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Crosstalk between nuclear and G protein-coupled estrogen receptors

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Abstract

In 2005, two groups independently discovered that the G protein-coupled receptor GPR30 binds estradiol in cultured cells and, in response, initiates intracellular signaling cascades [69, 81]. GPR30 is now referred to as GPER, the G-protein coupled estrogen receptor [65]. While studies in animal models are illuminating GPER function, there is controversy as to whether GPER acts as an autonomous estrogen receptor *in vivo*, or whether GPER interacts with nuclear estrogen receptor signaling pathways in response to estrogens. Here, we review the evidence that GPER acts as an autonomous estrogen receptor *in vivo* and discuss experimental approaches to test this hypothesis directly. We propose that the degree to which GPER influences nuclear estrogen receptor signaling likely depends on cell type, developmental stage and pathology.

Estrogens exert diverse effects on organ systems throughout the body by binding to estrogen receptors. Estrogen receptors alpha and beta (ERα, ERβ), members of the nuclear receptor family [6, 16], were initially cloned and characterized as ligand-dependent transcription factors [22, 23]. Yet we now appreciate that the same nuclear estrogen receptor proteins and their alternatively spliced variants can also activate signaling pathways in the cytosol and at the plasma membrane, separate from their function as transcription factors [7, 13, 39, 51, 60]. Ligand bound ERs can associate with the plasma membrane [1, 47, 59, 61, 67] and influence kinase signaling cascades via direct interaction with Src [50, 51], B-Raf [73] and the p85α subunit of phosphatidylinositol-3-OH-kinase [72]. ERα can also interact directly with G proteins [35, 52, 87, 88] and with the integral membrane protein metabotropic glutamate receptor type 1a [13, 37]. Whereas ERα and ERβ function in the nucleus and at the plasma membrane, the G protein-coupled estrogen receptor (GPER/GPR30) represents a new class of estrogen receptor, an integral membrane protein restricted to cell membranes that cannot directly regulate gene expression [18, 65, 69, 81]. While GPER, unlike ERα and ERβ, is not a transcription factor and thus cannot directly regulate gene expression, GPER may regulate gene expression indirectly, by activating proteins that change transcription factor activity [3, 34, 56, 66].

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MEMBRANE VS NUCLEAR ESTROGEN SIGNALING

ER splice variants in mammals

How do cells regulate ERα- and ERβ-dependent nuclear vs membrane associated signaling? This question remains an active area of inquiry. Alternative splicing of estrogen receptor genes has been demonstrated to influence membrane signaling. Alternative splicing of the human *ESR1* gene results in multiple isoforms of ERα protein, where different isoforms differentially associate with cell membranes (Figure 1). The full-length protein encoded by the human *ESR1* gene, hERα66, acts as a ligand-dependent transcription factor and, following post-translational modification, can anchor to the plasma membrane and activate signaling cascades [67, 68]. The alternatively spliced hERα46 protein lacks the N-terminal transactivation domain (AF-1 domain) [19] and is targeted to the plasma membrane of endothelial cells in a palmitoylation-dependent manner [39]. The alternatively spliced hERα36 protein lacks both N- and C-terminal transactivation domains (AF-1, AF-2 domains) [84] and is associated with the plasma membrane, where it activates MAPK pathway in a heterologous culture system (HEK293 cells) [85]. In heterologous cell culture systems, both hERα46 and hERα36 are less efficient transcription factors than hERα66 and are thought to predominantly function as membrane-associated estrogen receptors [39, 85]. When overexpressed in cultured HEK293 cells, hERα36 can act as a dominant negative and inhibit hERα66-and ERβ-dependent transcription in the nucleus [85]. ERα36 and ERα46 splice variants are expressed in mice and rats [29, 42], but their function is not well understood (Figure 1).

