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The extra-nuclear interactome of the estrogen receptors: implications for physiological functions.

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Abstract.

Over the last decades, a great body of evidence has defined a novel view of the cellular mechanism of action of the steroid hormone 17 β -estradiol (E2) through its estrogen receptors (i.e., ER α and ER β). It is now clear that the E2-activated ERs work both as transcription factors and extra-nuclear plasma membrane-localized receptors. The activation of a plethora of signal transduction cascades follows the E2-dependent engagement of plasma membrane-localized ERs and is required for the coordination of gene expression, which ultimately controls the occurrence of the pleiotropic effects of E2. The definition of the molecular mechanisms by which the ERs locate at the cell surface (i.e., palmitoylation and protein association) determined the quest for understanding the specificity of the extra-nuclear E2 signaling. The use of mice models lacking the plasma membrane ER α localization unveiled that the extra-nuclear E2 signaling is operational *in vivo* but tissue-specific. However, the underlying molecular details for such ERs signaling diversity in the perspective of the E2 physiological functions in the different cellular contexts are still not understood. Therefore, to gain insights into the tissue specificity of the extra-nuclear E2 signaling to physiological functions, here we reviewed the known ERs extra-nuclear interactors and tried to extrapolate from available databases the ER α and ER β extra-nuclear interactomes. Based on literature data, it is possible to conclude that by specifically binding to extra-nuclear localized proteins in different sub-cellular compartments, the ERs fine-tune their molecular activities. Moreover, we report that the context-dependent diversity of the ERs-mediated extra-nuclear E2 actions can be ascribed to the great flexibility of the physical structures of ERs and the spatial-temporal organization of the logistics of the cells (i.e., the endocytic compartments). Finally, we provide lists of proteins belonging to the potential ER α and ER β extra-nuclear interactomes and propose that the systematic experimental definition of the ERs extra-nuclear interactomes in different tissues represents the next step for the research in the ERs field. Such characterization will be fundamental for the identification of novel druggable targets for the innovative treatment of ERs-related diseases.

Keywords: 17 β -estradiol, estrogen receptor, extra-nuclear signaling, plasma membrane receptors, endocytosis

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1. Introduction.

*We shall not cease from exploration
And the end of all our exploring
Will be to arrive where we started
And know the place for the first time.
Through the unknown, remembered gate
When the last of earth left to discover
Is that which was the beginning.*

Little Gidding V – Four Quartets.
Sir T.S. Eliot (1943).

In ‘The Structure of Scientific Revolutions’, Dr. Thomas Khun has clarified that the definition of scientific truth does not solely derive by applying objective criteria, but it is established by a shared consensus of a scientific community. Thus, scientific knowledge does not progress linearly and continuously but rather it undergoes periodic paradigm shifts that open up new approaches to understanding what scientists would never have considered valid before (Khun, 1962). In other words, the understanding of a natural phenomenon is an integrated mixture between the ‘objective’ results arising from experimentations and their ‘social’ interpretation in the context of a specific scientific historical background.

The estrogen and estrogen receptor field represents a great example of how novel discoveries contribute to paradigm definitions and paradigm shifts. However, this same field also nicely shows how difficult is to introduce in the scientific community unconventional findings, which may not reconcile with the ‘social’ aspect of science but are fitting in the ‘objective’ understanding of the natural phenomenon.

In this respect, the Wikipedia definition of estrogens reads that ‘they are a group of compounds [*i.e.*, estrone (E1), estriol (E3) and 17 β -estradiol (E2), the most effective one] named for their importance in both menstrual and estrous reproductive cycles. They are the primary female sex steroids. [...] Their name comes from the Greek *οἶστρος* (oistros), literally meaning verve or inspiration but figuratively sexual passion or desire, and the suffix *-gen*, meaning producer of’. The basic textbook definition of estrogens, instead, reads that ‘they are steroid hormones produced in ovary and adrenal cortex and are the dominant steroid in females’ (Silverthorn, 2007).

As steroid hormones (*i.e.*, small lipophilic molecules that freely diffuse across cell membranes), estrogens act by engaging the estrogen receptors (ERs) that, as again defined by Wikipedia, ‘are a group of proteins found inside cells that are members of the nuclear hormone family of intracellular receptors. Once activated by estrogen, the ERs can translocate into the nucleus and bind to DNA to regulate the activity of different genes’. A very similar definition can be also found in textbooks where the ERs are ‘found within cells, either in the cytoplasm or in the nucleus. The ultimate destination of ER-hormone complexes is the nucleus, where the complex acts as a transcription factor, binding to DNA and regulating gene transcription’ (Silverthorn, 2007).

Consequently, the ‘social’ perception of estrogens is clearly that they are female sex steroid hormones acting exclusively in female reproductive tissues and organs by triggering the activity of their receptors, which are transcription factors. The effects of estrogens then derive from a change in the gene expression profile of the target cells that results in a phenotypic alteration of their specific cellular behavior.

Although these definitions are undoubtedly true, they represent only a part of the complex mode of estrogen action through the ERs (Table 1). Indeed, the actual inclusive definition of this particular scientific field that takes into account all the ‘objective’ knowledge accumulated during years should

read as follows. In target cells, estrogens specifically act through at least two ERs subtypes, which work both as transcription factors and as plasma membrane receptors and mediate the physiological effects of these sex steroid hormones in reproductive and non-reproductive tissues both in females and in males. This latter definition underscores a shift from a limited perspective (*i.e.*, traditional view) to a wider one in which estrogens in general and 17 β -estradiol (E2), in particular, are global regulators of integrated physiology and thus of body homeostasis.

