The F domain of estrogen receptor alpha is involved in species-specific, tamoxifen-mediated transactivation

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Running title: Species difference of ERα F domain functionality

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ABSTRACT

Estrogen receptor alpha (ERα) is a major transducer of estrogen-mediated physiological signals. ERα is a member of the nuclear receptor superfamily, which encompasses ligand-dependent transcription factors. The C terminus of nuclear receptors, termed the F domain, is the least homologous region among the members of this family. The ERα F domain possesses 45 amino acids; however, its function remains unclear. We noticed that the homology of the F domains between mouse and human ERαs is remarkably lower (75.6% similarity) than that between the entire proteins (94.7% similarity). To assess the functionality of the ERα F domains, here we generated chimeric ERα expression constructs with mouse-human exchanged F domains. Using cell-based in vitro assays, we analyzed the transcriptional, coactivator interaction, and ligand-binding domain dimerization activities of these mouse-human F domain-swapped ERαs. We found that the transcriptional activity of the mouse wild-type ERα is more potent than that of the human wild-type ERα in the human hepatoma cell line HepG2. 4-hydroxytamoxifen (4OHT)-mediated transcriptional activity of mouse-human F domain swapped ERαs was the inverse of the wild-type ERα activities but not estradiol-mediated transcriptional activities. Further experiments with constructs containing deletion or point mutations of a predicted β-strand region within the F domain suggested that this region governs the species-specific 4OHT-mediated transcriptional activity of ERα. We conclude that the ERα F domain has a species-specific function in 4OHT-mediated receptor transactivation and that mouse-human F domain-swapped ERα mutants enable key insights into ERα F domain structure and function.

Estrogen regulates multiple physiological functions in various tissues, including reproduction, bone density and metabolic regulation. Estrogen receptors (ERα and ERβ) are ligand dependent transcription regulators, which transmit estrogen signals to estrogen-mediated physiological functions (1). The phenotypes of ER null mutant mice have suggested that ERα plays a primary role in a number of estrogen mediated physiological responses compared to ERβ null mutant mice (2). Many of these phenotypes of
ERα activities are also observed in clinical cases of ERα insensitivity (3-5). Thus, ERα has been a major target for the development of therapeutic reagents, such as selective estrogen receptor modulators (SERM) and selective estrogen receptor degraders which control estrogen-mediated physiological responses.

ERα contains highly-conserved domain structures common among the nuclear receptor superfamily, designated A through F domains. The A/B domain, which contains transcription activation function 1 (AF-1), is localized on the N-terminus and the structure varies between the nuclear receptors. On the other hand, the C domain, also known as the DNA binding domain (DBD), and the E domain, also known as the ligand binding domain (LBD) are highly conserved between nuclear receptors. The E domain contains the ligand binding pocket and transcription activation function 2 (AF-2). The F domain is localized on the very end of the C-termini of some nuclear receptors and is the most variable domain between the family members. Even within orthologues of ERα, the homology of the F domain sequence is significantly lower than the other domains. For instance, the similarity between human and mouse ERα A/B domain, F domain and the whole protein are 90.6%, 75.6% and 94.7% respectively (Table 1).

Concerns have arisen over differential pharmacokinetics of estrogenic compounds across different species, which are relevant to the findings of differential activities of estrogenic compounds seen in experimental animal studies (6). Due to the higher homology of ERα protein among species, it has been thought that the differential metabolism of chemicals may be the cause of differential estrogenic activities rather than species differences of ERα structure. Indeed, the structural difference of ERα between animals has also been considered as a contributing factor to the differential estrogenic activities. However, there is limited evidence for differential functionality of ERα among different animal species (7). In addition, it is totally unknown whether the F domain, which is the least homologous region among the species, is involved in the differential functionality of ERα. The function of human ERα F domain has been analyzed by several groups using different experimental models (8-12). These reports have suggested that the human ERα F domain is needed for the partial agonist activity of 4-hydroxytamoxifen (4OHT) and the efficacy of estradiol (E2)-mediated transcription activity of ERα. However, the mechanism of the precise F domain functionality is still unclear.

The differential estrogenic activity of tamoxifen between human and mouse has been discussed. However, there are still no informative conclusions. We evaluated the transcription activities of human and mouse ERα using a cell based in vitro reporter assay and found differential transcription activities under equivalent conditions in a human hepatoma cell line. To confirm F domain’s contribution to this differential activity, we generated chimeric mouse-human F domain swapped ERα expression plasmids. Analyses suggested that the F domain governs the species difference of ERα activity and a specific region of F domain contributes to the differential functionality for E2 and 4OHT mediated transcription. Our strategy of exchanging the mouse-human F domains of ERα presents new insight into evaluating ERα F domain functionality and potentially for explaining differential estrogenic chemical activities among species.

**RESULTS**

**Differential transcription activity between mouse and human ERα in the human hepatoma cell line** - At first, we analyzed the estrogen responsive element (ERE)-dependent transcription activity of mouse ERα (mERα) and human ERα (hERα) using ERE-fused luciferase (luc) reporters (3xERE-TATA-luc and C3-110tk-luc). We chose both a mouse and a human cell line to account for both species, mouse fibroblast cell line (Balb3T3A31) and human hepatoma cell line (HepG2) were transfected with the reporter plasmid and the receptor expression plasmid (pcDNA3-mERα or pcDNA3-hERα) or the empty plasmid (pcDNA3). Transactivation levels were expressed as relative activity compared to the level of pcDNA3. The basal activity of mERα without
ligands was higher than hERα in the Balb3T3A31 cells but there was no difference in the maximum level of E2-mediated transactivation (Fig. 1A). In the Balb3T3A31 cells, the activity of mERα was blocked by 4OHT. On the other hand, the activity of hERα was not stimulated or blocked by 4OHT (Fig. 1B). In the HepG2 cells, E2 (Fig. 1C and 1E) and 4OHT (Fig. 1D and 1F) mediated transcription activity of mERα was significantly higher than hERα. Although the transcription activities of mERα and hERα were different in the HepG2 cells, there was no difference in the receptor protein expression levels (Fig. 1G).

**AF-1 activity was similar between mouse and human ERα in the HepG2 cells** - To understand the contribution of transactivation function domains (AF-1 and AF-2) in differential activity of mERα and hERα in the HepG2 cells, we analyzed AF-1 activities using the AF-2 truncated ERα mutants (mERα339 and hERα340) (Fig. 2A and 2B), which exhibits only a constitutive AF-1 mediated transactivation activity (13, 14). There was no difference between mERα339 and hERα340 in ERE-luc reporter activation function (Fig. 2C and 2D), suggesting that the AF-1 activity of mouse and human ERα is indistinguishable in the HepG2 cells.