In contrast to nuclear estrogen receptors that are alternatively spliced and signal in the nucleus and at cell membranes, GPER is a seven transmembrane GPCR with no known alternatively spliced isoforms. GPER has been detected at the plasma membrane and at intracellular membranes, such as the endoplasmic reticulum [2, 8, 9, 17, 20, 46, 62, 69, 86].

ER splice variants in teleosts

As in humans, many teleost fish species express alternatively spliced variants of ERα, including sea bream [64], killifish [24] and zebrafish [10] (Figure 1). Though six splice variants have been identified in zebrafish, none appear orthologous to the two physiologically relevant human ERα splice variants hERα36 and hERα46. Additionally, no functional role has been identified yet for any ERα splice variant in zebrafish. Thus, it is not known whether zebrafish ERα splice variants preferentially signal at the cytosol or cell membranes.

GPER signaling in teleosts

GPER mediates oocyte maturation in teleosts [44, 57, 58, 63]. Oocytes mature in the presence of progestins, while estrogens block maturation. In oocytes from Atlantic croaker (*Micropogonias undulates*), common carp (*Cyprinus carpio*) and zebrafish (*Danio rerio*), the GPER agonist G1 blocked progestin-induced maturation [44, 57]. In the absence of exogenous progestins, G1 reduced spontaneous maturation of croaker and zebrafish oocytes [57], while the GPER antagonist G15 increased spontaneous maturation of carp and zebrafish oocytes [44, 63], suggesting that estrogens act via GPER to block oocyte

maturation. In zebrafish oocytes, G15 blocked the inhibitory effects of estradiol. Additionally, reducing GPER function using either anti-sense morpholino oligonucleotides or pre-treatment with GPER antibodies blocked the inhibitory effects of estradiol [57, 63]. Knockdown of ERα did not alter the response to estradiol, suggesting that estradiol acts via GPER and not via ERα to block oocyte maturation [58]. However, the involvement of ERβ, or of alternatively spliced variants of ERα, has not been explored.

GPER signaling in mammals

Several groups have independently generated GPER mutant mice. Disruption of the first two transmembrane domains of the GPER gene by insertion of a lacZ reporter gene resulted in mice with no gross changes in fat mass, growth or reproductive ability [30]. Disrupting the GPER gene with a neomycin resistance cassette resulted in mice that were viable and fertile with no gross abnormalities in physical, immunological, or neurological development [83]. However, Haas et al. later reported that these mice exhibited increased body weight and abdominal fat compared to wildtype [25]. Two groups independently deleted the complete GPER open reading frame using Cre-mediated recombination: one by deleting the entire open reading frame [45], the other by deleting exon 3 entirely (open reading frame plus additional untranslated regions) [55]. The former mutant mice exhibited hyperglycemia, impaired glucose tolerance, reduced body growth, and increased mean arterial blood pressure associated with decreased insulin production in female mice [45], while the latter mutant mice exhibited no abnormalities in blood pressure, growth, fertility or expression of estrogen responsive genes [55]. Subsequent studies have identified potential roles for GPER, but few have utilized the available ERα and ERβ mutant mice, together with GPER, to directly test whether GPER and ERs interact in a number of physiological processes. Additionally, double and triple combination mutants would help further our understanding of these complex interactions between estrogen receptors.

EVIDENCE FOR GPER INTERACTING WITH ER SIGNALING

GPER activity leads to increased expression of ERα36

Evidence that GPER upregulates hERα36 expression comes from studies in cultured cells. In HEK293 and COS7 cells lacking endogenous GPER and hERα36 expression, overexpression of GPER increased expression of hERα36 but not hERα66 [34]. Conversely, in the SK-BR-3 breast cancer cell line, knockdown of GPER reduced hERα36 expression, while knockdown of hERα36 had no effect on GPER expression [34]. These results suggest that under certain contexts, GPER can upregulate ERα expression.

