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Nuclear receptors outside the nucleus: extranuclear signalling by steroid receptors

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Abstract

Steroid hormone receptors mediate numerous crucial biological processes and are classically thought to function as transcriptional regulators in the nucleus. However, it has been known for more than 50 years that steroids evoke rapid responses in many organs that cannot be explained by gene regulation. Mounting evidence indicates that most steroid receptors in fact exist in extranuclear cellular pools, including at the plasma membrane. This latter pool, when engaged by a steroid ligand, rapidly activates signals that affect various aspects of cellular biology. Research into the mechanisms of signalling instigated by extranuclear steroid receptor pools and how this extranuclear signalling is integrated with responses elicited by nuclear receptor pools provides novel understanding of steroid hormone signalling and its roles in health and disease.

Nuclear receptors, most notably steroid hormone receptors, have been investigated as models to understand the principles of the transcriptional regulation of mammalian development and adult physiology, and its impairment in disease states. According to the classic model of steroid receptor signalling, steroids enter cells through the plasma membrane and bind to their receptors localized in the cytoplasm (for example, androgen receptor (AR) and glucocorticoid receptor (GR)) or in the nucleus (for example, oestrogen receptor-α (ERα; encoded by *ESR1*) and ERβ (encoded by *ESR2*)). Next, steroid-bound receptors translocate to the nucleus (in the case of cytoplasm-localized receptors) and engage with specific nuclear DNA response elements to regulate the transcription of target genes. This drives changes in mRNA expression, which usually alter protein expression and ultimately affect cellular biology (FIG. 1a). Alternatively, steroid receptors can regulate transcription through a tethered mechanism, whereby receptors do not directly bind DNA but instead interact with other DNA-bound transcription factors or co-regulators[1] (FIG. 1b).

Importantly, a considerable amount of experimental data gathered over nearly 60 years demonstrates that steroid hormones also induce responses that are too rapid (measured in seconds rather than minutes) to be mediated through transcriptional regulation[2,3]. Early observations of such rapid signalling include oestrogen induced generation of cyclic AMP[4]

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and calcium flux in uterine epithelial cells[5] — responses that occur rapidly and engage membrane-localized effectors, suggesting that in this context oestrogen binds to a receptor at the endometrial cell membrane rather than in the nucleus[6]. It is now known that this is indeed the case. In fact, multiple steroid and non-steroid nuclear receptors have now been described at the plasma membrane, and their functions are increasingly being determined in mammals, including humans. Interestingly, extranuclear signalling by steroid receptor pools is not limited to animals: it has been shown that plant steroids (brassinosteroids) regulate flowering and fertility by signalling through tyrosine kinase receptors expressed at the cell plasma membrane[7]. This example of an ancient and conserved pool of steroid receptors at the plasma membrane suggests that steroid receptor signalling might have evolved from outside the nucleus. Perhaps the nuclear pool subsequently developed as the genome became more complex in some organisms, at which point multiple hormone receptor response elements throughout the genome began to be used to mediate complex steroid hormone effects on gene expression. In addition to the nucleus and plasma membrane, steroid receptors localize to the mitochondria, endoplasmic reticulum and other cellular organelles[8]. In some settings, these extranuclear receptor pools act coordinately with the classic nuclear pools. As an example, in prostate cancer, ARs at the epithelial cell plasma membrane rapidly signal to augment nuclear AR actions in promoting tumour proliferation[9,10]. In addition, receptors for oestrogen, progesterone and androgens have been reported to form complexes both at the cell membrane[11,12] and in the nucleus[13,14] in breast and prostate cancer epithelial cells, affecting tumour cell biology.

In this Review, we discuss the emerging roles of extranuclear pools of nuclear receptors, focusing on receptors localized to the plasma membrane, which often work in partnership with nuclear receptor pools but are also capable of acting independently to affect cell functions. As most of the data regarding extranuclear signalling have been obtained for steroid receptors, we focus here on these examples. The identity of these receptors, their intracellular trafficking and the signalling by their membrane pools are outlined. Their effects on protein modification and function, and their involvement in genetic and epigenetic regulation, are also reviewed. The impact that signalling from membrane steroid receptors has, including crosstalk with the nuclear receptors pools, as well as the unique, independent actions in normal development, biology and disease states, are stressed. Our accelerated knowledge of these functions is derived in part from newly developed mouse models and steroid receptor ligands. Appreciation of the coordinated signalling between extranuclear and nuclear steroid hormone receptors is particularly important for developing novel strategies to modulate physiological and pathological processes in which steroid signalling is involved.

Steroid receptors in the nucleus

All nuclear receptors have the same basic structure, which is crucial to understanding their nuclear functions (FIG. 2a). A and B domains at the amino terminus provide a binding site for transcriptional co-regulators[15]. C and D (hinge) regions in the protein interior enable DNA binding at discrete sequences, are involved in protein nuclear localization and/or influence binding of the receptor to other transcription factors in a tethered fashion[16]. E and F domains bind to additional co-regulators, often through an LXXLL motif in co-regulatory

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proteins[15]. The E domain also contains a ligand-binding sequence that structurally varies in different receptors to confer specificity to ligands and antagonists[17]. In sum, all these regions contribute to the steroid ligand-induced changes in gene expression occurring through the combined action of co-regulators and nuclear receptors bound at specific DNA sites.

Receptors at the plasma membrane

Although most steroid receptors are located in the nucleus of cells, it has been reported that a small subset (approximately 5%) of different steroid receptors localizes to the plasma membrane, including classic steroid receptors (the ERs, progesterone receptor (PR; encoded by *PGR*), AR, GR and mineralocorticoid receptor (MR)) and non-classic steroid receptors (thyroid hormone receptor (THR) and vitamin D receptor (VDR))[8] (TABLE 1). In addition, alternative membrane localized receptors for various steroid hormones have been described. Similarly to steroid receptors from the nuclear pool, which form complexes with other signalling molecules in the nucleus to regulate transcription, these membrane-localized steroid receptors interact with cell signalling machinery at the plasma membrane to promote membrane-initiated responses.

ERs

Both ER- and PR-null mice were generated in the mid-1990s[18,19], but neither model was initially investigated for how the lack of these receptors affected rapid actions of the respective steroids. Thus, the identity of extranuclear steroid receptors and their involvement in rapid steroid actions was enigmatic. Finally, with the use of antibodies to classic ERα, immunoreactivity was detected at the plasma membrane, supporting the idea that a pool of ERα localizes to this cellular compartment[20]. Functionally, binding of oestrogen to this cell membrane-localized protein was significantly diminished by antisense oligonucleotides targeting classic *ESR1* mRNA[21]. Moreover, on re-expression of classic ERα from a single plasmid in ERα-null cells, both nuclearand membrane-localized steroid receptors were produced, and 17β-oestradiol was able to elicit rapid signal transduction[22]. In addition, mass spectrometry of proteins isolated from distinct subcellular compartments of human breast cancer cells indicated the existence of both membrane and nuclear pools of ERα[23]. Collectively, these findings supported the idea that, indeed, membrane ERα and nuclear ERα are the same protein.

Notably, it has been shown that some oestrogenic compounds that did not significantly activate classic ERα-induced transcriptional responses typically elicited by the classic ligand, oestradiol, nonetheless produced protection against metabolic bone disease *in vivo*[24]. These effects were the result of rapid signalling and promoted the idea not only that membrane-localized ERα contributes to the cellular responses mediated by this sex steroid but also that the signalling instigated by this pool is possibly sufficient in some situations. The specificity of these compounds for the membrane receptor suggests that the ligand–receptor conformation at the membrane is different from the nuclear complex structure; however, what these differences are is still not understood.

Interestingly, many laboratories have indicated that, in addition to full-length, 66-kDa ERα, truncated forms of this receptor exist in various organs, often outside the nucleus. Both 46-kDa and 36-kDa ERα have been reported at the membrane, especially in breast cancer cell lines[25,26]. These proteins are produced by alternative promoter use in the *ESR1* gene, which generates splice isoforms and thereby truncated receptors[27,28]. However, confirmation that these truncated ER variants localize to the membrane of normal organs is not yet available. In addition, there is no conclusive evidence regarding the importance of the 36-kDa ER isoform for normal or abnormal biology. Also, low abundance of this endogenous receptor at the membrane suggests a rather limited role, if any, in oestrogen signalling from the membrane. By contrast, abundant 66-kDa, full-length ERα has been identified at the plasma membrane by many laboratories, in multiple animal and cell models, with evidence indicating that this form mediates most of the rapid actions of this sex steroid[8]. Similarly to ERα through *ESR1*, expression of wild-type *ESR2* mRNA in cells produces both membrane and nuclear pools of ERβ. Notably, when membrane localization of ERβ was prevented (by introducing mutations interfering with the trafficking of this receptor to the membrane; see below for details of receptor trafficking to the membrane), rapid signalling by oestrogen was perturbed[29].

AR

In addition to the ERs, classic AR is located at the plasma membrane. Membrane signalling through AR was first described in *Xenopus laevis* oocytes. In these cells, meiotic progression occurs via membrane-localized classic AR, which, on activation by a ligand, rapidly suppresses G protein signalling at the cell membrane with a concomitant decrease in intracellular cAMP. Decreased cAMP ultimately leads to activation of ERK signaling and meiotic progression. Notably, this essential process occurs without transcription[30–32], demonstrating that membrane-localized steroid receptors can affect biology completely independently of the nuclear receptor pool. Classic AR is also located in the cell membranes of prostate cancer and ovarian granulosa cells, where on hormonal stimulation it triggers signalling through kinases such as Src and ERK to mediate important processes, including proliferation and apoptosis[9,33,34].