**AF-2 activity was different between mouse and human ERα in the HepG2 cells** - Next, we evaluated the AF-2 activity of mouse and human ERα using the G5-luc reporter and Gal4DBD-fused ERα LBDs (pBIND-mEF, pBIND-hEF, pBIND-mΔEF and pBIND-hΔEF). As shown in Fig. 3A, E2-dependent transactivation function of mERα AF-2 (pBIND-mEF) was higher than hERα AF-2 (pBIND-hEF). The transcription activity was reduced by F domain truncation (pBIND-mΔEF and pBIND-hΔEF). Furthermore, we analyzed the ligand dependent coactivator (NCOA) recruitment activity to the AF-2 using the mammalian two-hybrid assay. The expression plasmids for Gal4DBD-fused nuclear receptors interaction domain (RID) of NCOA1, 2 or 3 (pM-SRC1-NR, pM-GRIP1-NR or pM-ACTR-NR) and the expression plasmids for VP16AD fused-ERα LBDs (pACT-mEF, pACT-hEF, pACT-mΔEF or pACT-hΔEF) were cotransfected with G5-luc reporter in the HepG2 cells. As shown in Fig. 3B, E2-dependent NCOA recruitment activity of mERα was higher than hERα. Furthermore, F domain truncation (pACT-mΔEF or pACT-hΔEF) reduced the activity of NCOA recruitment. These results were consistent with the E2-dependent activation levels of mouse and human ERα AF-2 (Fig. 3A). Such findings suggest that the E2-mediated differential activities of mERα and hERα correlate with the differential AF-2 activities of mouse and human ERα in HepG2 cells.

**F domain contributes to the differential transcription activity of mouse and human ERα** - The results of the F domain truncated mutant suggested that the F domain is highly associated with the AF-2 activity of mERα and hERα. To assess the role of the F domain in AF-2 activity, we generated ERα mutants which contain mouse and human exchanged F domains (Fig. 4A and 4B). HepG2 cells were transfected with an ERE-luc reporter plasmid and the expression plasmid for the F domain swapped receptor (mERα-hF, hERα-mF) or the control receptor (mERα-mF, hERα-hF). The E2-mediated activity of F domain swapped receptors showed in-between levels compare to the levels of control receptors (Fig. 4C and 4E). In response to 4OHT activities of mERα and hERα were totally inverted by F domain swapping (Fig. 4D and 4F). These results suggest that the F domain influences the differential activities of mERα and hERα to 4OHT.

**F domain is involved in the E2-dependent coactivator recruitment to the LBD** - We assessed the ligand dependent AF-2 activity of F domain swapped ERα using a G5-luc reporter and Gal4DBD-fused LBDs. The E2-dependent AF-2 activity of mERα-hF (pBIND-mhEF) was lower than the mERα-mF (pBIND-mhEmF). In contrast, the AF-2 activity of hERα-mF (pBIND-hmEF) was higher than the hERα-hF (pBIND-hhEF) (Fig. 5A, left panel). 4OHT did not activate G5-luc reporter through any Gal4DBD-fused LBDs (Fig. 5A, right panel). Furthermore, E2-dependent NCOA recruitment activity of mERα-hF LBD (pACT-mhEF) was lower than the mERα-mF LBD (pACT-mhEmF). In contrast, E2-dependent NCOA recruitment activity of hERα-mF LBD (pACT-hmEF) was higher than the hERα-hF LBD (pACT-hhEF) (Fig. 5B). These results clearly
suggest that the F domain modulates the E2-dependent coactivator interacting surface of ERα LBD.

**F domain is involved in the 4OHT-dependent LBD dimerization activity** - Because we have reported previously that the efficacy of LBD dimerization associates with the partial agonist activity of 4OHT (15), 4OHT-dependent LBD dimerization activity was analyzed by using the mammalian two-hybrid assay. HepG2 cells were cotransfected with the G5-luc reporter and plasmids for expressing Gal4DBD-fused mEmF, mEhF, hEhF or hEmF (pBIND-EF), in the presence of plasmids for expressing VP16AD alone (pACT) or VP16AD-fused mEmF, mEhF, hEhF or hEmF (pACT-EF). Cells were treated with 0.1-10 nM 4OHT or vehicle. Since the 4OHT-mediated dimerization activity of mErα LBD was significantly higher than hErα LBD, the results are shown separately for mErα and hErα (Fig. 6A and 6B). 4OHT-mediated dimerization activity of the mErα LBD (mEmF) was significantly reduced by the swapping to the human F domain (mEhF) (Fig. 6A). In contrast, the lower 4OHT-mediated dimerization activity of hErα LBD (hEhF) was increased by the swapping and incorporation of the mouse F domain (hEmF) (Fig. 6B). These results suggest that the 4OHT-mediated dimerization activities of mouse and human Erα LBD are governed by the F domain. These results coincide with the 4OHT-mediated ERE-dependent transcription activity of F domain swapped Erα mutants (Fig. 4D and 4F).

**Specific region of F domain potentiates 4OHT-mediated mouse Erα activity** - The function of possible structural regions of human Erα F domain have been reported, including a predictive β-strand in the C-terminal of the F domain, however actual conformation is still uncertain (10, 11). We analyzed the transcription activity of several Erα mutants focused on the element near a suggested β-strand in the F domain to confirm the functional relevance of this region. Three amino acids (tyrosine-tyrosine-isoleucine, YYI) were deleted from the F domain to disrupt the predicted β-strand structure (mErαΔYYI and hErαΔYYI) (Fig. 7A). The expression level of mutant receptor protein was lower than WT in both species (Fig. 7B). Although the protein level was lower than WT, mouse and human ErαΔYYI exhibited 4OHT-mediated transcription activity similar to WT (Fig. 7C). Interestingly, 4OHT-mediated transcription activity of mErαΔYYI was significantly decreased but not hErαΔYYI (Fig. 7D). Furthermore, we generated a mutant hErα protein expression plasmid which replaced the three amino acids on the flanking of YYI to the mouse corresponding residues. Namely, the lysine (K581) on the N-terminal flanking of YYI sequence was replaced to threonine (T) and the threonine-glycine (T585, G586) residues on the C-terminal flanking of YYI sequence was replaced to prolines (PP) (hErαK581T,T585P,G586P; denoted as hErαTyyiPP) (Fig. 7E). The 4OHT-mediated transcription activity of hErαTyyiPP was identical to hErα-hF (Fig. 7F). On the other hand, 4OHT-mediated transcription activity of hErαTyyiPP was significantly higher than hErα-hF and similar to mErα-mF (Fig. 7G). Furthermore, we analyzed the LBD dimerization activity of K581T,T585P,G586P mutated human Erα (hEFTyyiPP) using the mammalian two-hybrid assay. 4OHT-mediated dimerization activity of hEFTyyiPP was significantly higher than WT human Erα LBD (hEFTyyiPP) (Fig. 7H). These results suggest that the mouse specific residues around the predictive β-strand in the F domain are involved in the differential activities of 4OHT-mediated mErα and hErα transcriptional regulation.