Note that these results do not exclude GPER acting as an autonomous estrogen receptor. Temporal differences in GPER activity could explain how GPER can act autonomously and influence ER expression in the same cells. Immediately following GPER activation, GPER may activate G proteins and other signaling proteins independently of ERα. Such downstream signaling pathways may ultimately upregulate hERα36, but this may occur hours following initial GPER activity.

GPER and ERα proteins interact

Two independent studies provide evidence for a physical association between GPER and ERα proteins in human primary monocytes (hPM) and in a human endometrial adenocarcinoma cell line (Ishikawa cells) [62, 82]. Derived from normal men or premenopausal women, hPMs express hERα36 and GPER but not hERα66 or ERβ [62]. Exposure to lipopolysaccharides (LPS) induces expression of pro-inflammatory genes IL-6 and TNF-α, and this effect is blocked by pre-incubation with estradiol [62]. Interestingly, both ICI182,780 (ICI), an ER antagonist/GPER agonist, and G15, a GPER antagonist, block the effects of estradiol. If ICI is acting predominantly as a GPER agonist in this situation, then it is unclear how opposing actions on GPER – either inhibiting GPER with G15 or activating GPER with ICI — could block the effects of estradiol. Alternatively, if ICI is acting predominantly as an ER antagonist, then this suggests that both hERα36 and GPER are required for estradiol to block LPS-dependent IL-6 and TNF-α expression. To explore this further, the authors found that G1 pretreatment (GPER agonist) failed to reduce LPS-dependent IL-6 expression, suggesting that ICI182,780 acts predominantly as an ER antagonist rather than as a GPER agonist in this context [62]. These results also suggest that both hERα36 and GPER must be activated to block LPS-dependent IL-6 expression.

To test the hypothesis that hERα36 and GPER are physically associated to regulate the anti-inflammatory response in hPMs, Pelekanou and colleagues used proximity ligation assay, a histological method to visualize protein colocalization [75], and found that LPS treatment caused an association between hERα36 and GPER [62]. Additionally, hERα36 and GPER were associated with each other in the area of atherosclerosis plaques from human samples with coronary artery disease [62], suggesting that hERα36 and GPER inhabit the same membrane complex *in vivo*.

In Ishikawa cells, estradiol increases proliferation. Estradiol-dependent proliferation was blocked following silencing of GPR30 or ERα by RNA interference [82]. This suggests that both proteins are involved, but whether they interact or are parts of two independent pathways was not known. To test for a direct interaction, Vivacqua and colleagues used co-immunoprecipitation and found that ERα and GPER interact directly [82], supporting the hypothesis that GPER and ERα interact to regulate proliferation in Ishikawa cells.

Using the BG-1 ovarian cancer cell line, Albanito and colleagues demonstrated that both ERα and GPER are required for estradiol-dependent proliferation [3]. In BG-1 cells, estradiol treatment upregulates c-fos expression, increases levels of phosphorylated ERK1/2 and cell proliferation. These effects were blocked by antisense oligonucleotides targeting either ERα or GPER, demonstrating that ERα and GPER are necessary for estradiol-dependent increase in cfos, phosphorylated-ERK1/2 and proliferation, similar to what was reported in Ishikawa cells [82]. In contrast to studies in Ishikawa and hPM cells, it is not known whether ERα and GPER interact in BG-1 cells.

EVIDENCE FOR GPER ACTING AUTONOMOUSLY

GPER activity influences physiology throughout the body, including metabolic functions [4, 36, 71] and reproductive [11, 21] and cardiovascular systems [32, 40, 41, 48, 49]. A majority

of these studies rely solely on pharmacologic approaches to implicate GPER signaling. Pharmacologic approaches, while useful, are limited by non-specific effects. For example, G-1, developed as a selective GPER agonist that would not activate nuclear estrogen receptors [5], was later shown to influence nuclear estrogen receptor activity [74]. Without complementary genetic approaches, such as the use of estrogen receptor mutant animals or cell lines, it is difficult to investigate the degree to which GPER acts autonomously.