PRs

Although less well biochemically characterized than the ERs and AR, classic PR-A and PR-B at (or near) the plasma membrane regulate kinase signals, such as ERK and PI3K–AKT, thereby mediating biological processes, including breast cancer proliferation. Evidence suggests that extranuclear PRs often do not act alone in breast cancer cells, and may directly interact with extranuclear ERs and/or AR, as well as membrane-localized kinases, to promote important proliferative signals[12,35,36].

VDRs

In addition to the canonical steroid receptors, other members of the steroid receptor superfamily have been reported to localize to the membrane. This includes VDR, whose full-length and truncated classic, as well as non-classic, isoforms are known to localize to the membrane. For example, an alternative isoform of VDR that is expressed primarily in neonate mammals was found to mediate intestinal phosphorus absorption[37]. Phosphorus

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absorption in isolated intestinal epithelial cells was shown to result from rapid activation of protein kinase C (PKC) following exposure to vitamin D and its binding to the novel, membrane-localized receptor. This response to vitamin D stimulation was found to be additional to the function of the extranuclear pool of the classic VDR, which localizes to caveolae at the membrane (see below for details regarding receptor localization at the membrane) and has been shown to facilitate intestinal absorption of vitamin D in chick and rat intestinal caveolae-enriched cell membrane preparations[38].

THRs

Similarly to VDR, a truncated form of classic THRα is localized to the cell membrane. This truncated THRα signals through cyclic GMP to suppress increased apoptosis of osteocytes and osteoblasts seen in hypo hyroidism, preventing overall impaired bone formation[39]. In addition, full-length THRβ signalling through PI3K from the cytoplasm–membrane interface has been reported to be important for normal maturation of hippocampal neurons and synapse strength[40].

Alternative steroid receptors

Several alternative receptors for steroid hormones have been described and proposed to contribute to membrane-initiated signalling by these hormones. It has been reported that an orphan G protein-coupled receptor, G protein-coupled ER1 (GPER1; also known as GPR30), serves as a receptor for oestrogen and mediates oestradiol signalling from the cell membrane[41,42] or the endoplasmic reticulum[43]. Although these publications support the idea that oestrogen indeed binds to GPER1, several laboratories have used ERα-null cells that express endogenous GPER1 to test this further and have been unable to see appreciable specific oestrogen association with the membrane or membrane-associated signalling in response to oestradiol[23,44]. GPER1-knockout mice have been created[45,46], and the early models do not display abnormal phenotypes in response to various stresses or under basal conditions that result from loss of oestrogen action. Importantly, no overlap with the profound phenotypes observed in ERαor ERβ-knockout mice (BOX 1) has been conclusively shown. It is therefore likely that only in some cell types, such as hormone-responsive cancer cells, do GPER1 and membrane ERα collaborate to induce signal transduction from the membrane, activating downstream kinase cascades[47]. However, in opposition to this idea, some investigators have not seen a functional linkage between membrane-localized ERα and GPER1 in breast cancer and other cell types[48,49]. It should thus be considered that, despite recent studies suggesting important roles for this protein[50], these functions might be unrelated to direct oestrogen stimulation. Together, it is not yet established *in vivo* that GPER1 and membrane ERs meaningfully collaborate.

Progesterone has been shown to bind to and activates an alternative membrane receptor, known as progesterone receptor membrane component 1 (PGRMC1), to affect the growth and viability of various mammalian cells[51,52]. In addition, PGRMC1 was recently shown to regulate iron homeostasis in zebrafish and human hepatoma cells[53]. Another family of membrane PRs (mPRs) has been described, mainly in fish[54,55]. Although these receptors bear little sequence homology to G protein-coupled receptors, evidence suggests that they modulate G protein signalling on progesterone and other progestin binding. The importance

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of these receptors is unclear, as definitive mammalian genetic-deletion models for these putative receptors have not been created. However, progesterone has been shown to inhibit gonadotropin-releasing hormone in mice lacking all classic PR expression, perhaps acting via signalling through mPRs, suggesting that alternative membrane-localized PRs might also exist in mammals[56]. Additional work will be required to understand the relative importance of these multiple, putative steroid receptor proteins.

Recent evidence suggests that, like progesterone and oestradiol, androgens may bind to and activate an alternative receptor on the plasma membrane. G protein coupled receptor family C group 6 member A (GPRC6A), whose ligands seem to include osteocalcin, calcium and amino acids, has been shown to additionally engage androgens in many cell types to induce rapid G protein and kinase signalling (such as through ERK). Androgen-stimulated GPRC6A has been suggested to mediate insulin secretion from pancreatic β-cells and testosterone production from testicular leydig cells[57,58].

Receptor trafficking to the membrane

Sex steroid receptors, VDR and membrane-truncated THRα are known to traffic to the membrane after palmitoylation (that is, covalent attachment of a fatty acid, primarily palmitic acid (also known as palmitate)), which increases the hydrophobicity of the protein, thereby promoting its association with the membrane. For ERα, ERβ and probably other classic sex steroid receptors, the E domain (the ligand-binding domain) is necessary for trafficking to the membrane and therefore mediates the resulting generation of multiple rapid signals[29] (FIG. 2). Potential roles for the other receptor domains outside the nucleus are not well defined and represent an important area for further research.

In the case of sex steroid ligands, their binding to their respective receptors causes rapid receptor dimerization, which is required for both nuclear and membrane receptor activity[59]. Notably, however, palmitoylation occurs only on receptor monomers. In the case of ERα, when oestrogen binds to the total cellular receptor pool, it causes rapid dimerization of approximately 90% of receptors. This greatly limits the size of the ERα monomeric pool that can undergo palmitoylation. As palmitoylation is required to drive ERα to the membrane, this limits the number of receptors that traffic to this cellular site. As a result, the membrane ERα pool is limited and typically only approximately 5% of the total cellular pool of this receptor is found at the membrane[59,60]. The localization of the majority (~85%) of receptors to the nucleus is probably required to enable simultaneous binding to enhancers or promoters of many genes regulated by this sex steroid. In addition, approximately 10% of ERα molecules are localized to the mitochondria, where they serve largely unexplored functions (BOX 2).

The E domains of classic ERα, ERβ, PR-B and AR harbour a highly conserved, nine-amino-acid palmitoylation motif, including a Cys residue that is the site of attachment of palmitic acid[29,61,62] (FIG. 2b). Mutation of this Cys or other residues in the palmitoylation motifs of these receptors and expression of these mutants in various steroid receptor-null cells prevents membrane localization of these receptors.

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The mechanism of palmitoylation is best established for sex steroid receptors and is known to involve oligomerized heat shock protein 27 (HSP27). HSP27 associates with the palmitoylation motif in the E domain of cytoplasm-localized receptors, presumably opening up the structure of the receptor and thereby enabling the palmitoyl acyltransferases (PATs) zinc-finger DHHC domain-containing 7 (ZDHHC7) and ZDHHC21 (REF. 63) to access the palmitoylation motif. These PATs then attach the palmitic acid moiety to the N-terminal Cys of the motif, promoting the physical association of the steroid receptor with caveolin-1 (FIG. 2a). Caveolin-1 is the main structural coat protein of caveolae and, in line with this, membrane pools of steroid receptors are known to associate with these membrane domains. As an example, oestradiol is highly concentrated in isolated caveolae. It therefore readily engages ERα bound to caveolin-1, which serves as a scaffold for membrane-localized signalling molecules. Similarly, GR has been noted to localize to lipid rafts in association with caveolin-1 in neural progenitor and/or stem cells[64]. As noted above, classic VDR can also be found at caveolae[38]. Notably, caveolin-1 seems to be required for membrane localization of steroid receptors, as deletion of the membrane-localization sequence of caveolin-1 prevents this protein and the sex steroid receptors (as shown for the ERs) from localizing to the membrane[65].

Membrane steroid signalling outcomes

In contrast to nuclear steroid receptors, membrane-associated steroid receptors do not directly engage DNA to modulate transcription. However, they can elicit various cellular responses. These extranuclear actions of steroid receptors can be independent of the nuclear pool, inducing non-nuclear signalling cascades. Notably however, membrane-initiated signalling can also feed into transcriptional regulation mediated by various nuclear factors. In this case, the extranuclear and nuclear receptor pools can engage in crosstalk, collectively modulating the expression of steroid-responsive genes.

Activation of signalling cascades

Once receptors are located in membrane rafts and scaffolded to caveolin-1, steroid ligand binding activates rapid signals within seconds. This includes generation of cyclic nucleotides (cAMP and cGMP) and calcium flux. Rapid signalling results from physical interactions between steroid receptors and components of the multiprotein signalsome complex that vary by cell type and are currently poorly defined. The signalsome often contains specific G protein-α, -β and -γ subunits, Pro-, Gluand Leu-rich protein 1 (PELP1) and Src-homology 2 (SH2) domain-containing protein (SHC) scaffolding proteins, and kinases such as Src, the p85 subunit of PI3K and AKT (FIG. 2c).