**Mouse Erα is more potent than human Erα for estrogen responsive gene activation** - Lastly, we examined the efficacy of E2- or 4OHT-mediated endogenous gene activation through mouse and human Erα using mErα or hErα transfected HeLa cells (Fig. 8). Total RNA was extracted from the cells which were treated with 10 nM E2 or 4OHT for 24 h. As examples, the expression levels of SERPINB9 (P19) and TFF1 (P52) genes were analyzed. These genes are known estrogen responsive genes in several cell lines (16-19). The mRNA level of Erα regulated genes was normalized by the transcript of neomycin resistant gene (Neo), which is expressed from the pcDNA3 plasmid for normalizing the transfection efficiency. P19 and P52 genes were
activated by E2 in an ERα dependent manner (Fig. 8A and 8C). E2-induced mRNA levels of both genes were slightly higher in the mERα transfected cells than hERα transfected cells. PI9 gene was activated by 4OHT in an ERα dependent manner (Fig. 8B) but pS2 gene was not activated by 4OHT (Fig. 8D). Importantly, the 4OHT-induced PI9 mRNA level was significantly higher in the mERα transfected cells than hERα transfected cells (Fig. 8B), which is consistent with the in vitro observations in this report.

DISCUSSION

The differential estrogenic activity of tamoxifen in experimental animals has been reported (6). These findings, led to a question of the differential estrogenic activity of tamoxifen between the human and mouse species. However, the conclusion to explain these differential activities is still unclear. In particular, there is no finding that the structural difference between human and mouse ERα with the differential estrogenic activity of tamoxifen. The high homology between human and mouse ERα (94.7% similarity) has led to the thought that the function of these receptors is identical. However, we found that the efficacy of transcription activity of mERα is more potent than hERα. Specifically, the results of F domain swapped ERα mutants suggested that the F domain contributes to the tamoxifen-mediated differential transcriptional activity of mERα and hERα.

Our results suggested that the N-terminal transactivation function (AF-1) is the primary element for the 4OHT-dependent transcription activity of full-length ERα, following the previous reports (13, 14) and that the F domain plays a role in this regulation. We have previously reported that the SERM mediated partial agonist activity of ERα is correlated with the efficacy of LBD (EF domain) dimerization cooperating with ERE binding (15). As we demonstrate in this report, 4OHT-dependent dimerization of mouse ERα LBD is more potent than human ERα LBD, which is consistent with the 4OHT-dependent full-length ERα transcription activities. The species difference of 4OHT-mediated LBD dimerization efficiency was totally changed by the F domain swapping and that level reflects the full-length mutant ERα transcription activities. These results clearly suggest that the F domain structure is contributing to an important role in 4OHT-dependent LBD dimerization and partial agonist activity of ERα.

As we showed here, the PI9 gene was activated by 4OHT in the ERα-transfected HeLa cells but not pS2. This observation agrees with the promoter context dependent tamoxifen activity as previously reported (13). 4OHT-dependent PI9 gene expression was higher in the mERα than hERα transfected cells, suggesting that the agonist activity of 4OHT through mERα was more potent than hERα in the same promoter context. 4OHT activity via mERα was higher than hERα with each of the different promoters that we analyzed (3xERE-TATA, C3-110tk and SERPINB9). This may suggest that the species differences of 4OHT-mediated ERα activity is independent of the promoter context. In other words, structural differences between human and mouse ERα could cause the species differences of agonist activity of 4OHT.

F domain replacement did not affect E2-mediated full-length ERα transactivation strikingly, which was different from 4OHT-mediated regulation. However, the efficacy of E2-mediated NCOA interaction to the AF-2 was reversed by F domain swapping, and it was consistent with the E2-dependent AF-2 activity of F domain swapped ERα mutants. These results suggest that the F domain contributes to the modulation of the E2-dependent coactivator interacting surface of LBD, but it provides a limited contribution to the E2-dependent full-length ERα transactivation. The differential activities of F domain swapped ERα on the C3-110tk and 3xERE-TATA reporters imply that the E2-activated full-length ERα is functioning in a different manner on each responsive element. To verify this hypothesis, we performed further analysis using the reporters which contain 1, 2 or 3 consensuses ERE motifs fused with a thymidine kinase (tk) promoter (Fig. 9). E2-mediated full-length mouse ERα transcription activity was higher than human ERα for those reporters in
HepG2 cells. Interestingly, the profile of transcription activities of F domain swapped ERα mutants for 1xERE-tk reporter was similar to the profile of C3-110tk reporter. On the other hand, the profiles of 2xERE-tk and 3xERE-tk reporters were similar to 3xERE-TATA-luc. Importantly, sole activity of AF-1 and AF-2 was not observed on the 1xERE-tk reporter different from 2xERE-tk and 3xERE-tk reporters. These results suggested that the monomeric or multimeric binding of E2-mediated ERα dimer on the ERE regulates transcription activity in a different manner. Namely, the AF-2 (transactivation function of EF domain) works cooperatively with AF-1 when ERα is bound on a single ERE. On the other hand, AF-2 and AF-1 work additively expressing E2-mediated transcription activity when ERα is bound on multiple EREs. The characteristics of the F domain may be emphasized when ERα dimer is monomerically bound on the ERE.

Schwartz et al. have suggested that the residues of QKYYIT (hERα 580 to 585) in the human ERα F domain contribute to a possible β-strand structure (10). However, the function of this specific region is not well known. The computational prediction analysis for the protein secondary structure suggested that a β-strand is likely to exist in this region of mouse ERα F domain similar to human ERα. The results of the YYI sequence deleted mutant ERα suggested that this region is strongly correlated with the species difference of 4OHT-mediated ERα transcription activity. We recognized that the flanking amino acids of YYI are varied between species; the lysine (K), which is a 5’-flanking residue, is highly conserved among primates but most other mammals have threonine (T). On the other hand, the threonine (T), which is a 3’-flanking residue, is conserved among mammals except rodents; rodents have proline (P) instead of T. Further analyses using a human ERα mutant with the three-point mutation swapped to the mouse ERα residues in the predicted β-strand region (hERαTytyiPP) suggested that the characteristics of the amino acids flanking the YYI residues are important for the species-specific 4OHT-mediated transactivation function of ERα. Our strategy of exchanging the mouse-human F domain sequence may be useful to examine the structural features of the ERα F domain, since there is no published crystallographic structure of the F domain. Even though our study demonstrates a functional difference.