This definition derives from the concepts demonstrated by the contribution of different investigators in the last 60 years or so, which can be organized in historical periods. Clearly, such timeline does not have to be considered as composed by isolated periods in which only one specific line of research was pursued. Rather, we name them after the main discoveries that occurred in that particular period of time, but we additionally stress the concept that the accumulation of the knowledge of the different aspects of E2 signaling occurred (and still occurs) in parallel (Figure 1).

The Biochemical Age (1960s-1970s) represents the infancy of the estrogen and estrogen receptor field and has been instrumental in the definition that E2 specifically binds to a 'receptor protein' inside the target cell and also to understand that E2-induced administration causes changes in gene transcription because this hormone elicits transcriptional effects (Buller & O'Malley, 1976; Jensen & Jacobson, 1962; Noteboom & Gorski, 1965). This period set the pace for the Nuclear Age (1980s-2000) where most of the knowledge in the field accumulated. Indeed, by the end of the last century, it became clear that the 'receptor protein' in estrogen target cells is indeed one of the two ERs subtypes (*i.e.*, ER α and ER β) and that the effects of estrogens *in vivo* are dependent on both ER α and ER β that work as transcription factors in the nucleus (Brzozowski *et al*, 1997; Dupont *et al*, 2000; Green *et al*, 1986a; Green *et al*, 1986b; Kuiper *et al*, 1996; Lubahn *et al*, 1993; Ogawa *et al*, 1998; Schwabe *et al*, 1993a; Schwabe *et al*, 1993b).

The subsequent Extra-Nuclear Age (2001-2015) has demonstrated that the same ERs are located at the cell plasma membrane from which they signal for the activation of rapid effects (AKA extra-nuclear/non-genomic/membrane-initiated starting signals) that regulate diverse cellular processes, integrate with the ERs transcriptional actions and are operational *in vivo* (Acconcia *et al*, 2005a; Acconcia *et al*, 2004; Adlanmerini *et al*, 2014; Jakacka *et al*, 2002; La Rosa *et al*, 2012; Li *et al*, 2003; Pedram *et al*, 2014; Pietras & Szego, 1977; Razandi *et al*, 1999).

And yet, this might still represent only a part of the entire story as the research performed during the last five years (2016-2021) has shown the harmonization of the nuclear and extra-nuclear ERs field (*i.e.*, the age of cross-talk) thanks to the deep analysis of the phenotypes of the mice models in which the extra-nuclear E2 signaling has been selectively knocked out (Adlanmerini *et al*, 2014; Farman *et al*, 2018; Fontaine *et al*, 2020; Gustafsson *et al*, 2016; Nanjappa *et al*, 2016; Pedram *et al*, 2014; Vinel *et al*, 2016; Yu *et al*, 2020). Therefore, this period lays the foundations for the next future paradigm shifts. Indeed, reports indicate that E2 and the membrane-bound ER α could be internalized through an active mechanism for hormone uptake by complex endocytic trafficking routes (Adams, 2005; Hammes *et al*, 2005; Sampayo *et al*, 2018; Scheidt *et al*, 2010; Totta *et al*, 2016; Totta *et al*, 2015a; Totta *et al*, 2015b; Totta *et al*, 2014). Moreover, it is now clear that the extra-nuclear rapid effects mediated by the ERs can be specifically modulated by different ERs exogenous ligands (*e.g.*, xenoestrogen like nutritional flavonoids) determining a modification of ERs-based signaling to physiological functions (Acconcia *et al*, 2016; Acconcia *et al*, 2015; La Rosa *et al*, 2014; Marino *et al*, 2012).

Notwithstanding all this evidence, still, the concept that E2 elicits rapid actions modulating different aspect of cell physiology by acting through the same nuclear receptor, which is located outside of the nucleus is a debated issue as witnessed by the fact that in the last years research about the novel additional membrane receptor for estrogens (*i.e.*, GPR30 also known as GPER1) and its

connections with the E2-regulated effects in diverse cellular contexts (Pepermans *et al*, 2021) has exponentially exploded (Fig. 1B). The accumulation of novel information regarding the role of GPER1 in E2 signaling has driven the ‘social’ perception of E2 signaling towards the picture that, while the E2-dependent regulation of gene expression is due to the functions of the classic ERs, which can only work as nuclear transcription factors, the E2-elicited extra-nuclear effects are mostly ascribed to the activity of the non-classic seven-spanning membrane receptor for E2 (i.e., GPER1).

Perhaps, one of the reasons for which the above-mentioned ‘objective’ knowledge (i.e., the same nuclear ERs are also located at the plasma membrane and trigger E2 extra-nuclear signaling *per se*) is being underestimated resides in the fact that on the contrary to the knowledge regarding the molecular interactions the ERs exploit to regulate gene expression in the nucleus (i.e., the ERs nuclear interactome) (Cirillo *et al*, 2013; Gigantino *et al*, 2020; Giurato *et al*, 2018; Metivier *et al*, 2003; Nassa *et al*, 2011a; Nassa *et al*, 2011b; Reid *et al*, 2003; Tarallo *et al*, 2011), there is a lack of understanding of the molecular mechanisms activated by the extra-nuclear-localized ERs in association with other proteins (i.e., the ERs extra-nuclear interactome) for generating the diversity of rapid signal transduction pathways and physiological effects in the different cellular contexts.