ERα contacts and activates G proteins, designating it as a G protein-coupled receptor[66,67]. Depending on the cell type and context, oestrogen engagement of membrane receptors causes particular G protein activation, contributing to the specificity of membrane ER signalling. G protein activation generates cyclic nucleotides and calcium flux, activating proximal kinases such as Src, resulting in phospholipase activity and production of secondary signalling messengers, such as diacylglycerol and inositol-1,4,5-trisphosphate

(Ins(1,4,5)P_3). This contributes to the recruitment of additional linker and signalling proteins, expanding signal transduction.

As shown for ER, more-prolonged signalling to kinase cascades initiated by hormone-stimulated membrane receptors sometimes occurs from transactivating growth factor receptors (GFRs) (FIG. 2c), such as insulinlike growth factor 1 (IGF1) receptor (IGF1R) and epidermal GFR (EGFR), especially in breast and prostate cancer[67,68]. This transactivation occurs either directly, by formation of a complex between the steroid receptor and the GFR (the ligand-independent mechanism), or indirectly, via stimulation of the secretion of matrix metalloproteinases induced by signalling cascades initiated at the membrane following steroid stimulation, which leads to the release of GFR ligands that bind to and activate the receptor[67,68]. Transactivation of GFRs by membrane steroid receptors is required for initiation of, for instance, MEK–ERK and PI3K–AKT kinase cascades that participate in proliferative, invasive and migratory responses to steroid ligands.

Interestingly, in breast and prostate cancer cells, ER–PR and ER–AR protein complexes, respectively, are present at the plasma membrane and respond to both steroid ligands, resulting in signal transduction through kinase activation[11,12] (FIG. 2c). Finally, receptors rapidly relocalize in the cell on de-palmitoylation, often to endosomes. This can potentially induce further signalling[69], or direct the receptor for lysosomal degradation, thereby terminating signalling[69,70], or to the Golgi, followed by receptor re-palmitoylation and recycling to the membrane (FIG. 2a). Whether membrane receptors can move to the nucleus or vice versa is unknown.

Rapid signalling from PR-B to ERK via the Src kinase–RAS pathway has been described in breast cancer cells to result in enhanced proliferation of these cells and to depend on the membrane expression of ER and its interaction with PR-B. This process requires a physical interaction between Src and ER, presumably in the vicinity of the plasma membrane[12]. Although no structural studies have been performed to define how this signalling occurs, it probably shifts Src into an activated state. In part, this could occur from proximal ER signals, such as calcium flux, which activates the catalytic domain of Src. Interaction between the two steroid receptors requires a polyproline cluster in the N terminus of PR[35] that also mediates the physical interaction of this receptor with the sh3 domains of Src kinase[36]. Subsequent work showed that PR-B signals through increased WNT1 expression to induce EGFR, resulting in Src-dependent ERK activation in breast cancer cells that promoted growth in soft agar[71]. Similarly to ER, localization of PR-B to the plasma membrane and its resulting signalling were shown to result from palmitoylation, in this case of Cys820 by ZDHHC7 and ZDHHC21 PATs[60].

Although it is unclear whether classic MRs localize to the plasma membrane, rapid signalling in response to aldosterone (the main mineralocorticoid hormone) is established[72]. Aldosterone-induced signals, such as activation of PKA, PKC and PKD, directly or indirectly regulate the functions of the sodium/hydrogen exchanger 1, sodium/potassium pump and epithelial sodium channel in kidney and other cells. The E and F domains of MR are sufficient to induce this rapid signalling[73], and this includes EGFR transactivation, as described for all sex steroid membrane receptors (see the discussion above). Additionally,

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rapid aldosterone-induced PKC activation may couple the rapid signalling effects to transcriptional aldosterone responses mediated by nuclear MR pools[74]. Identification of residues and mechanisms mediating membrane localization of MR are important areas for research.

Signalling to the nucleus

Modification of transcriptional regulators—In addition to activating cytosolic signals (such as calcium flux), G protein signalling and protein kinases, membrane-localized steroid receptors signal to the nucleus to regulate gene expression. In particular, membrane-initiated signalling potentially affects many aspects of nuclear steroid receptor regulation of transcription (see also below).

An important mechanism of gene expression modulation by membrane-localized steroid receptors involves modification of the activity of transcription factors. This can occur through the effect of extranuclear signalling on transcriptional co-activators. On phosphorylation, many co-activators translocate to the nucleus and bind to target gene promoters and enhancers in conjunction with nuclear receptors to augment nuclear receptor action. Co-activator binding leads to the formation of multiprotein complexes, including histone acetyltransferases and other chromatin modifiers, transcription factors and components of the basal transcription machinery and RNA polymerase II. Phosphorylation of co-activators can arise directly, from membrane ERα, AR or PR signalling, or indirectly, from GFR transactivation by membrane steroid receptors, as often occurs in cancer cells[9,67,68,71,75]. Specifically, it has been proposed that the recruitment of nuclear receptor co-activator 3 (NCOA3; also known as SRC3) to DNA arises from extranuclear ER signalling[76,77], but this has not been definitively shown. Transcription factors also suppress gene expression. This could be through evicting co-activators and/or recruiting co-repressor proteins, such as nuclear receptor co-repressor 2 (NCOR2; also known as SMRT) and NCOR1, to chromatin — processes that entail similar rapid post-translational modifications[78,79]. Together, in the specific cell contexts under which membrane steroid receptors selectively signal, the resulting modifications of co-regulatory proteins at discrete residues dictate their function, thereby modulating nuclear receptor action.

Steroid signalling from the membrane also affects expression and cell localization, hence functions of transcription factors, including transcription factors that work in conjunction with nuclear steroid receptors, thereby contributing to the crosstalk between membrane and nuclear pools of these receptors (see also below). Membrane PR or ERα signalling enhances transcription factor expression and recruitment to promoters and enhancers of steroid hormone-regulated genes[80–82]. In some situations, membrane ERα signalling inhibits transcription factor gene or protein expression and activity, resulting in repression of cell function[83]. In part, this also occurs through signalling that alters transcription factor protein activity and subcellular localization, including nuclear exclusion. These mechanisms, for example, underlie the ability of oestrogen to suppress excess lipogenesis in several organs, including adipocytes[83–86]. It has been established that oestrogen inhibits excessive development of visceral fat in animal models and postmenopausal women. In the mature adipocyte, glucose is taken up and used for synthesis of triglyceride, which is stored as a

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source of energy. Membrane ERα signalling through AMP-activated protein kinase (AMPK) and PKA phosphorylates subunits of a key transcription factor, carbohydrate-responsive element-binding protein (ChREBP) at key Ser and Thr residues, preventing the insulin-stimulated translocation of this transcription factor to the nucleus. This precludes insulin-induced stimulation of ChREBP targets, including the fatty acid synthase and acetyl-CoA carboxylase genes that are crucial for lipid and fatty acid synthesis in the adipocyte (FIG. 3a). In this way, membrane ERα signalling, through modulation of transcription factor cellular localization and subsequent transcription, is sufficient to inhibit important aspects of insulin-stimulated adipocyte metabolism[83]. Similarly, membrane signalling by ERβ is involved in the regulation of gene expression through the modulation of transcription factor localization in the heart, preventing heart hypertrophy and fibrosis (see the section on Physiological implications below).

Epigenetic regulation of genomic loci—Signalling by nuclear receptors at the membrane has recently been shown to affect the epigenome. As an example, in cells of the developing uterine myometrium, the synthetic xenoestrogen diethylstilboestrol was shown to activate PI3K–AKT signalling (through undetermined pathways), causing inhibitory phosphorylation at Ser21 of the enhancer of Zeste homologue 2 (EZH2) histone methyltransferase[87]. As a result, repressive trimethylation of histone H3 at Lys27 (H3K27me3) was lifted and the expression of mRNAs, including mRNAs of ER-responsive genes, which are not normally expressed in the developing myometrium, was induced. The misexpression of ER-responsive genes caused disruption of tissue morphology and function, and increased the risk of tumour development (FIG. 3b). Similar epigenetic modifications were also found to be important for the ability of membrane ERα to regulate normal mammary gland development; in mice genetically engineered to lack the membrane receptor ERα pool, AKT signalling was impaired, leading to a lack of epigenetic input required for PR expression and to abnormal ductal branching[88].

Similarly, in the heart, membrane ERβ signalling interferes with angiotensin II (Ang II)-stimulated phosphorylation of class I (pro-hypertrophic) and II (antihypertrophic) histone deacetylases (HDACs), thereby modulating their cellular localization (promoting nuclear exclusion of class I and nuclear re-l ocalization of class II HDACs) and contributing to the anti-hypertrophic function of oestrogen in this organ[89] (see the section on Physiological implications below). Furthermore, in hormone-responsive tumours, membrane-localized sex steroid receptors induce kinase signalling, which affects multiple metabolic pathways in the cancer cells by altering chromatin compaction and the active epigenetic state of key genes, such as glucose transporters, glycolytic enzymes (including hexokinase) and mTOR complex 2 (REF. 90). As well as these examples, it is very likely that other signals from membrane steroid receptors have an impact on additional chromatin writers, erasers and readers.

Alteration of molecular properties or actions of nuclear steroid receptors—
Membrane-initiated steroid signalling is known to alter nuclear functions of the respective nuclear receptors, supporting the idea that both receptor pools engage in crosstalk, collectively regulating cellular responses to steroid stimulation. In some situations,

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signalling from membrane steroid receptors or GFRs modifies nuclear receptor proteins to enhance or inhibit their activity[75]. Many discrete residues in ER, AR and PR are phosphorylated, often enhancing but sometimes diminishing their transcriptional activity. In addition, phosphorylation of ERα or PR in response to ligands causes complexing of the two receptors, resulting in enhanced activation of the production of mRNAs, including those of genes that are not regulated by either receptor alone[13]. It is likely that these effects drive proliferation in hormone-responsive cancers, such as breast cancer and uterine malignancies.