Previous reports using the yeast two-hybrid assay have suggested that the F domain prevents the E2-mediated SRC1 (NCOA1) RID interaction to the human ERα LBD (11, 12). Those results are inconsistent with the transcription activity of F domain truncated ERα in mammalian cells (8, 11). We showed that the truncation of F domain clearly reduced E2-mediated NCOA RID interaction and AF-2 activity of mouse and human ERα in HepG2 cells. Our results suggested that the F domain facilitates the E2-mediated NCOA interaction to the AF-2 surface rather than preventing interaction. The contradiction between our results and previous reports which used a yeast system suggests that the function of F domain is cell type dependent. We showed here the differential activity of hERα and mERα in HepG2 cells (human hepatoma cell). We observed the same trends of ERα dependent gene regulation in a human cervical cancer cell line, HeLa cells. Thus far, only the mouse fibroblast cell line, Balb3T3A31 cells showed different results in our analyzed cell types. These results imply that species-specific factor(s) may exist for modulating the F domain function.

In summary, we revealed that the species-specific amino acids of the predicted β-strand region on the C-terminal of ERα F domain govern the species dependent tamoxifen mediated transcription activity. Our results also suggest that considerations should be made when analyzing SERM activity in mouse versus human samples because of the existence of differential F domain functionality between human and mouse ERα.

**EXPERIMENTAL PROCEDURES**

**Plasmid constructions** - The following plasmids were used for WT and C-terminal truncated ERα protein expression, pcDNA3-mERα, the plasmid contains full-length mouse ERα (mERα1-599); pcDNA3-mERα339, the plasmid contains 1 to 339 amino acids (AA) of
mouse ERα with extension of 10 extra AA (GPYSIVSPKC) in C-terminus derived from pcDNA3 sequence. pcDNA3-hERα, the plasmid contains full-length human ERα (hERα1-595); pcDNA3-hERα340, the plasmid contains 1 to 340 AA of human ERα with extension of 7 extra AA (CMPAGRI) in C-terminus derived from pcDNA3 sequence. The following plasmids were used for N-terminal truncated ERα protein expression, pcDNA3-121-mERα, the plasmid contains 121 to 599 AA of mouse ERα with extra MTM sequence in N-terminus; pcDNA3-117-mERα, the plasmid contains 117 to 595 AA of human ERα with extra MTM sequence in N-terminus. To generate human-mouse F domain swapped ERα expression plasmids, the control plasmids (mouse and human ERα expression plasmids which have an extra BamHI site between E and F domain) were created by PCR based site-directed mutagenesis and the following oligo DNAs were used for the mutagenesis, hERα_1657BamHI_S: 5' -CCG CCT ACA TGC GCC CGG ATC CCG TGG AGG GGC ATC C-3' and hERα_1657BamHI_AS: 5'-GGG TGC TCC CCC AGC GGA TCC GGG CGC ATG TAG GCG G-3' for human ERα, mERα_1669BamHI_S: 5'-CCG CCT TCA TGC CCC AGG ATC CCG CAT GGG AGT GCC CCC-3' and mERα_1669BamHI_AS: 5'-GGG GGC ACT CCC ATG CGG GAT CCT GGG GCA TGA AGG CGG-3' for mouse ERα. The reaction mixture contains the Pfu Turbo DNA polymerase (Agilent Technologies), a pair of sense (S) and anti-sense (AS) oligo DNAs, and the plasmid pGEM3zf-hERα_Xbal (the Xbal fragment from pcDNA3-hERα was subcloned into the Xbal site of plasmid). The XhoI fragment from pcDNA3-mERα was subcloned into the XhoI site of pcDNA3-mERα then generated pcDNA3-mERα1669BamHI (pcDNA3-mERα-mF); the direction of the inserted fragment was determined by NotI digestion. The pcDNA3-mERα-mF plasmid expresses A557G mutant mERα and the pcDNA3-hERα-hf plasmid expresses T553G mutant hERα. The function of mERα-mF and hERα-hf for the ligand-dependent transcription activity was identical to mERαWT and hERαWT respectively (Fig. S1). To generate pcDNA3-mERα-hF and pcDNA3-hERα-mF plasmids, the BamHI fragments from pcDNA3-mERα-mF and pcDNA3-hERα-hf were subcloned into the BamHI site of pcDNA3-hERα and pcDNA3-mERα respectively. The direction of the inserted fragment was determined by NotI digestion. The plasmids pcDNA3-mERαΔYYI and pcDNA3-hERαΔYYI were created by PCR based site-directed mutagenesis and the following oligo DNAs were used for the mutagenesis, hERα_Del_YYI_S: 5' -CAT CGG ATT CCT TGC AAA AGA CGG GGG AGG CAG-3' and hERα_Del_YYI_AS: 5'-CTG CCT CCC CCG TCT TTT GCA AGG AAT CGG ATG-3' for human ERα, del586-588_S: 5'-ACA TTC CTT ACA AAC CCC CCC GGA AGC AGA GG-3' and del586-588_AS: 5'-CTG CCT CCC CCG GGG GGG TTT GTA AGG AAT GT-3' for mouse ERα. pGEM3zf-hERα_Xbal and pBluescript-mERαWT_XhoI were used for a template respectively. The XhoI fragment from pGEM3zf-hERαΔYYI was subcloned into the XhoI site of pcDNA3-hERα then generated pcDNA3-hERαΔYYI; the XhoI fragment from pBluescript-mERαΔYYI was subcloned into the XhoI site of pcDNA3-mERα then generated pcDNA3-mERαΔYYI. The expression plasmid for hERαK581T,T585P,G586P (pcDNA3-hERαTyiiiPP) was created by PCR based site-directed mutagenesis. The following oligo DNAs were used for the first mutagenesis, hERα_T585P,G586P_S: 5'-GAA ACC CTC TGC TGC CTC CGG CGG GAT GTA ATA CTT TTT CAA GGA A-3' and hERα_T585P,G586P_AS: 5'-TTC CTT GCA AAA GTA TTA CAT CCC GCC GGA AGA GGG TTT C-3'. pGEM3zf-hERα_XbaI was used for a template to generate pGEM3zf-hERαPP_XbaI. The following oligo DNAs were
used for the second mutagenesis, hERa-PP_K581T_AS: 5′-CCG GCG GGA TGT AAT ACG TTT GCA AGG AAT GCG AT-3′ and hERa-PP_K581T_S: 5′-ATC GCA TTT CTT GCA AAC GTA TTA CAT CCC GCC GG-3′. pGEM3zf-hERaPP_Xbal was used for a template. The Xbal fragment from pGEM3zf-hERaTyyiPP_Xbal was subcloned into the Xbal site of pcDNA3-hERa then generated pcDNA3-hERaTyyiPP. The pGL3-Basic-TATA-Int-Luc reporter plasmid (3xERE-TATA-luc) (15) and the TK-C3ER1+2 reporter plasmid (C3-110tk-luc) containing the luciferase reporter gene fused with the tk promoter and the minimum estrogen responsive element (117 bp) of human compliment 3 (C3) gene (20) were used for reporter assay. The reporter plasmids 1xERE-TK-luc, 2xERE-TK-luc and 3xERE-TK-luc were used for reporter assay in Fig. 9 (gift from Dr. McDonnell, Duke). The plasmid pRL-TK renilla luciferase expression plasmid (Promega) was used for internal control. Plasmids used for mammalian two-hybrid assay are as follows, the plasmids pM-SRC1-NR, which contains 621 to 766 AA of human NCOA1, pMGrip1-NR, which contains 629 to 761 AA of mouse NCOA2, pM-ACTR-NR, which contains 611 to 784 AA of human NCOA3 (gift from Dr. McDonnell, Duke) (21), pBIND-mEF, pBIND-mEmF (15), pBIND-hEF, pBIND-hEmF, pBIND-hEΔF, pBIND-mEmF, pBIND-mhEf, pBIND-mhEf, pBIND-hmEmF, pBIND-hEΔF-TyyiPP and pBIND (Promega) were used for the bait; the plasmids pACT-mEF, pACT-mEΔF (15), pACT-hEF, pACT-hEΔF, pACT-hEmF, pACT-mEmF, pACT-hEΔF, pACT-hEΔF, pACT-mEΔF, pACT-hEf, pACT-hEf, pACT-mEf, pACT-hEf, pACT-hEf, pACT-EF-TyyiPP and pACT (Promega) were used for the prey; the plasmid pG5-Luc (Promega) was used for GAL4 binding element fused reporter gene. The ERα EF domain fused pACT and pBIND plasmids were generated as follows, the template DNAs pcDNA3-hERa-hF, pcDNA3-hERa-TyyiPP, pcDNA3-hERa-mF and pcDNA3-mera-mF were amplified by PCR using the following primer sets, hERa-LBD5′_Sall: 5′-GTG GCC GCT TCG CCT GCC TGG CCT TGT AAT ACG TTT GCA AGG AAT GCG AT-3′ and hERa-LBD3′_NotI: 5′-GGC GCC GCT TCG CCT GCC TGG CCT TGT AAT ACG TTT GCA AGG AAT GCG AT-3′ (hEf and hEF-TyyiPP), primers hERa-LBD5′_Sall and mE/F-3′_Kpnl: 5′-GGT ACC TGG GAG CTC TCA GAT CGT GTT GGG-3′ (hEmF), primers mE/F-5′_BamHI: 5′-GGG TCA AGC ACA CTG GCC AAG TCG AGA AGA ACA GCC TGG CCT TGT AAT ACG TTT GCA AGG AAT GCG AT-3′ and hERa-PP_K581T_S: 5′-CCG GCG GGA TGT AAT ACG TTT GCA AGG AAT GCG AT-3′ and hERa-PP_K581T_AS: 5′-ATC GCA TTT CTT GCA AAC GTA TTA CAT CCC GCC GG-3′. The amplified fragment was cloned into pCR2.1 by TA-cloning kit and sequenced. The inserted fragment was excised by Sall and NotI (hEfH, hEΔF and hEF-TyyiPP) or Sall and Kpnl (hEmF) then subcloned into the Sall and NotI or Sall and Kpnl sites of pACT or pBIND vectors. To generate the plasmid pACT-mEmF, pACT-mEf, pBIND-mEmF and pBIND-mEf, the BamHI fragment was excised from the pCR2.1-mEmF then subcloned into the BamHI site of pACT-hEf, pACT-hEf, pACT-hEf, pACT-hEf, pACT-hEmF and pBIND-hEmF plasmids. The direction of the inserted fragment was determined by NdeI digestion.

