 ERα Ishikawa cells were not elevated in H2NES ERα Ishikawa cells.

H2NES ERα activated the artificial 3X ERE fused reporter but not the reporter fused with an endogenous pS2 ERE sequence. In addition, H2NES ERα stably transfected Ishikawa cells lack expression of endogenous estrogen responsive genes. The consensus ERE is a 13-base pair perfect palindromic inverted repeat with a 3-base pair spacing of variable bases [41]. A 3X ERE reporter is simply the consensus ERE sequence repeated three times and inserted into the reporter plasmid. On the other hand, in an endogenous ERE such as pS2, the ERE has an imperfect palindrome or a cluster of half sites of the palindrome sequence [42]. Transcriptional activity of ERα can be decreased due to the reduction of ERE binding affinity of ERα [43]. An additional consideration is that transient transfected reporter genes do not contain chromatin structure. With this in mind, it is possible that H2NES ERα may not be able to stay in the nucleus long enough to modulate chromatin structure necessary for gene stimulation and bind to the low affinity EREs.

To determine if rapid action responses were maintained by H2NES ERα, WT ERα and H2NES ERα transfected HeLa cells were cultured in serum depleted medium to decrease phospho-p44/42 MAPK level, and then treated for 0, 3, 5, and 10 minutes with 100 nM E2. In both the WT and H2NES ERα transfected HeLa cells, an early increase in phospho-p44/42 MAPK level was observed by 5 min, which suggested that H2NES ERα maintains rapid action responses in the cytoplasm [39].

When the E2-mediated cell proliferation was assessed, H2NES ERα Ishikawa cells did not show E2-dependent cell growth when exposed to 10 nM E2 or vehicle for five days. WT and H1 ERα Ishikawa cells did exhibit E2-dependent cell proliferation. The cell proliferation assay was also performed with a DBD mutant, AA ERα [44], stably transfected Ishikawa cells which exhibited no proliferation. These results suggest that the loss of cell proliferation of H2NES ERα mutant was due to an exclusion from the nucleus and reduction of chromosomal ERE binding.

From the in vitro assays performed by Burns et al., it was concluded that H2NES ERα mutant maintained estrogen dependent rapid action but lacked the ability to activate estrogen dependent endogenous gene expression despite its transient presence in the nucleus. These results suggested that H2NES ERα is a useful model for analyzing the physiological actions linked to non-genomic ERα action.

3.2. In Vivo Phenotypic Observations of H2NES

To assess physiological application of the non-genomic signaling we generated H2NES mutant mice and performed preliminary characterization experiments (unpublished observations). Female H2NES mice are infertile, having hypoplastic uteri and hyperemic ovaries that lack corpora lutea, similar to αERKO female mice [45]. Male H2NES mice are also infertile and show testicular atrophy, similar to αERKO male mice [46]. Loss of estrogenic action has been associated with development of the metabolic syndrome including obesity and insulin resistance [47], and reduced bone mineral density [48, 49]. Our preliminary observations suggest that the phenotypes of H2NES mice are similar to αERKO mice. However, H2NES mice should retain the non-genomic ERα mediated signal transduction, thus this mouse model will be useful in further investigation of various estrogen actions involving biological responses.