To provide a more detailed example of the crosstalk between the nuclear and extranuclear steroid receptor pools, there is emerging evidence that membrane ER signalling enhances the residency or recruitment of nuclear ER to promoters and enhancers. Membrane-induced signalling by oestradiol and ERα (through an as-yet-unknown mechanism) activates p38 kinase, which then stimulates phosphorylation of Ser294 of the nuclear ERα[91]. As a result, the interaction between the nuclear pool of ER and the E3-ubiquitin ligase S-phase kinase-associated protein 2 (SKP2) is enhanced. On ubiquitylation, nuclear ERα is degraded, thereby contributing to the removal of nuclear ERα from promoters and enhancers of target genes, accounting for the expression of ER-target genes 'on' and 'off ' in a cyclical pattern[92]. In addition, membrane ERα signalling through ERK results in upregulation of the oestrogen-responsive gene *TFF1* (trefoil factor 1; also known as *pS2*)[88]. This occurs, at least in part, through ERK signalling-mediated recruitment of nuclear ERα to an oestrogen-response element on the promoter of this gene.

Another example of extranuclear, steroid-induced kinase signalling modulating nuclear recruitment and function of steroid receptors occurs in prostate cancer cells. In these cells, AR activation in the membrane leads to rapid transactivation of EGFR through membrane metalloproteinase-mediated release of EGFR ligands. This rapid activation of EGFR increases ERK signalling, which then mediates Ser phosphorylation of the scaffold molecule paxillin. Phosphorylated paxillin then enters the nucleus and interacts with the nuclear AR to help maintain nuclear localization and enhance AR-mediated transcription. In the nucleus, phosphorylated paxillin also stimulates the expression of ERK-dependent genes[9]. These effects promote cell proliferation (FIG. 3c). In fact, paxillin protein expression is upregulated in prostate cancer relative to benign prostate epithelium.

Crosstalk between membrane and nuclear pools of steroid receptors has also been reported for GR. Glucocorticoid binding to membrane-localized GR in neural progenitor and/or stem cells leads to rapid MAPK signalling and inhibition of gap junction connectivity between cells, which is necessary for proper tissue architecture and cell communication. Moreover, membrane-initiated glucocorticoid signalling was associated with modulating the overall GR transcriptome and was shown to promote the expression of glucocorticoid-responsive genes necessary for the anti-proliferative effects of glucocorticoids. These rapid and long-term effects of glucocorticoid signalling may, at least in part, be responsible for the negative effects of gestational glucocorticoid use on fetal neural development[64]. In addition, rapid gluco corticoid-mediated activation of ERK in the dentate gyrus of rodent brains modulates the effects of the transcription factor ELK1 and mitogenand stress-activated protein kinase 1 (MSK1; also known as RPS6KA5) on GR-mediated transcription and other transcriptional pathways, which may have important roles in stress responses[93]. Finally, in cells from male

mouse hypothalamus, glucocorticoids rapidly modulate G protein signalling and neurotransmitter actions at glutamate and γ-aminobutyric acid (GABA) synapses through glucocorticoid signalling initiated at the plasma membrane[94]. As well as the examples listed here, it is likely that membrane steroid receptor signalling modulates nuclear steroid receptor recruitment and target gene expression in other contexts and scenarios. Collectively, the crosstalk between extranuclear and nuclear steroid receptor pools might constitute an important regulatory paradigm in the cellular response to steroids.

Physiological implications

As mentioned in various examples above, membrane-initiated steroid signalling is frequently associated with proliferation-promoting effects and thus has been implicated in pathological growth in hormone-dependent tumours, such as breast and prostate cancers. Notably however, substantial evidence indicates that extranuclear steroid signalling from the membrane also regulates tissue and organ homeostasis throughout the body, as well as various aspects of metabolism. Understanding these processes has required the use of knockout and other important *in vivo* mouse models (BOX 1). Some of the examples of the physiological functions of membrane-initiated steroid signalling are discussed below.

Hormonal regulation of metabolism

Signalling from membrane ERα affects liver lipid metabolism—Determining the effect of rapid signalling initiated by the membrane-localized pool of steroid receptors requires selective mammalian models. This has been greatly facilitated by the generation of the membrane-only ERα (MOER) mouse, in which the gene expressing full-length ERα has been replaced with one expressing only the E domain of the receptor, thereby targeting the protein exclusively to the plasma membrane[95]. Cells isolated from organs of the MOER mouse responded to oestradiol with various rapid signals, including ERK1–ERK2 and PI3K–AKT activation, cyclic nucleotide generation and calcium flux. Importantly, rapid responses to oestradiol in wild-type and MOER mice, as well as in cells isolated from these animals, were comparable but absent in the full-knockout mice, supporting the idea that the E domain of the membrane receptor pool is sufficient for many extranuclear signals.

To determine the effect of membrane ERα signalling on gene transcription, wild-type, homozygous MOER and global ERα-knockout mice were ovariectomized (to eliminate endogenous oestrogen production) and injected with an ERα agonist. Microarrays of liver RNA showed significant suppression of mRNA expression on ERα stimulation mostly in wild-type mouse livers, indicating that the nuclear ERα pool is required for most genomic effects. However, 30 mRNAs were comparably suppressed by treatment with ERα agonist in livers of wild-type and MOER mice but were not suppressed in global ERα-knockout mice. These 30 mRNAs were mainly involved in lipid synthesis[84]. Comparable suppression of triglyceride, cholesterol and fatty-acid content in livers was seen in wild-type and MOER female mice, but not in global ERα-knockout mice. *In vitro* studies in hepatocytes from wild-type and MOER mice indicated that membrane ERα signalling leads to AMPK activation, causing phosphorylation of sterol regulatory element-binding protein 1 (SREBP1; also known as SREBF1) — the key transcription factor involved in sterol biosynthesis —

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thereby preventing SREBP1 nuclear localization. Specifically, AMPK-induced phosphorylation of SREBP1 prevented Golgi-located processing proteases S1P (also known as MBTPS1) and S2P (also known as MBTPS2) from physically associating with SREBP1, therefore precluding its proteolytic processing, which is necessary for nuclear translocation[84]. Thus, membrane ERα signalling exclusively inhibits some mRNAs and produces a metabolic phenotype that does not require nuclear ERα actions.

Membrane ERs regulate glucose metabolism—Oestrogen modulates multiple aspects of normal organ metabolism[96]. Recent studies suggest that oestrogen regulates glucose metabolism in mice and humans at least partly from membrane ER signalling. In particular, signalling from membrane ERα and ERβ has been shown to be important for promoting insulin sensitivity and insulin synthesis and secretion from betacells of the pancreas[80,97]. Specifically, membrane ERα signalling promotes the recruitment of the neurogenic differentiation factor (neuroD) transcription factor to the insulin promoter in pancreatic β-cells, thereby augmenting insulin synthesis. By contrast, membrane ERβ engages in stimulatory crosstalk with the atrial natriuretic peptide receptor in these same pancreatic cells, thereby stimulating the ATP-sensitive K^+ channels to release insulin.

The importance of membrane signalling through ER in glucose metabolism is particularly well evidenced in breast cancer cells (FIG. 4a). In these cells, glycolysis is the main source of ATP, cyclic nucleotides, NADPH and phospholipids, and it maintains reduced glutathione to mitigate oxidative stress. In high-glucose states, such as may occur in people with poorly controlled diabetes, membrane ER signalling through AKT enhances glucose uptake and glycolysis, contributing to the thriving of cancer cells[98]. Interestingly, high glucose levels and heightened insulin signalling promote aggressive development of breast cancer in mouse models[99,100], suggesting a possible function by which oestrogen and extranuclear ER stimulate the progression of this malignancy. Notably however, in tumours *in vivo*, inadequate blood flow, and therefore availability of nutrients such as glucose, frequently occurs, thereby limiting growth and promoting apoptosis. Modelling of decreased glucose availability in breast cancer epithelial cells has revealed that oestrogen, acting through ERα and ERβ, can also contribute to cancer growth in low-glucose conditions, in this case by activating AMPK[98]. AMPK activation causes suppression of AKT signalling and glycolysis at the same time, leading to the phosphorylation of pyruvate dehydrogenase, which increases its ability to process glucose through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation. This collectively promotes a metabolic shift away from glycolysis and into the TCA cycle, which results in more efficient usage of glucose to produce ATP (delivering energy for survival and growth) and the generation of citrate important for the synthesis of phospholipids (necessary to build new membranes for cell proliferation). In sum, these data indicate that membrane ER signalling stimulates metabolic plasticity that contributes to cancer cell survival and growth.