Therefore, we reviewed here the reported physical interactions of the ERs with other proteins in the extra-nuclear compartment to provide a picture of the extra-nuclear ERs interactomes and their possible physiological implications in the appearance of the E2-dependent effects.

2. The relationship among the pleiotropic effects of E2 and the extra-nuclear signaling.

The definition of a possible molecular link among the physiological pleiotropic functions of E2 with the cellular signaling diversity generated by the ERs in different contexts requires the discussion of the available phenomenological data on these aspects.

2.1 The pleiotropic effects of E2

E2 is a critical regulator of reproductive tissue physiology both in males and in females. This crystal-clear evidence continues to bias many scientists that consider these tissues as the only E2 target tissues.

Indeed, E2 exerts many effects that go far beyond the regulation of reproductive tissue physiology: in bone, E2 contributes to the maintenance of a healthy skeleton both in males and in females because it inhibits bone resorption (Cauley, 2015). The effect of E2 in the cardiovascular system are well known (Ueda *et al*, 2019) and can be direct on the blood vessel walls (Arnal *et al*, 2017) but also indirect since this hormone controls the serum lipid and cholesterol content (De Marinis *et al*, 2008; Pallottini *et al*, 2008). E2 is also a metabolic hormone as it regulates the integrated physiology of those tissues and organs that regulate lipid homeostasis (Pallottini *et al*, 2008) and a role for E2 in the regulation of skeletal muscle cell differentiation has also been reported (Galluzzo *et al*, 2009). Another important non-reproductive E2 target tissue is the central nervous system (McEwen & Alves, 1999) where E2 exerts many different physiological functions including the regulation of synapse formation (Brandt & Rune, 2020), neuronal cell survival (Ascenzi *et al*, 2016; De Marinis *et al*, 2013) as well as the control of many different neurotransmitter systems (Fink *et al*, 1996). The ability of E2 to regulate epithelial ion transport in the lungs, kidney, intestine sweat gland is critical for the regulation of whole-body fluid homeostasis and gut absorption/secretion balance (Alzamora *et al*, 2011a; Alzamora *et al*, 2011b; O'Mahony *et al*, 2009; Rapetti-Mauss *et al*, 2013) (O'Mahony *et al*, 2007; Saint-Criq *et al*, 2012) (Table 2). On the other hand, in male, E2 plays a physiologic role during prostate development by programming prostate stromal cells and directing early morphogenic events (Prins *et al*, 2006).

Thus, it should not be surprising that this hormone elicits also many different beneficial effects against different diseases including osteoporosis, cardiovascular and neurodegenerative diseases (Ascenzi *et al*, 2006). Finally, E2 also contributes to the modulation of the immune system physiology (Chotirmall *et al*, 2010; Kovats, 2015) and may also be considered as an interkingdom signaling (Hughes & Sperandio, 2008) molecule as it can affect also bacterial physiology (Chotirmall *et al*, 2012; Tyrrell & Harvey, 2020).

2.2 The E2 signaling diversity

All the above-mentioned physiological effects depend on the convoluted nature of the E2 signaling via the ERs as a function of the expression of the ER α and the ER β . Indeed, the tissue distribution of the ERs can consist in the single expression of one isoform but also in their co-expression. Also, the cellular abundance of ERs is not static but it varies depending on the time and the tissue physiological state (Nilsson & Gustafsson, 2011).

It is well known that such different receptor distribution generates different genome expression patterns in each E2-target tissue. Indeed, as transcription factors, in the nucleus, the E2-activated ERs bind to the estrogen-responsive elements (ERE) sequences within the promoter of E2-target genes. The E2:ERs complexes bound to the gene promoters become nucleation sites for many transcriptional co-activators or co-repressors that are required for the regulation of gene transcription. While co-repressors impede the activation of the molecular mechanisms required for the association of the basal transcriptional apparatus, the recruitment of co-activators (e.g., steroid receptor coactivators - SRC) to the activated E2:ERs complex allows for the engagement of those nuclear protein complexes containing enzymes for the DNA:histones unfolding and for the chromatin unmasking, thus determining the activation of DNA transcription (Wang *et al*, 2016). Gene transcription proceeds in a cyclical manner for which the receptors cycle on-and-off their target gene promoters in an oscillatory trend, which allows the recruitment in an ordered and sequential manner all the co-activator proteins required for RNA PolIII-induced productive mRNA synthesis (Metivier *et al*, 2003; Reid *et al*, 2003). The clearance of the activated receptor by the cellular proteolytic pathways (e.g., 26S proteasome for the ER α) guarantees the coordination of the transcriptional waves with the pulsatile nature of the E2 fluctuations in the bloodstream (La Rosa & Acconcia, 2011; Metivier *et al*, 2003; Reid *et al*, 2003). The ERs can increase the repertoire of the regulation of E2-target genes as they can bind to additional transcription factors like AP-1, SP-1, and NF-kB (Ascenzi *et al*, 2006), which in turn address the receptor to other non-ERE containing genes.