Comparing phenotypes among estrogen receptor deficient animals

The use of targeted, loss-of-function mutations in estrogen receptor genes is a powerful approach for identifying the function of estrogen receptors in animals. If estradiol administration causes a phenotype in wildtype animals and in animals deficient in ERα or ERβ, but estradiol administration fails to cause the same phenotype in animals deficient in GPER, then this suggests that estradiol acts via GPER but independently of nuclear estrogen receptors. This paradigm was tested using the cardiac ischemia-reperfusion injury model: ischemia causes cardiac damage, but estradiol administration reduces cardiac damage following ischemia [26, 53, 76]. To identify the receptor required for estradiol's protective effects, Kabir and colleagues subjected hearts from male mutant mice lacking either GPER, ERα or ERβ to ischemia-reperfusion injury in the presence of estradiol or vehicle. Estradiol treatment protected wildtype and ERα and ERβ mutant mice from injury, but had no effect on GPER mutant mice [33]. These results indicate that GPER, but not nuclear estrogen receptors, are required for estradiol-dependent protection of cardiac injury following ischemia in male mice. Furthermore, these results also support the hypothesis that GPER acts as an autonomous estrogen receptor *in vivo*.

Crucially, Kabir and colleagues used ERα knockout mice that have no detectable expression of ERα splice variants ERα36 and ERα46 [14], splice variants that encode membrane-associated ERα proteins [39, 84, 85]. Thus, it is difficult to argue that the protective effects of estradiol following cardiac ischemia are due to membrane-associated ERα signaling.

A second example comes from zebrafish embryos. Acute exposure to estradiol increased heart rate in zebrafish embryos. This effect was absent in GPER mutant zebrafish. In contrast, estradiol exposure increased heart rate in ERα and ERβ mutant embryos [Romano & Gorelick, bioRxiv preprint doi: https://doi.org/10.1101/088955], demonstrating that GPER, but not nuclear estrogen receptors, are required for estradiol-dependent increase of heart rate in zebrafish embryos.

Ethinyl estradiol (EE2) can reduce severity of established disease in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, when administered following the onset of disease symptoms [80]. EE2 treatment reduced disease severity in ERα −/− mice, but was not effective in GPER −/− mice [89], suggesting that EE2 acts via GPER, not ERα, to improve EAE symptoms. This protective effect was associated with increased production of the anti-inflammatory cytokine IL-10. Following treatment with EE2, IL-10 was increased in both wildtype and ERα −/− mice, but not in GPER −/− mice [89]. Though the complete story of how GPER mediates immune function is still unclear, this study suggests that GPER functions autonomously to increase IL-10 production and improves disease outcome in EAE. Note that ERβ function was not explored

in the context of EE2-induced reduction in EAE severity, thus it is possible that GPER and ERβ interact, or influence parallel pathways, to reduce EAE symptoms. To make matters more complex, the preceding model examined estrogen ability to reduce symptoms in established EAE. Other studies using genetic approaches have shown that estrogens, when administered prior to EAE onset, exhibit neuroprotective effects and that this is dependent on ERα [77, 78]. The degree to which GPER is involved in neuroprotection of EAE onset is not well understood.

GPER and ER signaling influence parallel signaling pathways to achieve the same result

Two examples of GPER and ER acting in parallel signaling pathways to achieve the same result come from studies investigating apoptosis in two distinct cell types: thymocytes and islet cells. Prolonged treatment with E2 can lead to thymic atrophy. This effect can only be partially attributed to ERα signaling [79] and subsequent studies identified an insignificant contribution from ERβ [15]. Using a genetic approach, E2-induced thymic atrophy was attenuated in both ERα −/− mice and GPER −/− mice [83]. However, further investigation demonstrated that ERα activity blocked thymocyte maturation, which triggered cell death, whereas GPER activity induced mature thymocytes to undergo apoptosis [83]. Consistent with these results, the GPER agonist G-1 induced thymic atrophy via apoptosis but did not block thymocyte maturation [83]. Together, these results indicate that GPER acts autonomously to induce thymocyte apoptosis, while ERα acts to block thymocyte maturation. Activation of either receptor, potentially acting via independent pathways, leads to thymic atrophy.