Tissue homeostasis

Membrane oestrogen signalling prevents heart muscle hypertrophy and fibrosis—It has been shown that signalling through membrane-localized ERβ is important for heart homeostasis, preventing hypertrophy and fibrosis of this organ (FIG. 4b). The

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membrane pool of this steroid receptor inhibits the activity of calcineurin (also known as protein phosphatase 3) in cardiac muscle cells (cardiomyocytes), which is involved in regulating their size (through regulation of growth) (FIG. 4b, left). In these cells, the hypertrophic peptide Ang II signals through Ang II receptor type 1 (AT1) to activate calcium flux, thereby stimulating the activity of calcineurin. Additionally, membrane ERβ signalling through PI3K stimulates expression of modulatory calcineurin-interacting protein 1 (MCIP1; also known as RCAN1), at both the mRNA and the protein levels. MCIP1 binds to cytoplasmic calcineurin at the catalytic subunit, preventing calcium-induced activation of the phosphatase. This in turn prevents dephosphorylation of transcription factor nuclear factor of activated T cells, cytoplasmic 3 (NFATc3), sequestering it in the cytoplasm and thereby preventing the stimulation of Ang II-induced genes that cause cardiomyocyte hypertrophy[101].

In addition, membrane ERβ signalling acts protectively against heart hypertrophy by affecting the expression and function of epigenetic modifiers — the HDACs (FIG. 4b, left). Hypertrophic peptides Ang II and endothelin 1 (ET-1; also known as EDN1) stimulate enhanced calcium-dependent activation of calcium–calmodulin-dependent kinase II (CaMKII), which phosphorylates the class II HDACs HDAC4 and HDAC5 to cause their exclusion from the nucleus. As a result, these HDACs cannot inhibit the nuclear actions of the transcription factor myocyte-specific enhancer factor 2 (MEF2), which stimulates hypertrophic gene expression. Membrane ERβ causes a $G_{\alpha i}$-induced inhibition of calcium flux, thereby preventing CaMKII activation and the downstream events that result in the retention of class II HDACs in the cytoplasm. As a result, HDAC4 and HDAC5 repress MEF2 function in the nucleus. Membrane ERβ also blocks the Ang II-stimulated PKC-PKD signalling axis that also phosphorylates and thereby promotes class II HDAC nuclear exclusion, but the mechanism by which membrane ERβ accomplishes this is unclear. Similarly, membrane ERβ blocks Ang II and ET-1-mediated activation of casein kinase 2 (CK2), which phosphorylates the pro-hypertrophic class I HDAC HDAC2, thereby promoting HDAC2 nuclear localization. As a result, HDAC2 is retained in the cytoplasm and its pro-hypertrophic activity is inhibited. Together, it seems that ERβ agonists could be one of the long-sought-after regulators of cardiac HDACs, preventing cardiac disease but not stimulating uterine or breast tissue proliferation mediated through canonical ERα.

Membrane signalling from ERβ also prevents heart fibrosis, by inhibiting the transition of fibroblasts into heart-resident myofibroblasts and the expression of fibrotic genes (FIG. 4b, right). Both of these effects are mediated by transforming growth factor-β (TGFβ) signalling, which in fibroblasts is promoted by Ang II. In this case, membrane ERβ signalling through cAMP and PKA inhibits TGFβ signalling-induced phosphorylation of transcription factors SMAD2 and SMAD3, thereby sequestering these transcription factors in the cytoplasm. This prevents SMAD2 and SMAD3 nuclear translocation and consequently the expression of fibrotic genes mediated by these transcription factors. By inhibiting elevation of TGFβ, membrane ERβ also prevents the transition of fibroblasts to heart-resident myofibroblasts. As a result, cardiac fibrosis can be prevented (both *in vitro* and *in vivo*)[102]. In these examples, membrane ERβ signalling is both required and sufficient for preventing the pathological state.

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Membrane ERα and LXRβ affect blood vessel repair—Oestrogen signalling through membrane-localized ERα in endothelial cells stimulates rapid activation of endothelial nitric oxide synthase, which then regulates arterial re-endothelialization and vasodilation[103] (FIG. 4c). Notably, in mice harbouring a mutation in the palmitoylation site of ERα, the ability of oestrogen to prevent acute vascular injury of the endothelium was absent[104]. This confirms that membrane ERα is necessary for this function of oestrogen. In the vasculature, the cholesterol metabolite 27-hydroxycholesterol (27HC) serves as an endogenous selective oestrogen receptor modulator (SERM)[105,106]. This cholesterol metabolite is produced by cholesterol 7α-hydroxylase in the endothelial cells, as well as in the liver, and acts as a competitive antagonist of the membrane pool of endothelial ERα, inhibiting the ability of oestrogen to stimulate nitric oxide formation and the concomitant re-endothelialization of the injured artery (FIG. 4c). In addition to its actions in endothelial cells, this endogenous SERM modulates ER signalling in breast cancer cells. Aromatase inhibitors used to reduce endogenous oestrogen production for the treatment of ERα-positive breast cancer commonly result in the acquisition of resistance to this therapy. This resistance has been associated with increased expression of 27HC in the tumour, and evidence suggests that this metabolite promotes ERα-mediated proliferation[107]. This indicates that breast tumours can produce the cholesterol metabolite to drive breast cancer progression when endogenous oestrogen production is insufficient. Signalling through ERK and PI3K from membrane ERα bound by 27HC probably contributes to the breast cancer epithelial cell proliferation, which contrasts with vascular endothelial cells, in which signalling in response to oestrogen is inhibited by this SERM.

Recent studies have shown that in the context of blood vessel repair membrane ERα does not operate alone but colocalizes with liver X receptor-β (LXRβ; also known as NR1H2)[108] (FIG. 4c). In line with this, the activation of LXR was shown to induce PI3K–AKT signalling, which was the first demonstration of a function for extranuclear LXR. This led to enhanced nitric oxide formation and aortic ring relaxation, and promoted carotid artery re-endothelialization. Notably, this extranuclear signalling by LXR required Ser118 phosphorylation of ERα, which facilitated the association of these two receptors, presumably occurring directly at the plasma membrane. Furthermore, this Ser118 phosphorylation of the membrane pool of ER was demonstrated to depend on LXR activation and to be necessary for stimulating the activity of endothelial nitric oxide synthase in response to both ER and LXR ligands[108]. This suggests that, as in the case of the nuclear ERα, in which Ser118 phosphorylation promotes transcriptional activity, this specific phosphorylation also regulates membrane steroid receptor function. In sum, these data show that membrane ERα and membrane LXR act together to regulate arterial homeostasis (FIG. 4c).

Organogenesis and organ functions

Importance of membrane ER signalling for uterine development—Many non-steroidal nuclear receptors contribute to the development and functions of organs[109–111]. In addition, most steroid receptors contribute to organ development[18,112,113]. To investigate roles of extranuclear steroid receptors, two groups developed mice lacking only membrane-localization of ERα[88,104] by replacing the wild-type *ESR1* gene with a gene expressing

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ERα containing a palmitoylation-site mutation (in which Cys451 was changed to Ala) (FIG. 2). Although these transgenic mice lack membrane expression of ERα, abundant nuclear ERα is present, as palmitoylation does not have an impact on nuclear localization. Cells from these nuclear-only ERα (NOER) homozygous mice failed to respond to oestradiol with kinase signalling that was comparably present in wild-type or heterozygous NOER mouse cells[88].

Various organ dysfunctions and abnormal phenotypes in the female homozygous NOER mice included infertility, owing to aberrant development of both the uterus and the ovaries. In addition, key developmental genes were not stimulated by oestradiol in multiple organs in these mice[88]. For instance, normal uterine development involves increased expression of connexin 43, lactoferrin, oxytocin and *IGF1* mRNAs; expression of all these genes, except for *IGF1*, was considerably reduced in NOER compared with wild-type mouse uteri. Inability of nuclear ERα alone to stimulate *PGR* mRNA expression contributed to an abnormal mammary phenotype in these female mice similar to that seen in mice with complete *PGR* gene deletion[114] (BOX 1). All abnormalities were reversed in heterozygous NOER female mice, comparable to wild-type mice.

Evidence suggests that ERβ, through non-genomic signalling from extranuclear pools, is also involved in pathological endometrial growth. ERβ signalling prevented tumour necrosis factor-induced apoptosis and inflammation of epithelial cells, and stimulated proliferation and invasion, in a mouse model of endometriosis[115]. Based on this work, the authors proposed that an inhibitor of the NCOA1 (also known as SRC1) transcription factor, which functionally complexes with ERβ outside the nucleus, could be used together with an ERβ antagonist in a future therapy for this painful disorder.

Membrane AR promotes follicle development in the ovar y—For many decades, androgen excess has been known to be detrimental to ovarian health, causing diseases such as polycystic ovary syndrome (PCOS), the leading cause of infertility in women. However, recent evidence suggests that physiological levels of androgen play a critical part in normal ovarian function by regulating the growth of ovarian follicles, mediated in large part through extranuclear AR[33,34]. Specifically, androgens advance ovarian follicle development from the pre-antral to the antral stage, which is the last step before oocytes are released during ovulation (FIG. 4d). Similarly to the scenario in prostate cancer cells discussed above (FIG. 3c), androgens bind to classic extranuclear AR in ovarian granulosa cells to promote transactivation of EGFR through the rapid release of membrane-bound EGFR ligands. On activation, EGFR triggers ERK signalling through interactions with the scaffold molecule paxillin. This signalling cascade then promotes increased expression of follicle-stimulating hormone receptor (FSHR) in a transcription-independent fashion (most likely by increasing translation). Additionally, as discussed above, ERK mediates Ser phosphorylation of paxillin. Phosphorylated paxillin then enters the nucleus and assists nuclear AR, in this case stimulating transcription of microRNA miR-125b, which suppresses apoptosis. Suppression of apoptosis, plus enhancement of the signalling mediated by FSH owing to the increase in FSHR expression, promotes follicle growth and improves ovulation[33,34]. Thus, extranuclear signalling and nuclear AR signalling collaborate to promote normal ovarian function (FIG. 4d).