The E2-evoked nuclear actions (i.e., nuclear mechanism) are integrated by the rapid effects of E2 (i.e., extra-nuclear signaling) that are generated by the activation of the same nuclear ERs, which are located outside the nuclear envelope (Acconcia *et al*, 2005a; Acconcia *et al*, 2004; La Rosa *et al*, 2012; Marino *et al*, 2006; Pietras & Szego, 1977; Razandi *et al*, 1999; Szego & Davis, 1967) (Norfleet *et al*, 1999; Pappas *et al*, 1995). Interestingly, the ‘extra-nuclear estrogen signaling’ pathway has been recently added as a category in the <https://reactome.org/> database (stable identifier: R-HSA-9009391).

The ‘extra-nuclear estrogen signaling’ is independent of the ERs receptor transcriptional activity, it originates in seconds to minutes after E2 administration to ERs expressing cells and can be activated by compounds in which E2 has been immobilized to a specific matrix (e.g., E2-BSA; E2-dendrimers) (Acconcia & Marino, 2011; Kim *et al*, 2015). A vast arrays of cytoplasmic signal transduction cascades have been identified as the main actors of the E2:ERs extra-nuclear signaling [e.g., Ca²⁺ liberation from intracellular stores (Chaban *et al*, 2004; Doolan & Harvey, 1996; Roper *et al*, 2002); phospholipase C (PLC)/protein kinase C (PKC) (Distefano *et al*, 2002; Marino *et al*, 2001); Src/extracellular-activated kinase (ERK) (Castoria *et al*, 2001; Marino *et al*, 2002; Marino *et al*, 2003; Migliaccio *et al*, 2000), phosphatidyl-inositol 3 kinase (PI3K)/AKT (Marino *et al*, 2002; Marino *et al*, 2003; Simoncini *et al*, 2000), p38/mitogen-activated protein kinase (MAPK); Janus kinase/signal transducers and activators of transcription (JAK/STAT) (Bjornstrom & Sjoberg, 2002);

p21-activated kinase 1 (Pak1) (Rayala *et al.*, 2006); focal adhesion kinase (FAK) (Acconcia *et al.*, 2006a; Sanchez *et al.*, 2010); RhoA/ROCK- 2/moesin (Simoncini *et al.*, 2006); Ataxia Telangiectasia Mutated/Checkpoint Kinase 1 (ATM/CHK2) and Ataxia Telangiectasia and Rad3-Related Protein/Checkpoint Kinase 1 (ATR/CHK1) (Caldon, 2014; Jimenez-Salazar *et al.*, 2021; Pescatori *et al.*, 2021)] (Fig. 2). Ultimately, extra-nuclear signaling could result in distinct transcriptional events, different from those induced by the classical activation of ERE-containing genes. A deep analysis on the permissive and potentiating effects of extra-nuclear signaling on the nuclear effects of E2 actions has been the focus of excellent reviews and will not be discussed here (Hammes & Levin, 2019; Levin, 2005; Levin & Hammes, 2016).

2.3 The dynamics of E2 signaling

The activation of such signaling pathways has been linked to the regulation of the above-mentioned physiological effects of the hormone.

The Src, ERK/MAPK, PI3K/AKT, and p38/MAPK regulate the balance between the E2-induced proliferative and anti-proliferative effects (Castoria *et al.*, 2001; Marino *et al.*, 2002; Marino *et al.*, 2003; Migliaccio *et al.*, 2000). The integrin-linked kinase (ILK), the FAK, the RhoA/ROCK-2/moesin, and WAVE1 activation mediates the effects of E2 on cytoskeleton remodeling and cell migration (Acconcia *et al.*, 2006a; Acconcia *et al.*, 2006b; Sanchez *et al.*, 2010; Simoncini *et al.*, 2006). An Src/Shc/ERK signaling pathways is required for the regulation of E2 effects in bone (Kousteni *et al.*, 2001) while a PLC/PKC pathway is active in liver-derived cells for the regulation of LDL receptor and thus lipid metabolism (Distefano *et al.*, 2002; Marino *et al.*, 2001) and E2-dependent effects on vascular physiology (e.g., dilation and re-endothelization) are regulated by PI3K/eNOS (Guo *et al.*, 2005; Haynes *et al.*, 2002; Simoncini *et al.*, 2002) (Fig. 2).

2.4 The topology of the origin of the extra-nuclear E2 signaling.

The extra-nuclear E2 effects are generated because E2 engages the ERs that are located at the cell plasma membrane (Fig. 2).

One of the main puzzles the field of ERs has faced during the years was the mechanism through which E2 could activate the extra-nuclear signaling via receptors that belong to the nuclear receptor protein superfamily and mainly work as transcription factors in the nucleus (Ascenzi *et al.*, 2006). Indeed, strong evidence allowed the assumption that the activations of the above-mentioned signaling cascades could depend on a transcriptional feedback loop determined by the ability of E2 to increase the cellular expression of both growth factor receptors (i.e., insulin-like growth factor receptor - IGF1R; epidermal growth factor receptor – EGFR) and growth factors (e.g., IGF1). In this model, the increased amount of growth receptors to the cell surface and the increased secretion of its cognate ligands would have been the cause for the E2-dependent increase in the activation status of the cytoplasmic kinases (Salvatori *et al.*, 2003; Schiff *et al.*, 2004).