Cell culture and transfection condition for luciferase assay - HepG2 cells (human hepatocellular carcinoma) were cultured in phenol red-free α-MEM (Invitrogen) supplemented with 10% FBS (Gemini-Bio) and 1% penicillin-streptomycin solution (Sigma-Aldrich). Balb3T3A31 cells (mouse fibroblast) were cultured in phenol red-free α-MEM supplemented with 10% FBS, 2 mM sodium pyruvate (Sigma-Aldrich) and 1% penicillin-streptomycin solution. For transient transfections, the cells were cultured in phenol red-free medium supplemented with 10% charcoal-stripped FBS (Gemini-Bio) and seeded in 24-well plates at a density of 1.2x10⁵ cells/well (HepG2) and 0.8x10⁵ cells/well (Balb3T3A31). The cells were transfected with the following DNA mixture for 6 h using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. For reporter assay, a DNA mixture containing 50 ng of expression plasmids for WT or mutated ERα, 100 ng of reporter plasmids for 3xERE-TATA-luc or C3-110tk-luc, and 100 ng pRL-TK was transfected in each well. For assessment of AF-2 activity, the
DNA mixture contained 50 ng expression plasmids for GAL4 DBD fusion proteins (pBIND) and 100 ng pG5-Luc reporter plasmid was transfected in each well. pBIND plasmid contains renilla luciferase expression unit for transfection normalization. For assessment of LBD dimerization, the DNA mixture contained 50 ng expression plasmids for GAL4DBD fusion proteins (pBIND), 50 ng expression plasmids for VP16 activation domain (AD) fusion proteins (pACT) and 100 ng pG5-Luc reporter plasmid was transfected in each well. For assessment of coactivator interaction, the DNA mixture contained 100 ng expression plasmids for GAL4DBD fused NCOA NR-box (pM), 50 ng expression plasmids for VP16AD fusion proteins (pACT), 50 ng pRL-TK and 100 ng pG5-Luc reporter plasmid was transfected in each well.

Luciferase assay - The cells were cultured in fresh medium supplemented with the ethanol as a vehicle, E2 (Steraloids) or 4OHT (Sigma Aldrich) 6 h after transfections. Luciferase and renilla luciferase activities were assayed 18 h after treatments using Dual-luciferase Reporter Assay System (Promega). Luciferase activity was normalized for transfection efficiency using renilla luciferase as an internal control. All results are representative of at least two independent experiments and represent the mean ± S.D. of triplicate samples.

Western blot assay - The transfected cells on 24-well plate were washed with PBS and 100 µl of 2x Laemmli sample buffer (Bio-Rad) near 100°C was added to the wells. The cells were pipetted vigorously and then into a 1.5-ml centrifuge tube. The tubes were heated at 100°C for 10 min, cooled on ice, and stored at −80°C until samples were analyzed on SDS-PAGE. Proteins were resolved by SDS-PAGE with 4-12% gradient gel (Invitrogen) and subsequently transferred to nitrocellulose membrane. Blots were incubated overnight in 4°C with primary antibody for ERα (1:600; H-184, Santa Cruz Biotechnology) or β-actin (1:2000; AC-74, Sigma). The blots were washed then incubated with IRDye 800CW-conjugated anti-rabbit antibody (LI-COR Biosciences) for ERα or with IRDye 680RD-conjugated anti-mouse antibody (LI-COR Bioscience) for β-actin. The signals were visualized by Odyssey infrared imaging system (LI-COR Biosciences). BenchMark protein ladder (Invitrogen) was used for evaluating the molecular weight.