Treating mice with streptozotocin (STZ) causes pancreatic islet cell apoptosis and is commonly used to generate animal models of type 1 diabetes [38]. Estradiol (E2) can protect islet cells from STZ-dependent apoptosis, however this protection is only partially mediated by nuclear estrogen receptors. In ERα −/− or ERβ −/− mice, STZ + E2 exhibited partial protection of pancreatic β-cell apoptosis compared to STZ + E2 in wildtype mice [38], suggesting that multiple estrogen receptors cooperate to mediate the protective effects of E2 in the pancreas. Consistent with this idea, in ERα/ERβ double knockout mice, E2 only partially protected islet cells from STZ-dependent apoptosis [43], implicating a third estrogen receptor, likely to be GPER. E2 failed to protect GPER deficient female mice from STZ-dependent apoptosis [43]. Further, G1 protected cultured wildtype islet cells from STZ-induced oxidative stress [43]. Islet cells cultured from GPER mutant mice were no longer protected by G1, but the protective effects of E2 were still present in GPER deficient islets [43]. These results suggest that ERα, ERβ and GPER respond to E2 to protect pancreatic islet cells from oxidative stress, but that GPER plays the dominant role. It is possible that GPER acts via a separate, independent pathway from ERα and ERβ to protect islet cells from STZ-dependent apoptosis. This idea is supported by the fact that in ERα/ERβ double knockout mice, E2 is only partially protective.

Comparing gene expression following differential estrogen receptor activity

Notas and colleagues used cDNA microarrays to compare gene expression following pharmacologic inhibition of ERα versus GPER in multiple cells lines [54]. Identifying transcripts uniquely regulated by GPER would support the hypothesis that GPER can act

independently of ERα. Cell lines were incubated with a membrane-impermeable estradiol-BSA conjugate (E2-BSA), in the presence or absence of ICI182,780 (ER antagonist/GPER agonist) or G15 (GPER antagonist). In T47D and SKBR3 cell lines, which express both GPER and hERα36, 17 out of 403 transcripts (4%) and 33 out of 393 transcripts (8%) were significantly and uniquely inhibited by G15. These transcripts were upregulated at least 1.5x by E2-BSA and downregulated by E2-BSA + G15 but not downregulated by E2-BSA + ICI182,780. The majority of transcripts were inhibited by both ICI and G15, suggesting some interplay between GPER and ERα. However, it is important to note that the presence of transcripts uniquely downregulated by G15 suggests that GPER can act as an autonomous estrogen receptor. The fact that this response is different between different tumor cell lines supports the idea that the degree to which GPER acts autonomously of ERα depends on the cell type.

This genomic approach has several limitations. G15 may also have some activity against ERα [12], meaning that the few transcripts uniquely regulated by G15 could be GPER-independent. The use of microarrays to compare gene expression is more biased than using RNAseq, which has the potential to reveal not only additional differentially expressed genes but also differences in expression of noncoding RNAs. Ultimately, comparing gene expression among ER and GPER mutant cells may be a powerful approach to identify genes uniquely regulated by a specific estrogen receptor.

Is cross-talk between GPER and ER signaling influenced by cell type, developmental stage or pathology?

Evidence from knockout mice and cultured cells suggests that GPER can act as an autonomous receptor and can also interact with nuclear estrogen receptors (Figure 2). However, the degree to which GPER acts autonomously likely depends on cell type, differentiation status and pathology, i.e. whether the cell is quiescent, proliferative or cancerous.