However, as pointed above, the extra-nuclear E2 signaling precedes the ERs transcriptional actions and occurs with a kinetic incompatible with the activation of the transcriptional functions. Therefore, the most plausible hypothesis was the possibility that cells would express an estrogen receptor at the plasma membrane.

Careful inspection of the literature reveals that E2 rapid effects were already reported before (Szego & Davis, 1967) the discovery of E2-induced transcriptional actions (O'Malley & McGuire, 1968). Later E2 binding sites to the outer surface of E2-target cells were identified (Pietras & Szego, 1977) and subsequently demonstrated to be the same nuclear ERs localizing at this subcellular compartment (Pedram *et al.*, 2006; Razandi *et al.*, 1999).

The mechanism by which the nuclear ERs associate with plasma membrane was then discovered. Our group initially hypothesized that the ERs (as well as other nuclear receptors) could associate to the inner leaflet of the plasma membrane by lipid modifications (i.e., palmitoylation) that would have facilitated their plasma membrane insertion by increasing their hydrophobicity (Acconcia *et al.*, 2003). Evidence for this pioneering hypothesis was initially reported in an ER α isoform (i.e.,

ER46) (Li *et al.*, 2003) and later discovered in our laboratory for the ER α (Acconcia *et al.*, 2004) and the ER β (Galluzzo *et al.*, 2007). In humans, ER α palmitoylation occurs on the cysteine (C) residue 447 and mutation of the ER α palmitoylation site [i.e., to alanine (A) – C447A mutant] prevents receptor localization to the plasma membrane, the activation of the rapid signaling cascades (*e.g.*, ERK/MAPK, PI3K/AKT), the activation of ER α -mediated gene transcription and promoter occupancy and, consequently, the E2-induced effects. Remarkably, the fact that these molecular phenotypes were phenocopied by the chemical inhibition of the palmitoyl-acyl-transferase (PAT) strongly implicated this enzyme as the regulator of ER α palmitoylation (Acconcia *et al.*, 2005a; Acconcia *et al.*, 2004; La Rosa *et al.*, 2012; Marino *et al.*, 2006). Other groups corroborated these discoveries, identified the PATs required for receptor palmitoylation and further extended the concept that palmitoylation is a conserved mechanism for steroid hormone receptor plasma membrane localization and function (Pedram *et al.*, 2012; Pedram *et al.*, 2007).

Notwithstanding all these ‘objective’ evidence, the ‘social’ perception of the ER paradigm still held up with the strongest argument being the fact that the physiological function *in vivo* was not yet demonstrated.

Ten years after the initial discovery that the plasma membrane localization of the ERs was dependent on palmitoylation the definitive proof was reported that the extra-nuclear E2 effects are physiologically relevant in an animal model. Indeed, two independent research groups generated knock-in mice models lacking the ER α palmitoylation site and consequently the extra-nuclear effects of E2 (Adlanmerini *et al.*, 2014; Pedram *et al.*, 2014). Despite differences in these two mice models, the analysis of their phenotypes demonstrated that the extra-nuclear E2 actions depend on the plasma membrane localization of the receptor *in vivo*, integrate with the transcriptional actions elicited by the ER α located in the nucleus, and are relevant for organ development and functions (Adlanmerini *et al.*, 2014; Pedram *et al.*, 2014).

The link between the E2-dependent activation of the extra-nuclear signaling pathways and the plasma membrane localization of the ER α through palmitoylation was further demonstrated by evaluating the above-mentioned physiological effects of E2 in different tissues in the mice models mutated in the ER α palmitoylation site. Results demonstrate that extra-nuclear E2 signaling is active for the physiology of the ovary (Adlanmerini *et al.*, 2014; Pedram *et al.*, 2014), bone, uterus, thymus (Farman *et al.*, 2018; Gustafsson *et al.*, 2016; Pedram *et al.*, 2014; Vinel *et al.*, 2016), mammary gland (Fontaine *et al.*, 2020; Pedram *et al.*, 2014), testis (Nanjappa *et al.*, 2016) and also controls both E2-dependent vascular actions (Adlanmerini *et al.*, 2014), and the feeding behavior (Yu *et al.*, 2020).

3. The problem of the cell-type specificity of the extra-nuclear E2 signaling.

Altogether the evidence reported above represents a paradigm shift, which moves the E2 field from an exclusively ERs transcriptional-driven view to an integrated ERs molecular mechanism for which the nuclear and extra-nuclear actions do not exist anymore as separate aspects of receptor functioning but rather represent the unique and exquisite intertwined mode of E2 cellular effects. However, skepticism is still in place, as demonstrated by the fact that a growing body of literature is supporting the concept that the E2-elicited extra-nuclear effects are ascribed to the membrane protein GPER1 (Pepermans *et al.*, 2021) (Fig. 1B).

One possible reason for which the debate is still open can be ascribed to the fact that both E2, its ERs, and their consequent extra-nuclear molecular mechanisms are strictly cell-type dependent and tissue-specific as demonstrated by the analysis of the different phenotypes in the mice models defective in the membrane ER α signaling (Adlanmerini *et al.*, 2014; Farman *et al.*, 2018; Fontaine *et al.*, 2020; Gustafsson *et al.*, 2016; Nanjappa *et al.*, 2016; Pedram *et al.*, 2014; Vinel *et al.*, 2016; Yu *et al.*, 2020).