Condition for examining the ERα dependent endogenous gene expression - HeLa cells (human cervical cancer cell) were cultured in phenol red free α-MEM supplemented with 10% FBS, 2 mM sodium pyruvate and 1% penicillin-streptomycin solution. When the cells were transfected with DNAs, the cells were cultured in phenol red-free medium supplemented with 10% charcoal-stripped FBS and seeded in 6-well plates at a density of 4.5x10^5 cells/well. The cells were transfected with the 2 µg of pcDNA3-mERα, pcDNA3-hERα or pcDNA3 for 6 h using Lipofectamine 2000. The cells were cultured with fresh phenol red-free medium supplemented with 10% charcoal-stripped FBS and ligand for 24 h. The cells were homogenized with TRIzol reagent (Invitrogen) for extracting RNA. Total RNA was treated with TURBO DNase (Invitrogen) then reverse-transcribed by Superscript II (Invitrogen) to generate the cDNA with the manufacturer’s instruction. The quantitative PCR was perfomed by using the Fast CYBR Green Master Mix (Applied Biosystems) with cDNA and the gene specific forward and reverse primers. The sequence of primers is listed below, hSERPINB9_F: 5’-TCT TTG GAG AGA AAA CTT GTC AGT-3’, hSERPINB9_R: 5’-AAC AAC TAC TCT TCA ATT TTA CCT TCG G-3’, pS2_F: 5’-CCC GTG GAA AGA CAG AAT TGT-3’, pS2_R: 5’-GTC TAA AAT TCA CAC TCC TCT TCT GG-3’, h36B4_F: 5’-GGA CAT GTT GCT GGC CAA TAA-3’, h36B4_R: 5’-GGG CCC GAG ACC AGT GTT-3’, Neo_5’: 5’-GGC TAT GAC TGG GCA CAA CAG ACA ATC-3’ and Neo_3’: 5’-TAC TTT CTC GGC AGG AGC AAG GTG AG-3’. The Ct value was calculated by a mathematical model to quantify the relative mRNA level (22). The level of Neo mRNA, which is expressed from the pcDNA3 plasmid, was analyzed for normalizing the transfection efficiency. The data was obtained from three independent experiments.
Statistical analysis - ANOVA with Tukey’s multiple comparison test was performed by GraphPad Prism (GraphPad software). p < 0.05 was considered statistically significant.

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Conflict of interest: The authors declare that they have no conflict of interest with the content of this article.

Author contributions: YA conceived the idea, conducted the experiments, analyzed the results, and wrote the paper. KSK managed the project and wrote the paper.

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**FOOTNOTES**

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The abbreviations used are: ER, estrogen receptor; SERM, selected estrogen receptor modulator; AF, transactivation function; DBD, DNA binding domain; LBD, ligand binding domain; 4OHT, 4-hydroxytamoxifen; E2, estradiol; AA, amino acids; S, sense; AS, anti-sense, tk, thymidine kinase; C3, complement 3; AD activation domain; ERE, estrogen responsive element; luc, luciferase; RID, receptors interaction domain
TABLE 1. Homology between human and mouse ERα protein
The percentage of similarity and identity of whole protein and domains between human and mouse ERα protein is shown. T-Coffee multiple sequence alignment test was performed by using MacVector program.

|       | similarity | identity |
|-------|------------|----------|
| whole | 94.7       | 89.0     |
| A/B   | 90.6       | 81.8     |
| C     | 100        | 100      |
| D     | 98.2       | 85.5     |
| E     | 98.8       | 96.7     |
| F     | 75.6       | 62.2     |
FIGURE LEGENDS

FIGURE 1. Balb3T3A31 cells were cotransfected with the reporter plasmid (3xERE-TATA-luc), reference plasmid (pRL-TK) and expression vectors for full-length human or mouse ERα (hERαWT or mERαWT) and empty expression plasmid (pcDNA3). Cells were treated with vehicle (0 nM), estradiol (E2, A) or 4-hydroxymetamofixen (4OHT, B). Treated concentrations are described in figures. HepG2 cells were cotransfected with the reporter plasmids (3xERE-TATA-luc, C and D; or C3-110tk-luc, E and F), pRL-TK and expression vectors for full-length ERα or empty expression plasmid (pcDNA3). Cells were treated with vehicle (0 nM), E2 (C and E) or 4OHT (D and F). The luciferase activities for each panel are represented as relative activity over pcDNA3 in each concentration. The activity is represented as mean ± S.D.. Two-way ANOVA was performed to indicate significant difference between mERα and hERα activities, a; hERα activity against vehicle, b; mERα activity against vehicle, c; p < 0.05. G, whole cell lysates extracted from the plasmid-transfected HepG2 cells were analyzed by immunoblotting with anti-ERα antibody to demonstrate expression levels of ERα WT. β-actin was used as a loading control (Actin). Representative western blot is shown.

FIGURE 2. A, ERα consists of six domains named A to F. A/B domain possesses AF-1 (AF-1). E domain possesses ligand-dependent transactivation function (AF-2). mERα339, the mouse ERα mutant with entire AF-2 truncation; hERα340, the human ERα mutant with entire AF-2 truncation. B, whole cell lysates extracted from the plasmid-transfected HepG2 cells were analyzed by immunoblotting with anti-ERα antibody to demonstrate expression levels of WT and C-terminal truncated ERα. β-actin was used as a loading control (Actin). Representative western blot is shown. HepG2 cells were cotransfected with the reporter plasmids (3xERE-TATA-luc, C; or C3-110tk-luc, D), pRL-TK and expression vectors for full-length ERα (hERαWT or mERαWT), C-terminal truncated ERα (hERα340 or mERα339) or empty expression plasmid (pcDNA3). Cells were treated with vehicle (-) or 10 nM 4OHT (+). The luciferase activities for each panel are represented as relative activity over pcDNA3. The activity is represented as mean ± S.D.. One-way ANOVA was performed to indicate significant difference against pcDNA3, a; WT ERα activity against vehicle, b; p < 0.05.