Comparing the function of GPER in different cancer cell lines, we see that GPER requires ERα for the estradiol-dependent proliferation of ovarian cancer cells (BG-1) and endometrial adenocarcinoma cells (Ishikawa)[82], but not to induce cell proliferation in breast cancer cells (SKBR3) [3]. This suggests that GPER/ER interactions and crosstalk may depend on the cellular context of the tumor or cell type. In contrast, GPER signals autonomously to induce apoptosis in thymocytes [83] and to protect pancreatic islet cells from oxidative stress-induced apoptosis [38], both *in vivo* and in cultured cells. This suggests that GPER acts autonomously in different cell types, and that GPER can act to induce opposite effects depending on the cell type in which it is acting. In these examples, nuclear estrogen receptors activated signaling in a parallel pathway to achieve a similar effect. This indicates that GPER and nuclear ERs are sometimes present in the same cell or tissue type, yet signal independently of each other.

FUTURE DIRECTIONS: MERGING PHARMACOLOGIC AND GENETIC APPROACHES

With existing pharmacologic and genetic tools, it is possible to investigate the degree to which GPER acts autonomously of nuclear estrogen receptors *in vivo*. Comparing phenotypes among ER- and GPER-deficient mice, as Kabir and colleagues did for cardiac ischemia [33], is a powerful approach that can be applied to any phenotype of interest exhibited by GPER-deficient mice. To control for genetic compensation, one could compare phenotypes between GPER-deficient mice [45] and mice with homozygous mutations in both nuclear estrogen receptors alpha and beta [14]. Additionally, because estrogen signaling is conserved among vertebrates, many of these approaches could be carried out in zebrafish, where CRISPR-Cas technology allows rapid and straightforward generation of embryos with multiple loss-of-function mutations [31]. Comparing gene expression between wildtype, GPER mutant, ERα mutants, ERβ mutants and compound mutant animals will be a powerful approach to identify genes regulated by specific estrogen receptors *in vivo* and to identify the degree to which GPER and nuclear ER signaling overlap.

Small molecules that specifically target GPER, with low or no affinity for nuclear estrogen receptors, can be used to identify genes specifically regulated by GPER in cultured cells, isolated tissues and whole animals. Pharmacologic approaches avoid genetic compensation, which can be induced by deleterious mutations but are less likely to be induced by gene knockdown or temporary perturbation of receptor activity [70]. Comparing gene expression in a whole animal, tissue or cell type of interest following exposure to GPER-, ERα- or ERβ-specific agonists (such as G1, PPT and DPN [5, 28]) could provide important insights into GPER- versus ERα- versus ERβ-dependent gene expression. For example, administering such chemicals to different groups of mice, and then comparing gene expression in the liver, would generate an interesting list of candidate genes regulated by specific estrogen receptor types *in vivo*. Varying the dose and duration of treatment could be used to infer whether gene expression changes are regulated by a specific estrogen receptor directly or indirectly. Such gene expression studies could be performed on multiple different organisms in parallel, to compare the degree to which estrogen receptor regulate unique or overlapping genes in different species [27].

Pharmacologic approaches are limited by non-specific effects. However, non-specific effects could be controlled by combining pharmacologic and genetic approaches. In the previous example, a specific GPER agonist, such as G1, could be administered to wildtype and to GPER mutant animals. By comparing gene expression in the liver between wildtype and GPER-deficient animals following G1 exposure, it should be possible to identify genes non-specifically regulated by G1 and exclude them from future analyses. Analogous approaches could be used to identify genes non-specifically regulated by ERα- and ERβ-specific agonists and antagonists.

Biochemistry and cell biology should also be used to explore associations between GPER and ER proteins *in vivo*. Immunoprecipitation approaches can be used to test GPER and ER interactions *in vivo*, while newer histologic methods, such as proximity ligation, can be used to detect small amounts of GPER and ER proteins in fixed tissue and determine whether

they interact. While these approaches were used to show GPER-ERα interactions in human primary monocytes and in cultured Ishikawa cells [62, 82], there are many more cell types that express both GPER and nuclear estrogen receptors that have yet to be assayed for interactions among estrogen receptors. It will be interesting to explore how cell type and ligand regulate interactions between GPER and nuclear estrogen receptors.