3.3. Possible Limitations of H2NES Studies

Despite H2NES ERα's applications to studying non-genomic estrogenic effects, there are some possible limitations of this model due to the mutations created in the H2NES. The D-domain is a site for many post-translational modifications, such as acetylation, sumoylation, ubiquitination, methylation and phosphorylation [32–37, 50] Acetylation of lysine residues in the D-domain is essential for ERα hormone sensitivity and ligand dependent and independent gene regulation function [34, 50]. The residues 266 and 268 lysines of human ERα (270 and 272 lysines of mouse ERα) are acetylated by p300 and this lysine acetylation modulates ligand dependent ERα gene regulation activity [50]. These residues are mutated to alanines in H2NES, which may correlate with the loss of nuclear function of H2NES ERα. The residues 251 to 305 of human ERα (255 to 309 in mouse ERα) are deemed sufficient for sumoylation events to occur on ERα, with sumoylation of lysine residues 266, 268, 299, 302 and 303 being especially important [37]. The mutation of sumoylation sites of ERα, including a mutant which has 266 and 268 lysines to arginines, prevented SUMO modification and impaired ERα-induced transcription without influencing ERα cellular localization [37]. It is possible that the sumoylation-mediated regulation of ERα is disrupted in H2NES ERα due to mutations at residues 266 and 268. Romancer et al. reported that 260 arginine of human ERα (264 arginine of mouse ERα) is methylated by PRMT1 [35]. This methylation event is required for mediating the extranuclear function of the receptor by triggering its interaction with the p85 subunit of PI3K and Src. The residue of 260 arginine (267 arginine on mouse ERα) is mutated to alanine in H2NES. This mutation might be a limitation of the H2NES mouse model because it may not promote such non-nuclear rapid action(s). Major phosphorylation events in the hinge region occurs at residues 305 (309 in mouse ERα) [32] and 294 (298 in mouse ERα) [33], which are not mutated in H2NES. Further analysis of post-translational phosphorylation of H2NES is necessary, though these functions may be normal in H2NES mice. Examining the repercussions of the possible lack of post-transcriptional modification contributing to normal ERα function will be needed in the future to further assess the credibility of the H2NES mouse model.

4. Future Studies and Conclusions

In vitro evidence suggests that H2NES ERα could be a suitable model for non-genomic estrogenic effects. Two previous studies assessing its activity support the conclusion that H2NES ERα lacks the ability to modulate endogenous gene expression but possesses rapid action without any truncation of the ERα protein. Its apparent inability to elicit a genomic estrogen response seems to be rooted in the strong NES signal incorporated into the NLS in the hinge region, ensuring that it is rapidly shuttled out of the nucleus. Recent observations of the preliminary animal study indicate that the phenotypes of H2NES mouse resemble αERKO mice, suggesting that the genomic function of ERα is indispensable. However, further studies are needed to assess tissue specific differences between H2NES mice and αERKO mice, to elucidate the ERα mediated non-genomic signaling in the tissues. H2NES mice may be an alternative new in vivo model to uncover non-genomic estrogen signaling mechanisms.

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References

1. Szego CM, Davis JS. Adenosine 3′,5′-monophosphate in rat uterus: acute elevation by estrogen. Proc Natl Acad Sci U S A. 1967; 58(4):1711–8. [PubMed: 4295833]
2. Pietras RJ, Szego CM. Endometrial cell calcium and oestrogen action. Nature. 1975; 253(5490): 357–9. [PubMed: 1167402]
3. Pietras RJ, Szego CM. Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells. Nature. 1977; 265(5589):69–72. [PubMed: 834244]
4. Le Mellay V, Grosse B, Lieberherr M. Phospholipase C beta and membrane action of calcitriol and estradiol. J Biol Chem. 1997; 272(18):11902–7. [PubMed: 9115251]
5. Razandi M, et al. Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERalpha and ERbeta expressed in Chinese hamster ovary cells. Mol Endocrinol. 1999; 13(2):307–19. [PubMed: 9973260]
6. Simoncini T, Genazzani AR. Non-genomic actions of sex steroid hormones. Eur J Endocrinol. 2003; 148(3):281–92. [PubMed: 12611608]
7. Pedram A, Razandi M, Levin ER. Nature of functional estrogen receptors at the plasma membrane. Mol Endocrinol. 2006; 20(9):1996–2009. [PubMed: 16645038]
8. Revankar CM, et al. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science. 2005; 307(5715):1625–30. [PubMed: 15705806]
9. Gros R, et al. GPR30 expression is required for the mineralocorticoid receptor-independent rapid vascular effects of aldosterone. Hypertension. 2011; 57(3):442–51. [PubMed: 21242460]
10. Langer G, et al. A critical review of fundamental controversies in the field of GPR30 research. 2010
11. Martensson UE, et al. Deletion of the G protein-coupled receptor 30 impairs glucose tolerance, reduces bone growth, increases blood pressure, and eliminates estradiol-stimulated insulin release in female mice. Endocrinology. 2009; 150(2):687–98. [PubMed: 18845638]
12. Isensee J, et al. Expression pattern of G protein-coupled receptor 30 in LacZ reporter mice. Endocrinology. 2009; 150(4):1722–30. [PubMed: 19095739]
13. Otto C, et al. GPR30 does not mediate estrogenic responses in reproductive organs in mice. Biol Reprod. 2009; 80(1):34–41. [PubMed: 18799753]
14. Mangelsdorf DJ, et al. The nuclear receptor superfamily: the second decade. Cell. 1995; 83(6): 835–9. [PubMed: 8521507]
15. Katzenellenbogen BS, et al. Molecular mechanisms of estrogen action: selective ligands and receptor pharmacology. J Steroid Biochem Mol Biol. 2000; 74(5):279–85. [PubMed: 11162936]
16. Kushner PJ, et al. Estrogen receptor pathways to AP-1. Journal of Steroid Biochemistry and Molecular Biology. 2000; 74(5):311–317. [PubMed: 11162939]
17. Madak-Erdogan Z, et al. Nuclear and extranuclear pathway inputs in the regulation of global gene expression by estrogen receptors. Mol Endocrinol. 2008; 22(9):2116–27. [PubMed: 18617595]
18. Pedram A, et al. Developmental phenotype of a membrane only estrogen receptor alpha (MOER) mouse. J Biol Chem. 2009; 284(6):3488–95. [PubMed: 19054762]
19. Aronica SM, Kraus WL, Katzenellenbogen BS. Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. Proc Natl Acad Sci U S A. 1994; 91(18):8517–21. [PubMed: 8078914]
20. Ke FC V, Ramirez D. Membrane mechanism mediates progesterone stimulatory effect on LHRH release from superfused rat hypothalami in vitro. Neuroendocrinology. 1987; 45(6):514–7. [PubMed: 3302745]
21. Stevis PE, et al. Differential effects of estradiol and estradiol-BSA conjugates. Endocrinology. 1999; 140(11):5455–8. [PubMed: 10537181]