FIGURE 3. A, HepG2 cells were cotransfected with pG5-luc and the expression vector for the Gal4DBD-fused ERα LBD (pB-mEF, pB-mEΔF, pB-hEF or pB-hEΔF). Cells were treated with vehicle (0 nM) or E2 (1 - 100 nM). The luciferase activities are represented as fold activation over vehicle (0 nM). The activity is represented as mean ± S.D.. B, HepG2 cells were cotransfected with pG5-luc, pRL-TK, and the expression vectors for the Gal4DBD-fused NCOA NR-box (pM-SRC1-NR, pM-GRIP1-NR or pM-ACTR-NR) in the presence of expression vectors for VP16AD (pACT) or VP16AD-fused ERα LBD (pA-mEF, pA-mEΔF, pA-hEF or pA-hEΔF). Cells were treated with vehicle (0 nM) or E2 (10 and 100 nM). The luciferase activities are represented as relative activity over pACT in each concentration. The activity is represented as mean ± S.D.. Two-way ANOVA was performed to indicate significant difference against vehicle (0 nM), a; between mE and mEAF activities in each concentration, b; between hE and hEAF activities in each concentration, c; p < 0.05.
FIGURE 4. A, schematic diagram of human/mouse F domain swapped ERα constructs. B, whole cell lysates extracted from the plasmid-transfected HepG2 cells were analyzed by immunoblotting with anti-ERα antibody to demonstrate expression levels of WT and F domain swapped ERα. β-actin was used as a loading control (Actin). Representative western blot is shown. HepG2 cells were cotransfected with the reporter plasmid (3xERE-TATA-luc, C and D; or C3-110tk-luc, E and F), pRL-TK and expression vectors for ERα mutants (mERα-mF, mERα-hF, hERα-hF or hERα-mF) or empty expression plasmid (pcDNA3). Cells were treated with vehicle (0 nM), E2 (C and E) or 4OHT (D and F). Treated concentrations are described in figures. The luciferase activities are represented as relative activity over pcDNA3 in each concentration. The activity is represented as mean ± S.D.. Two-way ANOVA was performed to indicate significant difference between mERα-mF and mERα-hF in each concentration, a; between hERα-hF and hERα-mF in each concentration, b; p < 0.05.

FIGURE 5. A, HepG2 cells were cotransfected with pG5-luc and the expression vector for the Gal4DBD-fused ERα LBD (pB-mEmF, pB-mEmF, pB-hEmF or pB-hEmF). Cells were treated with vehicle (0 nM), E2 (1 - 100 nM) or 4OHT (1 - 100 nM). The luciferase activities are represented as fold activation over vehicle (0 nM). The activity is represented as mean ± S.D.. B, HepG2 cells were cotransfected with pG5-luc, pRL-TK, and the expression vectors for the Gal4DBD-fused NCOA NR-box (pM-SRC1-NR, pM-GRIP1-NR or pM-ACTR-NR) in the presence of expression vectors for VP16AD (pACT) or VP16AD-fused ERα LBD (pA-mEmF, pA-mEmF, pA-hEmF or pA-hEmF). Cells were treated with vehicle (0 nM) or E2 (10 and 100 nM). The luciferase activities are represented as relative activity over pACT in each concentration. The activity is represented as mean ± S.D.. Two-way ANOVA was performed to indicate significant difference against vehicle (0 nM), a; between mEmF and mEmF activities in each concentration, b; between hEmF and hEmF activities in each concentration, c; p < 0.05.

FIGURE 6. A, HepG2 cells were cotransfected with pG5-luc and expression vector for Gal4DBD-fused mERα LBD in the presence of expression vector for VP16AD (pB-mEmF+pA or pB-mEmF+pA) or VP16AD-fused mERα LBD (pB-mEmF+pA-mEmF or pB-mEmF+pA-mEmF). Cells were treated with either vehicle (0 nM) or 4OHT (0.1–10 nM). The luciferase activity is represented as relative activity that set as 1 in the vehicle (0 nM) treated pB-mEmF+pA or pB-mEmF+pA transfected cells for pB-mEmF+pA-mEmF or pB-mEmF+pA-mEmF respectively. The activity is represented as mean ± S.D.. Two-way ANOVA was performed to indicate significant difference of mEmF dimerization activity against vehicle (0 nM), a; mEmF dimerization activity against vehicle (0 nM), b; between mEmF dimerization and mEmF dimerization activities in each concentration, c; p < 0.05. B, HepG2 cells were cotransfected with pG5-luc and expression vector for Gal4DBD-fused hERα LBD in the presence of expression vector for VP16AD (pB-hEmF+pA or pB-hEmF+pA) or VP16AD-fused hERα LBD (pB-hEmF+pA-hEmF or pB-hEmF+pA-hEmF). Cells were treated with either vehicle (0 nM) or 4OHT (0.1–10 nM). The luciferase activity is represented as relative activity that set as 1 in the vehicle (0 nM) treated pB-hEmF+pA or pB-hEmF+pA transfected cells for pB-hEmF+pA-hEmF or pB-hEmF+pA-hEmF respectively. The activity is represented as mean ± S.D.. Two-way ANOVA was performed to indicate significant difference of hEmF dimerization activity against vehicle (0 nM), a; hEmF dimerization activity against vehicle (0 nM), b; between hEmF dimerization and hEmF dimerization activities in each concentration, c; p < 0.05.
FIGURE 7. A, the amino acid sequence of mouse and human F domain. Deleted tyrosine-tyrosine-isoleucine residues (YYI) are denoted as red letters. B, whole cell lysates extracted from the plasmid-transfected HepG2 cells were analyzed by immunoblotting with anti-ERα antibody to demonstrate expression levels of WT and YYI deleted ERα. β-actin was used as a loading control (actin). Representative western blot is shown. HepG2 cells were cotransfected with the reporter plasmid (3xERE-TATA-luc), pRL-TK and the expression vector for WT or YYI deleted ERα (mERαΔYYI, hERαΔYYI) or empty expression plasmid (pcDNA3). Cells were treated with vehicle (0 nM), E2 (C) or 4OHT (D). Treated concentrations are described in the figures. The luciferase activities for each panel are represented as relative activity over pcDNA3 in each concentration. The activity is represented as mean ± S.D.. Two-way ANOVA was performed to indicate significant difference, *; P < 0.05, **; P < 0.01, ****; P < 0.0001.