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Highlights

- GPER acts as an autonomous estrogen receptor in vivo
- GPER also interacts with nuclear estrogen receptors
- Whether GPER influences nuclear estrogen receptor signaling may depend on cell type

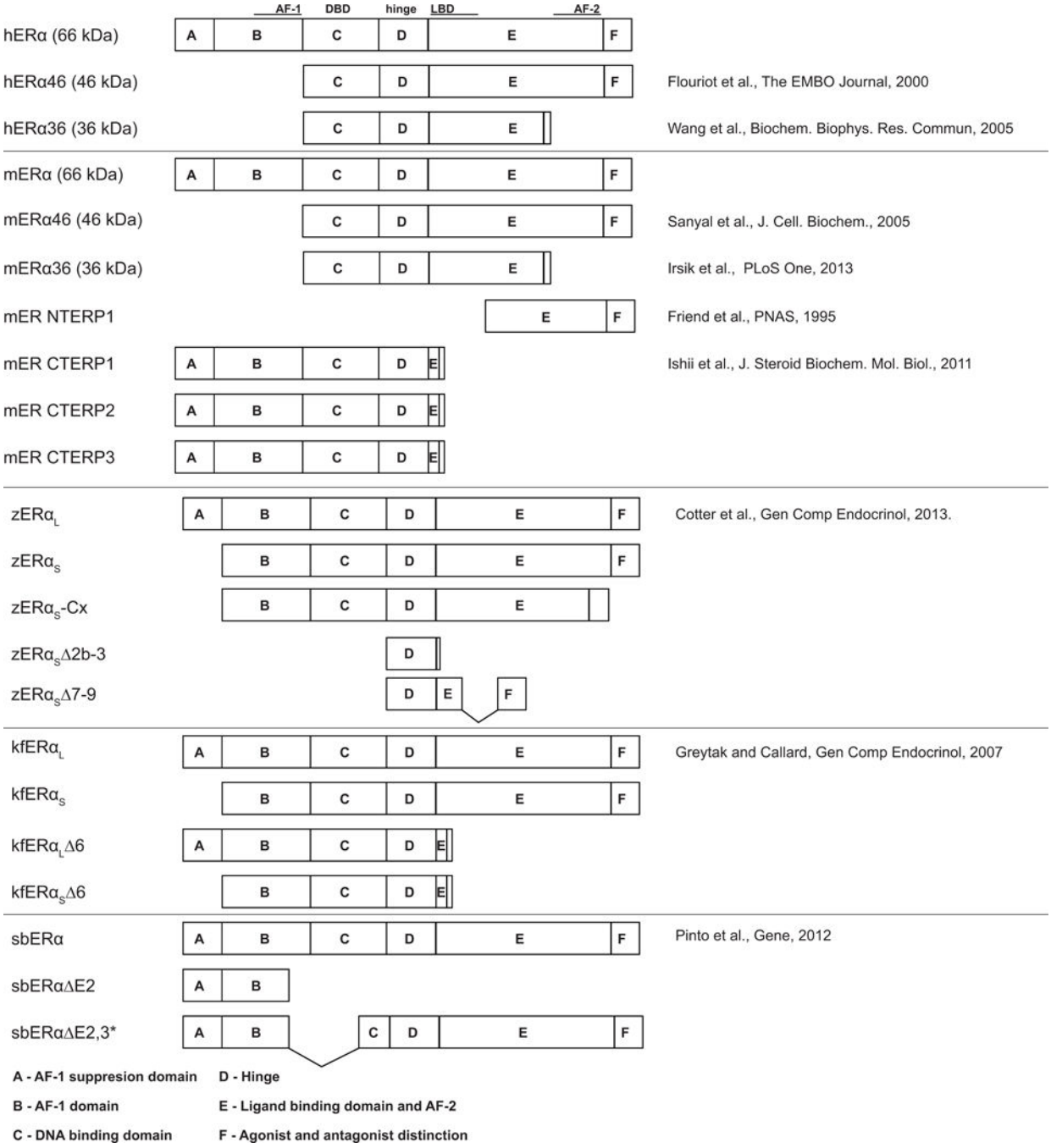

Figure 1. Estrogen receptor alpha (ERα) splice variants

Depicted protein structures of full length and spice variants in human (h), mouse (m), zebrafish (z), killifish (k) and sea bream (sb). Functional regions (based on Krust et al, EMBO J 1986): AF-1, activating function 1 