22. Chambliss KL, et al. Non-nuclear estrogen receptor alpha signaling promotes cardiovascular protection but not uterine or breast cancer growth in mice. J Clin Invest. 2010; 120(7):2319–30. [PubMed: 20577047]
23. Harrington WR, et al. Estrogen dendrimer conjugates that preferentially activate extranuclear, nongenomic versus genomic pathways of estrogen action. Mol Endocrinol. 2006; 20(3):491–502. [PubMed: 16306086]
24. Bartell SM, et al. Non-nuclear-initiated actions of the estrogen receptor protect cortical bone mass. Mol Endocrinol. 2013; 27(4):649–56. [PubMed: 23443267]
25. Chambliss KL, et al. Nonnuclear Estrogen Receptor Activation Improves Hepatic Steatosis in Female Mice. Endocrinology. 2016; 157(10):3731–3741. [PubMed: 27552247]
26. Pedram A, et al. A conserved mechanism for steroid receptor translocation to the plasma membrane. J Biol Chem. 2007; 282(31):22278–88. [PubMed: 17535799]
27. Adlanmerini M, et al. Mutation of the palmitoylation site of estrogen receptor alpha in vivo reveals tissue-specific roles for membrane versus nuclear actions. Proc Natl Acad Sci U S A. 2014; 111(2):E283–90. [PubMed: 24371309]
28. Pedram A, et al. Membrane-localized estrogen receptor alpha is required for normal organ development and function. Dev Cell. 2014; 29(4):482–90. [PubMed: 24871949]
29. Couse JF, Korach KS. Contrasting phenotypes in reproductive tissues of female estrogen receptor null mice. Ann N Y Acad Sci. 2001; 948:1–8. [PubMed: 11795387]
30. Zwart W, et al. The hinge region of the human estrogen receptor determines functional synergy between AF-1 and AF-2 in the quantitative response to estradiol and tamoxifen. J Cell Sci. 2010; 123(Pt 8):1253–61. [PubMed: 20332105]
31. Ylikomi T, et al. Cooperation of proto-signals for nuclear accumulation of estrogen and progesterone receptors. EMBO J. 1992; 11(10):3681–94. [PubMed: 1327748]
32. Cui Y, et al. Phosphorylation of estrogen receptor alpha blocks its acetylation and regulates estrogen sensitivity. Cancer Res. 2004; 64(24):9199–208. [PubMed: 15604293]
33. Williams CC, et al. Identification of four novel phosphorylation sites in estrogen receptor alpha: impact on receptor-dependent gene expression and phosphorylation by protein kinase CK2. BMC Biochem. 2009; 10:36. [PubMed: 20043841]
34. Wang C, et al. Direct acetylation of the estrogen receptor alpha hinge region by p300 regulates transactivation and hormone sensitivity. J Biol Chem. 2001; 276(21):18375–83. [PubMed: 11279135]
35. Le Romancer M, et al. Cracking the estrogen receptor's posttranslational code in breast tumors. Endocr Rev. 2011; 32(5):597–622. [PubMed: 21680538]
36. Berry NB, Fan M, Nephew KP. Estrogen receptor-alpha hinge-region lysines 302 and 303 regulate receptor degradation by the proteasome. Mol Endocrinol. 2008; 22(7):1535–51. [PubMed: 18388150]
37. Sentis S, et al. Sumoylation of the estrogen receptor alpha hinge region regulates its transcriptional activity. Mol Endocrinol. 2005; 19(11):2671–84. [PubMed: 15961505]
38. Rai D, et al. Distinctive actions of membrane-targeted versus nuclear localized estrogen receptors in breast cancer cells. Mol Endocrinol. 2005; 19(6):1606–17. [PubMed: 15831524]
39. Burns KA, et al. Selective mutations in estrogen receptor alpha D-domain alters nuclear translocation and non-estrogen response element gene regulatory mechanisms. J Biol Chem. 2011; 286(14):12640–9. [PubMed: 21285458]
40. Burns KA, et al. Research resource: comparison of gene profiles from wild-type ERalpha and ERalpha hinge region mutants. Mol Endocrinol. 2014; 28(8):1352–61. [PubMed: 24947674]
41. Peale FV Jr, et al. Properties of a high-affinity DNA binding site for estrogen receptor. Proc Natl Acad Sci U S A. 1988; 85(4):1038–42. [PubMed: 3422476]
42. Berry M, Nunez AM, Chambon P. Estrogen-responsive element of the human pS2 gene is an imperfectly palindromic sequence. Proc Natl Acad Sci U S A. 1989; 86(4):1218–22. [PubMed: 2919170]
43. Klinge CM, et al. Estrogen response element sequence impacts the conformation and transcriptional activity of estrogen receptor alpha. Mol Cell Endocrinol. 2001; 174(1–2):151–66. [PubMed: 11306182]