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Conclusions and perspectives

Significant research using *in vitro* and *in vivo* strategies has provided overwhelming evidence that steroid receptors exist in multiple subcellular locations and that they are required for full steroid hormone action during development and organ homeostasis. These receptors exist in most organs and cells and therefore their roles are diverse and widespread. In some cases, extranuclear steroid receptors function directly at the plasma membrane (or in other extranuclear compartments, such as mitochondria) and elicit transcription-independent responses (in particular, activation of cell signalling cascades) that regulate important biological processes. Alternatively, extranuclear receptors can influence gene expression and can engage in crosstalk with nuclear receptor pools to collectively regulate physiological and pathological processes.

Although some details of extranuclear receptor trafficking, function and effects on cell and organismal physiology are known, future studies are needed to fully understand the signalling instigated by these extranuclear pools. Strategies to achieve this goal will involve the development of better labelling techniques of individual receptors and their subcellular pools, as well as more advanced microscopy methods to follow cellular trafficking to distinct locations. Furthermore, we need a better understanding of signal transduction from extracellular compartments, particularly to the nucleus. This includes studies of how the dynamics and timing of membrane-generated signals are coordinated with chromatin states and with the regulated recruitment of nuclear receptors and co-regulatory proteins to drive changes in gene expression. Finally, researchers must continue to develop sophisticated *in vivo* models, allowing better understanding of the cooperation between nuclear and extranuclear steroid receptor pools. Shedding light on these aspects will provide us with a better understanding of steroid hormone signalling. In this way, we can develop new means of interceding with the pathological signals and maximizing the beneficial effects of steroid hormones, thereby opening possibilities to flexibly modulate a myriad of important biological processes, including reproduction, metabolism and cancer.

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Glossary

Co-regulators

Proteins that complex with other transcriptional regulatory proteins to alter gene expression

Epigenetic regulation

Modification of gene expression that does not involve the modification of the genetic code itself

G protein

Guanine nucleotide-binding protein that initiates signal transduction

Ovarian granulosa cells

Cells that surround and support the oocyte in ovarian follicles and contribute to steroidogenesis and follicle growth

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Caveolae

Lipid-containing invaginations at and within the plasma membrane in which caveolins serve as structural coat proteins and as scaffolds for many signalling molecules

Osteocytes

Multinucleated bone cells that break down bone matrix

Osteoblasts

Bone cells that generate new bone matrix

Leydig cells

Androgen-producing cells in the testes

Corpus luteum

A hormone-secreting structure that develops from the remains of the ovarian follicle after ovulation

Enhancers

Short regions of DNA that are bound by proteins (transcriptional regulators) to stimulate transcription

SH3 domains

Src-homology domains that are sites of physical protein–protein interactions

Histone acetyltransferases

Enzymes that add acetyl groups to proteins, most notably histones, thereby contributing to epigenetic regulation of gene expression

Ductal branching

The extending of hollow milk ducts through the mammary glands (like branches on a tree)

Histone deacetylases

(HDACs). Enzymes that remove acetyl groups from histones, thereby contributing to epigenetic regulation of gene expression proteins

Chromatin writers, erasers and readers

Proteins that, respectively, place, remove and interpret chemical modifications on histone proteins

Gap junction

A specialized intercellular connection between cells

Dentate gyrus

Region of the hippocampus in the brain that is thought to regulate memories and other functions

Hypothalamus

Region of the brain that links the nervous system to the endocrine system by signalling with the pituitary

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Selective oestrogen receptor modulator

(SERM). A molecule similar but distinct from oestradiol that interacts with oestrogen receptors as either an agonist or an antagonist, depending on the tissue context

Aortic ring

An angiogenesis model using cultured aortic cells

Carotid artery

An artery coming off the aorta that supplies the brain with blood

Endometriosis

Unregulated proliferation of the uterine endometrium outside the uterus

Ovarian follicles

Spheroid regions within the ovary that each contain and nurture an oocyte until ovulation and also produce sex steroids

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Box 1

Phenotypes of mice with perturbed oestrogen receptor pools

Comparable to total oestrogen receptor-α (ERα)-deletion mice (ERKO mice), both membrane-only ERα (MOER) and nuclear-only ERα (NOER) transgenic mice show infertility (see the table). This is attributed to maldevelopment of the ovaries, with impaired ovarian follicle formation, haemorrhagic cysts obliterating the normal cells and lack of corpus luteum formation. As a result of the corpus luteum deficiency, progesterone levels in the blood are very low, and the females do not undergo oestrus cycling and ovulation, contributing to the observed infertility. In addition, the epithelium of the uterus is hypoplastic in these animals and does not respond to oestradiol, indicating a poorly functional endometrium. This also contributes to the infertility. In both MOER and NOER mice, serum oestradiol levels are at least twofold the levels in wild-type mice, and luteinizing hormone (LH) in the pituitary (which is responsible for regulating oestrogen production in the ovary) is not suppressed, indicating loss of the negative-feedback regulation of LH by oestradiol. This is consistent with the findings in ERKO mice and indicates that both receptor pools are required for negative-feedback suppression of LH. Other pituitary hormones are also affected. Secretion of prolactin (a hormone involved in lactation) is diminished in NOER mice compared with wild-type mice, in which only ovariectomy and the loss of oestrogen result in decreased prolactin in the blood. These results indicate that the membrane receptor pool is necessary for the regulation of prolactin synthesis and secretion from the pituitary gland. In the mammary gland, the inability of oestrogen to stimulate the expression of progesterone receptors or progesterone production (from the ovary) results in a clear phenotype of diminished ductal side branching and loss of terminal-end bud formation. This phenotype is also seen in female ERKO mice and indicates that both ERα pools contribute to proper development of the mammary gland. Apart from female infertility and abnormal mammary gland development, other phenotypes associated with the deletion of ER pools have been described (see the table).

Phenotype (compared with wild-type)	MOER	NOER	ERKO
Pituitary hormone secretion • Luteinizing hormone increased • Prolactin decreased	Yes	Yes	Yes
Increase in serum oestrogen levels	Yes	Yes	Yes
Decrease in serum progesterone levels	Yes	Yes	Yes
Abnormal ovarian development • Abnormal follicles • Lack of corpus lutea	Yes	Yes	Yes
Female infertility	Yes	Yes	Yes

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Mammary gland maldevelopment and malfunction • Lack of ductal branching • Impaired end termini formation	Yes	Yes	Yes
Hypoplasia of uterine endometrium	Yes	Yes	Yes
Male infertility and sperm abnormalities	Yes	Yes	Yes
Impairment of oestrogen-induced protective response to arterial injury	Yes	No effect	Yes
Oestrogen-induced suppression of lipid synthesis in the liver and adipocytes	Yes	No effect	No effect
Visceral adiposity	Yes	Yes	Yes
Decreased total mRNA expression in various organs	Many various mRNAs	A few selected mRNAs	Most mRNAs

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Box 2

Mitochondrial steroid receptors

The presence of mitochondrial glucocorticoid receptor (GR) and oestrogen receptors (ERs) has been reported in various cell types[116,117]. Mitochondrial GR, ERα and ERβ seem to be the classic receptors but it is unclear how they localize to this organelle. Both steroid receptors regulate oxidative stress that often originates in the mitochondria[118,119]. In rodent breast cancer xenograft models, it has been shown that the cytotoxic response to tamoxifen (a selective ER modulator) is in fact mediated by the membrane pool of ERβ. In responsive cells, tamoxifen engages mitochondrial ERβ as an antagonist. In this way, this drug acts to inactivate manganese superoxide dismutase through a mechanism involving nitration of this enzyme, subsequent production of superoxide and upregulation of superoxide-induced apoptosis[120]. By contrast, tamoxifen resistance to these effects occurs because changes in ERβ structure and/or interactions with surrounding molecules lead to tamoxifen acting more like an ERβ agonist than an antagonist. It is likely that mitochondrial pools of other nuclear receptors exist and have important cellular functions, but this area is currently largely understudied.