domain; DBD, DNA binding domain; LBD, ligand binding domain; AF-2, activating function 2 domain. Blank regions indicate amino acid sequence unique to the splice variant, except for kfERα$_L$Δ6 and kfERα$_S$Δ6 where the sequence following the E domain is identical. NTERP, N terminally truncated ER protein; CTERP, C terminally truncated ER protein.

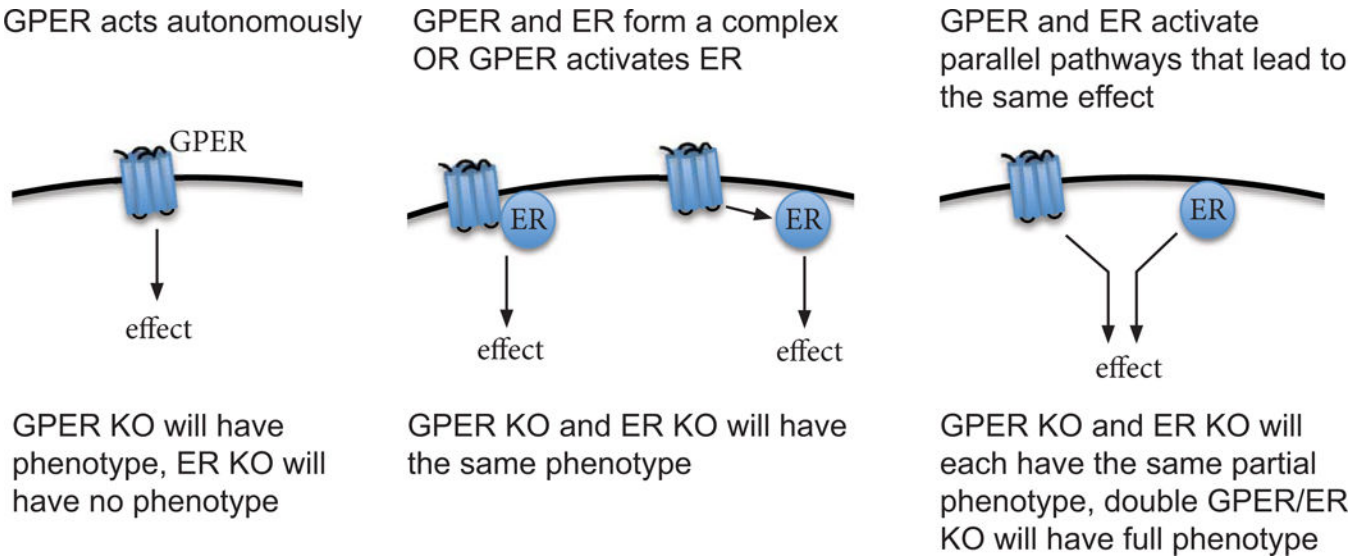

Figure 2. Models for autonomous GPER signaling versus GPER-ER signaling interactions

KO, knockout animal; GPER, G protein-coupled estrogen receptor; ER, nuclear estrogen receptor alpha or beta