44. Jakacka M, et al. Estrogen receptor binding to DNA is not required for its activity through the nonclassical AP1 pathway. J Biol Chem. 2001; 276(17):13615–21. [PubMed: 11278408]
45. Couse JF, et al. Tissue distribution and quantitative analysis of estrogen receptor-alpha (ERalpha) and estrogen receptor-beta (ERbeta) messenger ribonucleic acid in the wild-type and ERalpha-knockout mouse. Endocrinology. 1997; 138(11):4613–21. [PubMed: 9348186]
46. Hess RA, et al. A role for oestrogens in the male reproductive system. Nature. 1997; 390(6659): 509–12. [PubMed: 9393999]
47. Hart-Unger S, et al. Hormone signaling and fatty liver in females: analysis of estrogen receptor alpha mutant mice. Int J Obes (Lond). 2017; 41(6):945–954. [PubMed: 28220039]
48. Parikka V, et al. Estrogen responsiveness of bone formation in vitro and altered bone phenotype in aged estrogen receptor-alpha-deficient male and female mice. Eur J Endocrinol. 2005; 152(2):301–14. [PubMed: 15745940]
49. Lindsay R, et al. Long-term prevention of postmenopausal osteoporosis by oestrogen. Evidence for an increased bone mass after delayed onset of oestrogen treatment. Lancet. 1976; 1(7968):1038–41. [PubMed: 57448]
50. Kim MY, et al. Acetylation of estrogen receptor alpha by p300 at lysines 266 and 268 enhances the deoxyribonucleic acid binding and transactivation activities of the receptor. Mol Endocrinol. 2006; 20(7):1479–93. [PubMed: 16497729]