Interestingly, although, to date, there is not any report, which systematically addresses the underlying molecular details for the context-dependent effects of E2, the foundations of this

pleiotropic nature can be linked both to the dynamics of the movements of the ERs biochemical structure and to the nature of the E2 stimulation.

3.1 The ERs biochemical structure.

The ER α and the ER β belong to the nuclear receptor superfamily of proteins with which they share the same biochemical anatomy. The primary protein sequence is organized into six modular domains (named from A to F) (Fig. 3A upper picture). The C domain, which is also known as the DNA binding domain (DBD), contains two ‘zinc-finger’ motifs that mediate the physical contact of the receptors with the DNA helix. The E domain, which is also known as the ligand-binding domain (LBD), contains one β -sheet wrapped in 12 α -helices, binds E2, and is required for receptor activation. The C and E domains are connected by the D domain (i.e., hinge) that is the site of many different receptor post-translational modifications (Le Romancer *et al.*, 2011). The A/B domain confers to the receptors different functions including the control of ERs transcriptional activity while the F domain is a biochemical module with various functions (Ascenzi *et al.*, 2006; Patel & Skafar, 2015).

Although a detailed description of the biochemical structural properties of the ERs is given in excellent focused reviews (Ascenzi *et al.*, 2006; Katzenellenbogen *et al.*, 2018), it is worth pointing out here that the entire three-dimensional structure of ERs is not available. Possibly bioinformatic prediction of the ERs structure could be now pursued by exploiting the artificial intelligence machine learning-based platform AlphaFold (Senior *et al.*, 2020).

However, it is known that the folded C and E domains are interspersed within the other domains, which are intrinsically disordered regions (IDRs) (Fig. 3A, lower picture).

IDRs, which are highly abundant in the eukaryotic proteome especially in chromatin-associated proteins, render the overall structure of the protein extremely flexible and increase the repertoire of the protein interacting partners, thus allowing the same protein to be included in diverse signal transduction pathways. IDRs are also themselves the target of many different post-translational modifications (PTMs) (e.g., phosphorylation) (Bosso *et al.*, 2019).

In the ERs, the structural plasticity is indeed given to its IDRs: it has been shown that the A/B domain of the receptor assumes a defined three-dimensional structure depending on the protein interacting partner it is associated with (Yi *et al.*, 2015) and that the unfolded receptor portions are the target of diverse PTMs (e.g., A/B and D domains) (Le Romancer *et al.*, 2011), which direct the recruitment of the ERs to specific interacting partners (e.g., phosphorylation-dependent recruitment of transcriptional co-activators) (Dutertre & Smith, 2003).

Besides, notwithstanding their organized structure, both the DBD and the LBD are plastic parts of the ERs as DBD changes its conformation as a function of the association to specific DNA sequences and the LBD assumes a different folding as a function of the compound lodging in its ligand-binding pocket (Ascenzi *et al.*, 2006).

3.2 The ERs structural plasticity in the subcellular compartments.

Although the detailed description regarding the structural signal transduction allowing the modification of the ERs IDRs as a function of the allosteric transitions in the DBD and/or the LBD is still not known, the structural dynamics of the ERs is witnessed by their ability to assume different conformations in a spatial-dependent manner. Indeed, the systematic characterization of panels of different antibodies directed against different epitopes of the ERs demonstrated that ERs expose different epitopes in different subcellular compartments (Dan *et al.*, 2003; Welsh *et al.*, 2012) (Fig. 3B).

In 2003, Dan and co-workers used a panel of 3 anti-ER α and 3 anti-ER β antibodies to characterize the subcellular localization of these two proteins in endothelial cells (Dan *et al.*, 2003). By using a combination of deconvolution, 3-D imaging, and nuclear labeling, the Authors demonstrated that the majority of extra-nuclear ER α expose different epitopes from their intranuclear counterparts. Moreover, they provided evidence that this phenomenon is cell type-independent but cell-specific as some antibodies that recognized the receptor outside of the nucleus in some cell lines

were not able to detect it in other cell lines (Dan *et al.*, 2003). Subsequently, another technical work tested a panel of ER α -specific antibodies directed against different regions of the ER α and found that while antibodies recognizing the A/B domain of the receptor detected only the nuclear ER α , the antibodies binding to epitopes of the receptor that are located within the E domain spotted ER α both in the nuclear and in the extra-nuclear compartments (Welsh *et al.*, 2012). This differential epitope-dependent localization was further demonstrated in different cell types (Totta *et al.*, 2014) (Fig. 3B). These data show that ER α assumes different conformations both if it is inside or if it is outside of the nucleus and among cell types.

Although ER β was found to be predominantly nuclear and did not show a complex epitope-dependent localization as for the ER α (Dan *et al.*, 2003), criticisms have been put forward regarding the quality of the anti-ER β antibodies used (Andersson *et al.*, 2017a, b). However, on the basis that both ERs share a very high degree of sequence homology and structural conformations (Ascenzi *et al.*, 2006), it is reasonable to believe that also ER β can expose different epitopes in different subcellular compartments.

3.3 The nature of the E2 stimulation.

Another variable that impinges on the context-specific effects of E2, ERs, and their relative mechanisms of action is the E2 concentration- and time-dependent effects.