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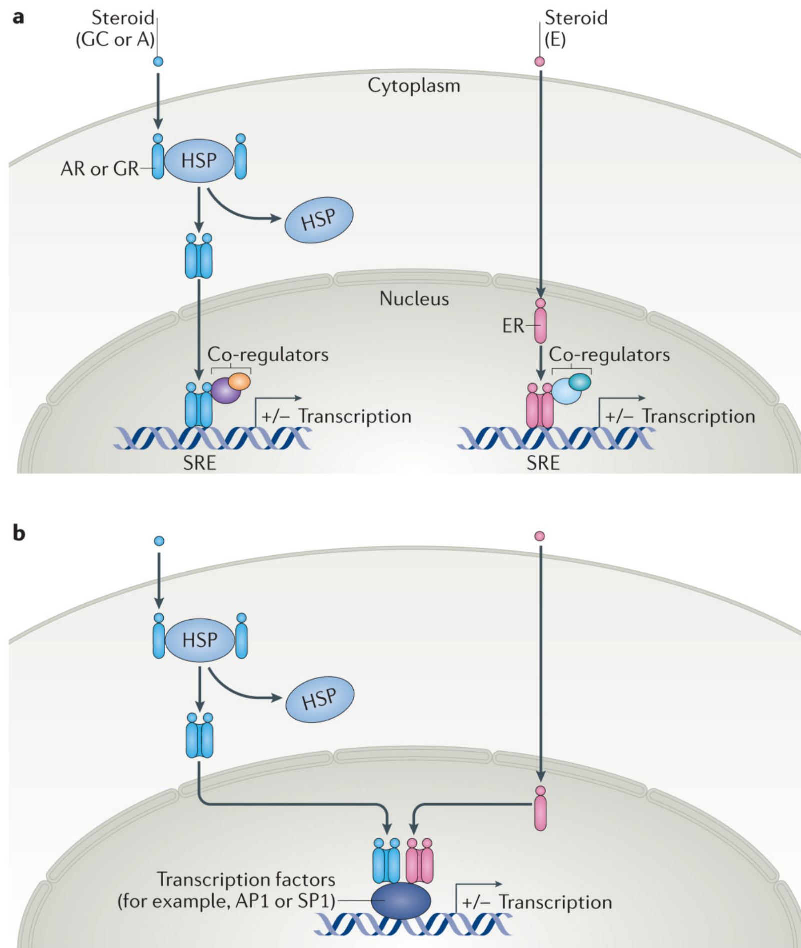

Figure 1. Nuclear steroid signalling

a | Classic steroid sinalling pathway. Steroids enter cells through mechanisms that are still not understood. Some steroid receptors, such as glucocorticoid (GC) and androgen (A) receptors (GR and AR, respectively), are primarily in the cytoplasm as monomers bound to heat shock proteins (HSPs). Others, such as the oestrogen (E) receptor (ER), are located as monomers primarily in the nucleus, although a small percentage may also be bound to HSPs in the cytoplasm (not shown). In the case of GC and A, steroid binding to cytoplasmic receptors triggers release from the HSPs, receptor dimerization, alterations in receptor conformation and nuclear localization. In the case of E, the sex steroid binds to nuclear receptors to promote dimerization and changes in receptor conformation. In all cases, nuclear dimerized receptors then bind to specific steroid-response elements (SREs) and interact with various co-regulators to modulate gene transcription through either repression or activation. **b** | Tethered steroid signalling. Nuclear steroid receptors can also modulate gene expression without direct DNA binding. In this case, they bind to other transcription factors, such as AP1 or SP1, to either repress or activate transcription.

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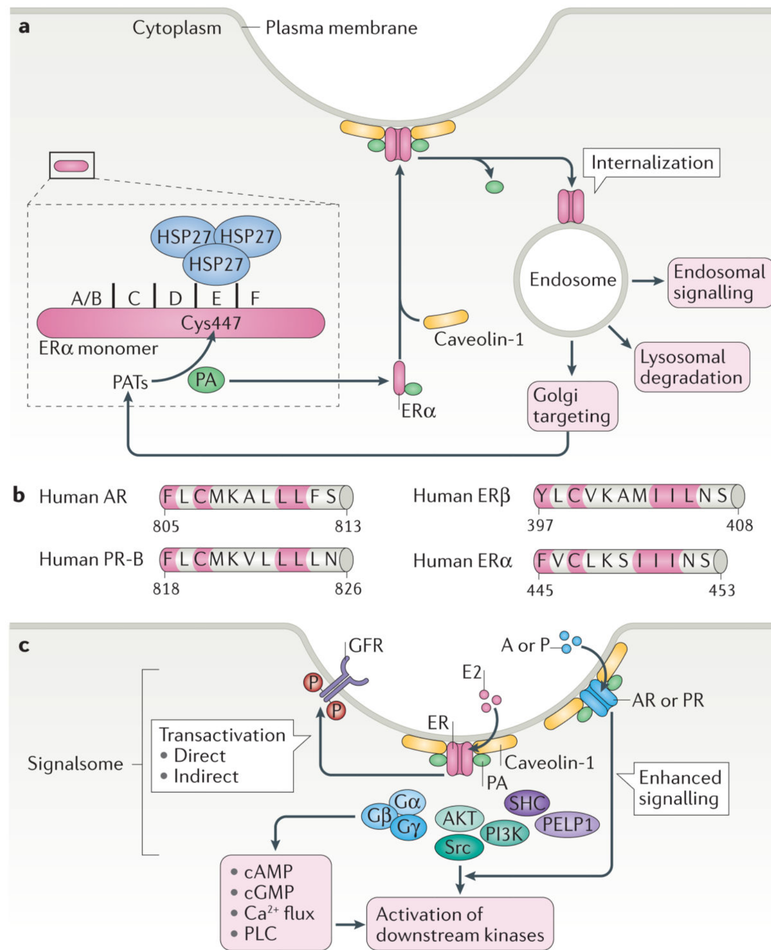

Figure 2. Events dictating trafficking of oestrogen receptor-α to and from the plasma membrane and its signal transduction

a | Monomeric oestrogen receptor-α (ER α_B) is sequentially bound by heat shock protein 27 (HSP27) molecules and palmitoyl acyltransferases (PATs) that attach palmitate (PA) to Cys447 of the E domain of this steroid receptor. Following palmitoylation, the steroid receptor associates with caveolin-1, which targets palmitoylated receptors to the caveolae of the plasma membrane, where they engage in signalling (see below in part **c**). Following de-palmitoylation, ERα undergoes internalization into endosomes, which can potentially result in alternative, endosomal signalling, lysosomal degradation of the receptor (and thus termination of signalling) or targeting of the internalized receptors to the Golgi for a subsequent round of palmitoylation. **b** | The palmitoylation motif is highly conserved in sex steroid receptors, and mutation of any of the residues indicated in pink severely restricts palmitoylation and subsequent trafficking of the receptors to, and their function at, the plasma membrane. **c** | Steroid receptors at the membrane are part of a large signalling complex, referred to as the signalsome, and mediate various signalling responses. For example, membrane-localized ERα can bind to the steroid hormone oestradiol (E2). Subsequent signal transduction from the membrane occurs through the physical interaction of membrane ERα with G proteins and kinases (such as AKT, Src and PI3K), which induce downstream signalling events (such as calcium flux, the generation of cyclic nucleotides and

the activation of phospholipase C (PLC), leading to further signal propagation by various downstream kinases), as well as through the interaction of membrane ERα with linker proteins (including Pro-, Glu- and Leu-rich protein 1 (PELP1) and Src-homology 2 domain-containing protein (SHC)), which enhance signalling by mechanisms that are unclear. Steroid receptors at the membrane can also collaborate with growth factor receptors (GFRs) and have been implicated in GFR transactivation (directly by inducing their phosphorylation (P) or indirectly by promoting the expression and/or activation of metalloproteinases, resulting in the release of GFR ligands from the extracellular matrix; see also FIG. 3c) and thus in signalling from these receptors. In addition, ERα can associate with other membrane-localized sex steroid receptors (such as androgen receptor (AR) and progesterone receptor (PR)) and cooperate in signal transduction on ligand binding.

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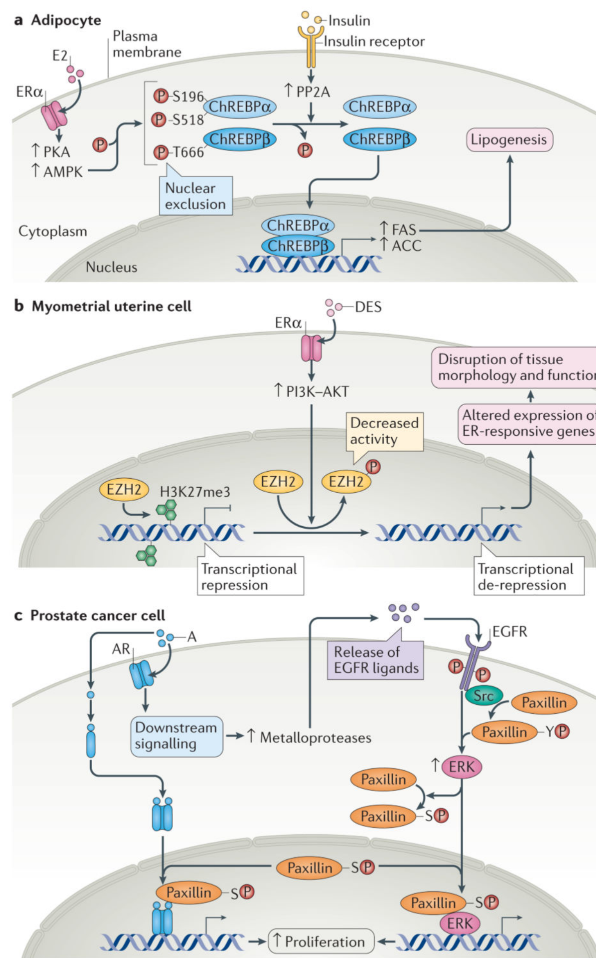