FIGURE 8. HeLa cells were transfected with the expression vectors for WT ERα (hERα or mERα) or empty expression plasmid (pcDNA3). The transfected cells were treated with vehicle, 10 nM E2 or 10 nM 4OHT for 24 h. SERPINB9, TFF1 or Neo mRNA was measured by qPCR. The expression level of SERPINB9 (A, B), TFF1 (C, D) mRNA was normalized by the Neo mRNA level. The results of ERα transfected cells and pcDNA3 transfected cells were shown separately, since the expression level of Neo mRNA was significantly different between these cells. The mRNA level is represented as relative level over vehicle treated hERα transfected cells. The mRNA level of pcDNA3 transfected cells is represented as relative level over vehicle. E, the Neo mRNA level is shown. Each data is the average of triplicate determinations represented as mean ± S.D.. Two-way ANOVA was performed to indicate significant difference, *, P < 0.05, **; P < 0.01, ****, P < 0.0001.
FIGURE 9. **Top panel**, HepG2 cells were cotransfected with the reporter plasmids (1xERE-tk-luc, 2xERE-tk-luc or 3xERE-tk-luc), pRL-TK and expression vectors for C-terminal truncated ERα (hERα340 or mERα339) or empty expression plasmid (pcDNA3). The luciferase activity is represented as relative activity over pcDNA3 in each reporter. The activity is represented as mean ± S.D.. One-way ANOVA was performed to indicate significant difference against pcDNA3 in each reporter, *a*; *p* < 0.05. **Middle panel**, HepG2 cells were cotransfected with the reporter plasmids, pRL-TK and expression vectors for N-terminal truncated ERα (117-hERα or 121-mERα) or empty expression plasmid (pcDNA3). Cells were treated with vehicle (0 nM), or E2. Treated concentrations are described in the figures. The luciferase activities are represented as relative activity over pcDNA3 in each concentration. The activity is represented as mean ± S.D.. Two-way ANOVA was performed to indicate significant difference between mERα and hERα activities, *a*; hERα activity against vehicle, *b*; mERα activity against vehicle, *c*; *p* < 0.05. **Bottom panel**, HepG2 cells were cotransfected with the reporter plasmids, pRL-TK and expression vectors for ERα mutants (mERα-mF, mERα-hF, hERα-hF or hERα-mF) or empty expression plasmid (pcDNA3). Cells were treated with vehicle (0 nM) or E2. The luciferase activities are represented as relative activity over pcDNA3 in each concentration. The activity is represented as mean ± S.D.. Two-way ANOVA was performed to indicate significant difference between mERα-mF and mERα-hF in each concentration, *a*; between hERα-hF and hERα-mF in each concentration, *b*; *p* < 0.05.
Figure 1, Arao Y & Korach K

**A** Balb3T3A31 – 3xERE - E2

**B** Balb3T3A31 – 3xERE - 4OHT

**C** HepG2 – 3xERE - E2

**D** HepG2 – 3xERE - 4OHT

**E** HepG2 – C3-110tk - E2

**F** HepG2 – C3-110tk - 4OHT

**G**

|        | pcDNA3 | mERaWT | hERaWT |
|--------|--------|--------|--------|
| ERα    | ![ERα](image) |
| Actin  | ![Actin](image) |

**Figure. 1, Arao Y & Korach K**
Figure 2, Arao Y & Korach K

A

|       | AF-1 | AF-2 |
|-------|------|------|
| mERαWT| 1    | 1    |
| mERα339| 1    | 339  |
| hERαWT| 1    | 546  |
| hERα340| 1    | 595  |

B

Relative activity

C

3xERE

D

C3-110tk

Figure 2, Arao Y & Korach K
Figure 3, Arao Y & Korach K

A

mouse ERαLBD

human ERαLBD

Fold activation

E2 (nM)

pB-mEF pB-mEΔF pB-hEF pB-hEΔF

0 1 10 100

pB-mEF pB-mEΔF pB-hEF pB-hEΔF

0 1 10 100

0 10 20 30 40

Relative activity

B

NCOA1

NCOA2

NCOA3

Relative activity

E2

pM-SRC1-NR pM-GRIP1-NR pM-ACTR-NR

0 1 10 100

0 10 20 30 40

Figure 3, Arao Y & Korach K
Figure. 4, Arao Y & Korach K

A

mERαWT

hERαWT

mERα-mF

mERα-hF

hERα-hF

hERα-mF

B

ERα

Actin

pcDNA3

mERα-mF

mERα-hF

hERα-hF

hERα-mF

C

HepG2 – 3xERE - E2

D

HepG2 – 3xERE - 4OHT

E

HepG2 – C3-110tk - E2

F

HepG2 – C3-110tk - 4OHT

Figure. 4, Arao Y & Korach K
Figure 5, Arao Y & Korach K

**Panel A**

E2 (nM) vs. 4OHT (nM) fold activation

**Panel B**

Relative activity

**Legend**

- E2
- 4OHT
- pBIND
- pB-mEmF
- pB-mEhF
- pB-hEhF
- pB-hEmF
- NCOA1
- NCOA2
- NCOA3
- pACT
- pA-mEmF
- pA-mEhF
- pA-hEhF
- pA-hEmF
- pM-SRC1-NR
- pM-GRIP1-NR
- pM-ACTR-NR

Letters (a, b, c) indicate statistically significant differences.
Figure 6, Arao Y & Korach K
Figure. 7, Arao Y & Korach K

A

mERaWT PASRMGVPPEPSQTQLATTSTSSAHSLQTYIIPPEAEFGPTNI
mERαΔYYI PASRMGVPPEPSQTQLATTSTSSAHSLQTYIIPPEAEFGPTNI

hERaWT PrrSGGASVEETDSH-LAGSTSSSHSLQKYIITGEAEFPATV
hERαΔYYI PrrSGGASVEETDSH-LAGSTSSSHSLQKYIITGEAEFPATV

B

C

HepG2 - 3xERE - E2

D

HepG2 - 3xERE - 4OHT

E

mERα WT PASRMGVPPEPSQTQLATTSTSSAHSLQTYIIPPEAEFGPTNI
hERα WT PrrSGGASVEETDSH-LAGSTSSSHSLQKYIITGEAEFPATV

F

HepG2 - 3xERE - E2

G

HepG2 - 3xERE - 4OHT

H

HepG2 - M2H assay

4OHT

Figure. 7, Arao Y & Korach K
Figure 8, Arao Y & Korach K
### AF-1 activity

| pcDNA3 | mERα339 | hERα340 | pcDNA3 | mERα339 | hERα340 | pcDNA3 | mERα339 | hERα340 |
|--------|----------|----------|--------|----------|----------|--------|----------|----------|
| 1xERE-tk | ![](chart1.png) | ![](chart1.png) | 2xERE-tk | ![](chart1.png) | ![](chart1.png) | 3xERE-tk | ![](chart1.png) | ![](chart1.png) |

**Figure. 9, Arao Y & Korach K**

### AF-2 activity

**1xERE-tk**
- 121-mERαa
- 117-hERαa,c
- pcDNA3

**2xERE-tk**
- 121-mERαa,c
- 117-hERαa,c
- pcDNA3

**3xERE-tk**
- 121-mERαa,c
- 117-hERαa,c
- pcDNA3

### Full-length activity (AF-1 + AF-2)

**1xERE-tk**
- hERα-hFa,b
- hERα-mF
- mERα-mF
- mERα-hF
- pcDNA3

**2xERE-tk**
- hERα-hF
- hERα-mF
- mERα-mF
- mERα-hF
- pcDNA3

**3xERE-tk**
- hERα-hF
- hERα-mF
- mERα-mF
- mERα-hF
- pcDNA3

**Figure. 9, Arao Y & Korach K**
The F domain of estrogen receptor alpha is involved in species-specific, tamoxifen-mediated transactivation
Yukitomo Arao and Kenneth S. Korach

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