Indeed, E2 plasma levels physiologically fluctuate (from low pM to 1 nM at the ovulation peak) (Zittermann *et al.*, 2000) and *in vitro* the concentration-dependent effects of E2 on, for instance, gene transcription or DNA synthesis are described by a bell-shaped curve in which low- and supra-physiological concentrations of E2 (*i.e.*, pM and μ M, respectively) have the same effects (Pescatori *et al.*, 2021). Similar behavior has been recently reported also in animal models where different tissues are differentially sensitive to low or high levels of E2 (Fontaine *et al.*, 2020). Additionally, real-time live-cell analyses of E2:ER α -mediated ERE-based transcription indicates that the transcriptional activity of the E2:ER α complexes is rapid and maximal already at low doses (*e.g.*, 1 - 10 pM) as early as 3 hours after E2 administration (Cipolletti *et al.*, 2020), thus suggesting that the time of hormonal exposure rather than its concentration could be a more stringent pre-requisite for receptor function. Interestingly, by using a two-pulse E2 schedule followed by E2 withdrawal in a concentration-dependent manner, it was shown that extra-nuclear E2 actions were a pre-requisite for the activation of the E2-induced transcriptional activity, thus showing that short time of extra-nuclear signaling activation potentiates transcriptional functions (Vasudevan *et al.*, 2001).

Therefore, the differential extra-nuclear responses could be also ascribed to the different effects of the E2 time and concentration exposure to different cellular or tissue contexts.

4. The ER α and ER β interactomes.

Because the ERs do not possess any intrinsic enzymatic activity, the only possible manner through which these receptors can account for the vast array of the E2 effects is their ability to differentially bind to specific protein interactors.

4.1 The nuclear binding partners of the ERs.

The ER α and the ER β interacting partners in the nucleus (*i.e.*, nuclear ERs interactome) have been reported and studied primarily in breast cancer cells both in the presence and in the absence of ligand stimulation. According to the ligand administration the nuclear ERs interactomes change so that the ligand-bound ER interactome is very different from the apo-ER interactome (Cirillo *et al.*, 2013; Gigantino *et al.*, 2020; Giurato *et al.*, 2018; Nassa *et al.*, 2011a; Nassa *et al.*, 2011b; Tarallo *et al.*, 2011).

Although a systematic description of nuclear interactomes is out of the scope of the present review, it is worth supporting this concept by reporting the differential effects of 4OH-tamoxifen (4OH-Tam) in the breast and endometrial tissues (Ascenzi *et al.*, 2006). 4OH-Tam is the prototype selective estrogen receptor modulator (SERM). It directly binds to the ER α and inhibits its

transcriptional activity. However, while 4OH-Tam is an antagonist of the receptor in the breast tissue, it is an agonist of the ER α in the endometrial tissue (Lumachi *et al.*, 2011). The molecular basis underlying this dual effect is dependent on the ability of the 4OH-Tam to induce an ER α conformation that recruits transcriptional co-repressors in the breast while the same 3D-folding can recruit transcriptional co-activators in the endometrial cells (Ascenzi *et al.*, 2006; Brzozowski *et al.*, 1997). In this way, the same conformation of the receptor in a different cellular context originates two different cellular outcomes.

4.2 The extra-nuclear binding partners of the ERs.

To date, an organized view of the extra-nuclear binding partners of the ER α and the ER β is not available but their characterization would be paramount in understanding the molecular basis of the specificity and the selectivity of the pleiotropic nature of the extra-nuclear E2 effects. Notably, the association of several extra-nuclear localized proteins to the ERs has been reported, although most of the evidence refers to the ER α extra-nuclear binding partners while fewer ER β interactors outside the nucleus have been identified and characterized, possibly because of anti-ER β antibodies are less reliable than the anti-ER α ones (Andersson *et al.*, 2017a, b).

4.2.1 Signaling molecules and adaptors

Many signaling molecules have been identified as functional binding partners, which directly implicate the receptor in the kinase pathways activated by E2. In particular, the ER α physically interacts with components of the ERK/MAPK and PI3K/AKT pathways.

4.2.1.1 Src and PI3K

The non-receptor tyrosine (non-RTK) kinase Src physically associates to the E domain of the ER α (Migliaccio *et al.*, 1998) (Fig. 4A). This physical association is triggered by E2 administration and prevented by the pure anti-estrogen ICI182,780 (Migliaccio *et al.*, 1998). The formation of this complex however also occurs in basal conditions and leads to two important consequences. On one hand, it mediates the E2-dependent activation of the ERK/MAPK kinase pathway (Migliaccio *et al.*, 1996) and on the other hand, it determines the phosphorylation of the ER α on the tyrosine (Y) residue 537 (Arnold *et al.*, 1995a), which in turn increases the receptor transcriptional activity (Arnold *et al.*, 1995b). Remarkably, it is now known that the mutation of the Y537 to serine (S) (i.e., Y537S) within the ER α represents one of the most common receptor variant driving metastatic breast cancer (Busonero *et al.*, 2019), thus underscoring the importance of Src activity for E2-dependent cell proliferation.