Figure 3. Membrane-localized steroid receptors can modulate gene expression in the nucleus

a | In adipocytes, following binding of the steroid hormone oestradiol (E2), oestrogen receptor-α (ERα) engages in phosphorylation (P) of Ser (S) and Thr (T) residues in subunits of carbohydrate-responsive element-binding protein (ChREBP) — a key transcription factor regulating the expression of genes involved in lipid biosynthesis. This phosphorylation (occurring through the activation of downstream kinases protein kinase A (PKA) and AMP-activated protein kinase (AMPK)) counteracts the dephosphorylation events (involving protein phosphatase 2 (PP2A)) stimulated by the binding of insulin to the insulin receptor and precludes ChREBP nuclear translocation. This decreases the expression of genes involved in lipogenesis, such as fatty acid synthase (*FAS*) and acetyl-CoA carboxylase (*ACC*; also known as *ACAC*). **b** | When bound to the xenoestrogen diethyl-stilboestrol (DES) in myometrial uterine cells, ERα activates the PI3K-AKT signalling pathway, which results in phosphorylation of an epigenetic modifier, enhancer of Zeste homologue 2 (EZH2), which functions as a histone methyltransferase and introduces repressive trimethylation of histone H3 at Lys27 (H3K27me3). Phosphorylation of EZH2 decreases its histone methyltransferase activity and leads to derepression of genes, including ER-responsive genes. This altered expression of ER-responsive genes has been shown to disrupt uterine tissue morphology and function, increasing susceptibility to tumorigenic

transformation. **c |** Extranuclear and intranuclear steroid signals can also cooperate in regulating gene expression. In prostate cancer cells, androgen (A) binds to membrane-localized androgen receptor (AR). This binding results in downstream signalling that activates metalloproteinases, which then act to release ligands of the membrane epidermal growth factor receptor (EGFR) from the extracellular matrix. This leads to transactivation of EGFR to trigger AKT (not shown) and ERK signalling. Activation of ERK signalling in part requires Src-mediated Tyr (Y) residue phosphorylation of paxillin. When ERK is activated, it regulates Ser phosphorylation of paxillin. Ser phosphorylated paxillin then enters the nucleus and assists in modulating both nuclear AR-mediated transcription and nuclear ERK-mediated transcription, promoting cell proliferation. Membrane AR signalling, in collaboration with nuclear signalling, therefore regulates prostate cancer epithelial cell growth.

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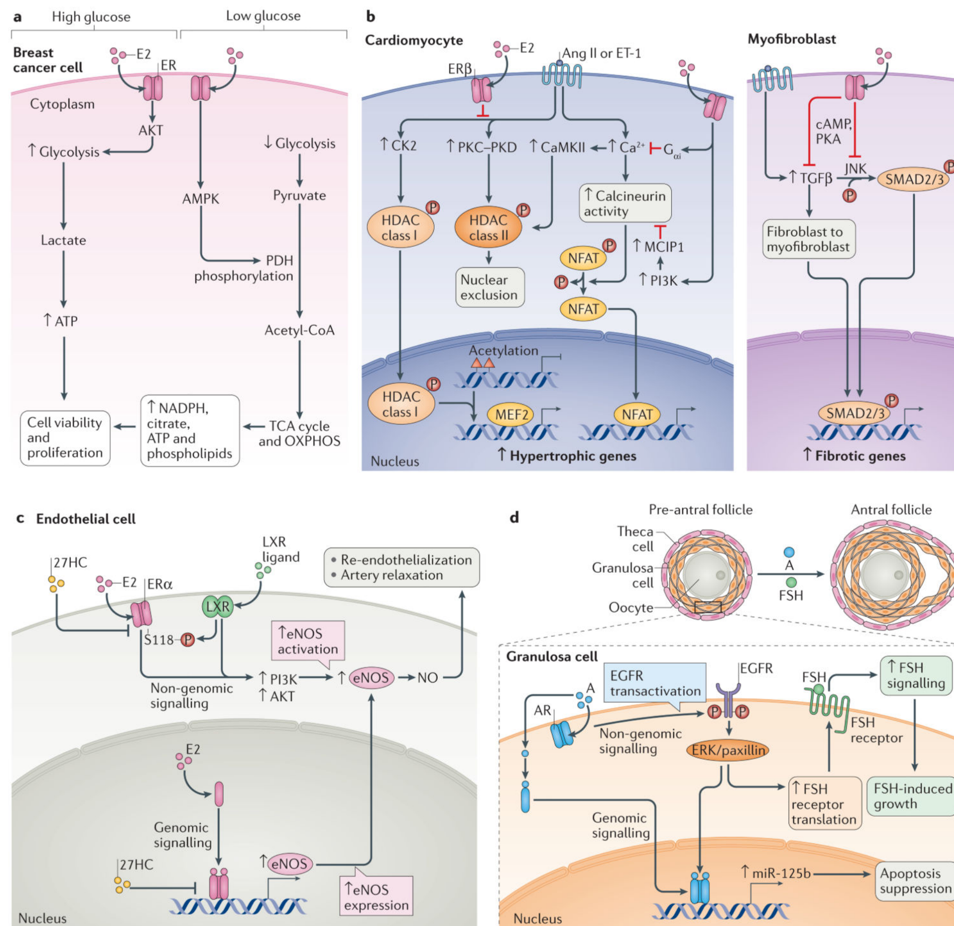

Figure 4. Membrane steroid signalling in the regulation of metabolism, organ homeostasis and organogenesis

a | In breast cancer cells, membrane oestrogen receptor-α (ERα) and ERβ respond to the steroid hormone oestradiol (E2) to provide adaptation to glucose availability. When glucose is abundant, membrane ERs, through AKT activation, promote glucose uptake and use for glycolysis (the main source of energy for tumour growth), whereas when glucose levels are low, membrane ERs activate AMP-activated protein kinase (AMPK), which stimulates pyruvate dehydrogenase (PDH) to promote the metabolism of glucose through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS), thereby enhancing cell viability under nutrient stress. **b** | In cardiomyocytes (left panel), membrane ERβ signalling upregulates modulatory calcineurin-interacting protein 1 (MCIP1), which binds to and inhibits calcineurin. Consequently, the transcription factor nuclear factor of activated T cells (NFAT) is not efficiently dephosphorylated and cannot translocate to the nucleus in response to pro-hypertrophic peptides angiotensin II (Ang II) and endothelin 1 (ET-1), meaning that it cannot engage in the transcription of hypertrophic genes. Ang II and ET-1 also activate calcium–calmodulin-dependent kinase II (CaMKII) and the protein kinase C (PKC)–PKD signalling axis, which both lead to the phosphorylation (P) of the anti-hypertrophic class II histone deacetylases (HDACs), leading to their nuclear exclusion. Membrane ERβ counteracts this phosphorylation. Similarly, membrane ERβ blocks Ang II-

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and ET-1-mediated activation of casein kinase 2 (CK2), which phosphorylates the pro-hypertrophic class I HDAC HDAC2, thereby promoting its nuclear localization. As a result, HDAC2 is retained in the cytoplasm and cannot deacetylate and activate pro-hypertrophic gene targets. In cardiac myofibroblasts (right panel), Ang II and ET-1 stimulate transforming growth factor-β (TGFβ) signalling, inducing the fibroblast to myofibroblast transition and the phosphorylation of the SMAD2 and SMAD3 transcription factors that is required for their nuclear translocation and subsequent stimulation of fibrosis-inducing genes. Membrane ERβ signalling inhibits this TGFβ axis, thereby preventing heart fibrosis. **c** | In endothelial cells, membrane ERα collaborates with membrane liver X receptor-β (LXRβ) and nuclear ERα signalling to stimulate the activity of endothelial cell nitric oxide synthase (eNOS), which leads to the generation of nitric oxide (NO) that then promotes artery relaxation and re-endothelialization of blood vessels, allowing endothelial repair after injury. 27-hydroxycholesterol (27HC) inhibits the actions of both the membrane and the nuclear ERα pools, acting as an endogenous selective ER modulator in this context. **d** | Androgens (A) enhance the effects of the pituitary hormone follicle-stimulating hormone (FSH) on the development of ovarian follicles. This requires both nuclear (genomic) and extranuclear (non-genomic) androgen receptor (AR) actions. Membrane AR transactivates epidermal growth factor receptor (EGFR), thereby activating ERK signalling and phosphorylation of paxillin (see also FIG. 3c). Through as-yet-unknown mechanisms, this then leads to increased translation of the FSH receptor. ERK activation and paxillin phosphorylation also lead to enhanced nuclear AR-mediated transcription of the microRNA miR-125b, which suppresses the expression of pro-apoptotic genes. This integrated response to androgens promotes follicle survival and growth. JNK, c-JUN N-terminal kinase (also known as MAPK); MEF2, myocyte-enhancing factor 2.

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Table 1

Membrane localization and signalling of steroid hormone receptors

Receptor	Isoforms	Downstream signals
Oestrogen receptor-α	66 kDa, 46 kDa and 36 kDa	• G protein activation: calcium flux, cyclic nucleotide production (cAMP and cGMP) • Kinase activation
Oestrogen receptor-β*	57 kDa	• G protein activation: calcium flux, cAMP and cGMP • Kinase activation
Progesterone receptor	A (94 kDa) and B (116 kDa)	• Kinase activation
Androgen receptor	110 kDa	• G protein activation • Kinase transactivation
Glucocorticoid receptor	α (97 kDa), β (94 kDa) and possible splice variants of these isoforms	• Calcium flux • Kinase activation
Mineralocorticoid receptor	Splice variants of 107 kDa and 105.4 kDa isoforms	• Calcium flux • Kinase activation • Nitric oxide production
Thyroid hormone receptor	• α: 30 kDa, 33 kDa, 43 kDa and 48 kDa • β1: 30 kDa and 53 kDa	• Calcium flux • Cyclic nucleotide production (cAMP and cGMP) • Kinase activation
Vitamin D receptor	48 kDa and possible splice variants of this isoform	• Calcium flux • Kinase activation

*Also found in mitochondria, where it stimulates nitric oxide production.