The evidence implicating the ER α in the PI3K/AKT pathway came from the work of Dr. Simoncini (Simoncini *et al.*, 2000) who reported for the first time that the receptor binds in an E2-dependent manner to the p85 regulatory subunit of the PI3K (Fig. 4A). E2 increased ER α :p85 association, which was required for AKT activation, eNOS-dependent activity, and the E2 protective effects on the vasculature. As for any extra-nuclear activated pathway, such signaling cascade was independent of gene transcription and inactivated by ER α inhibitors (e.g., ICI182,780) (Simoncini *et al.*, 2000). Remarkably, this initial discovery was then later confirmed in many different cellular contexts and it is now known that the PI3K/AKT pathway is a conserved extra-nuclear E2-activated kinase cascade for the regulation of many E2-dependent effects (e.g., cell proliferation and cell migration) (Acconcia & Marino, 2011). Other components of the PI3K/AKT pathway have been reported to bind ER α . The integrin-linked kinase (ILK) has been shown to bind the receptor both in vitro and in cell lines (Fig. 4A). E2-induced the dissociation of the ER α :ILK, thus allowing ILK activation and the PI3K/AKT/ILK-dependent regulation of cell migration (Acconcia *et al.*, 2006b). The ER α is also a substrate for glycogen synthase kinase-3 (GSK-3 β), which is a known AKT substrate. It has been shown that the GSK-3 β :ER α complex (Fig. 4A) is required for ER α stabilization under basal conditions and modulates ER α transcriptional activity upon E2 administration (Medunjanin *et al.*, 2005).

Interestingly, the E2-dependent activation of the PI3K/AKT pathway is independent of the SH domain of p85 (Simoncini *et al.*, 2000). As the SH domain-dependent association requires the interacting protein to be phosphorylated on a Y residue (Huang *et al.*, 2008), that evidence implied that the Src-dependent phosphorylation of the ER α was not required for the E2-dependent activation of the PI3K/AKT pathway, thus suggesting that the receptor could regulate in parallel both the ERK/MAPK and the AKT signaling cascade.

4.2.1.2 Receptor tyrosine kinase (RTKs) and Shc

RTKs represent signaling docking sites for the parallel E2-dependent activation of the ERK/MAPK and PI3K/AKT pathways. The IGF-1 receptor (IGF1-R) becomes phosphorylated upon E2 administration (Richards *et al.*, 1996; Richards *et al.*, 1998) and E2-induced IGF1-R phosphorylation requires the physical interaction of ER α with IGF-1R (Kahlert *et al.*, 2000), because phosphorylated IGF-1R also recruits the p85 subunit of the PI3K and E2 triggers ER α :p85 association (Simoncini *et al.*, 2000), the receptor becomes translocated to the this RTK. The cytoplasmic adaptor protein Shc that interacts with the IGF1-R has been shown to bind to ER α and to mediate the association of the receptor with IGF1-R (Fig. 4A). E2 triggers the formation of the ER α /Shc/IGF1-R complex and requires the ER α activity as ICI182,870 blocks the formation of this macromolecular complex. Shc is then believed to recruit a macromolecular complex containing also Grb2/Sos to IGF1-R in an E2-dependent manner, thus leading to the activation of ERK/MAPK (Boonyaratanakornkit, 2011; Song *et al.*, 2004).

4.2.1.3 p38/MAPK

The activation of the extra-nuclear E2 signaling by ER β has received less attention and in turn, fewer extra-nuclear ER β interactors have been reported. Nonetheless, work performed in our laboratory has identified the p38/MAPK as a conserved extra-nuclear ER β -activated pathway. Indeed, E2 activates this signaling kinase in different ER β -expressing cells (e.g., ER β -transfected HeLa cells, rat myoblasts, colon adenocarcinoma cells, and neuroblastoma cells) (Acconcia *et al.*, 2005b; De Marinis *et al.*, 2010; Galluzzo *et al.*, 2007; Galluzzo *et al.*, 2009). Analysis of the mechanisms through which this hormone allows the p38/MAPK activation resulted in the definition that the ER β physically associates in a ligand-dependent manner to p38/MAPK (Fig. 4A). This interaction is then required for the E2 anti-proliferative and pro-apoptotic effects mediated by the E2:ER β complex (Galluzzo *et al.*, 2007).

4.2.1.4 Other receptors

Other extra-nuclear interactors of the ER α and the ER β are the same ER α and ER β that can either homo- and heterodimerize at the level of the plasma membrane. Indeed, it has been shown that endogenous ERs at the plasma membrane exist as homodimers when cells are treated with E2, and dimerization is required for the ER α -dependent activation of extra-nuclear E2 signaling (Razandi *et al.*, 2004). Interestingly, the impact of ER β homodimers, the ER α /ER β heterodimers as well as their ligand-dependent modulation in the extra-nuclear compartment has yet to be defined.

A direct interaction between ER α and GPCRs (G $_{\alpha i}$ and G $_{\beta \gamma}$) has also been demonstrated to mediate E2-induced extra-nuclear signaling (Kumar *et al.*, 2007), and the association of ER α with $\beta 1$ -integrin was reported to control E2-dependent effects on the extracellular matrix (Sampayo *et al.*, 2018) (Fig. 4A).

4.2.2 Plasma membrane scaffolding proteins

Specific plasma membrane proteins have been reported to associate with both ER α and ER β and to control extra-nuclear E2 signaling.

4.2.2.1 Caveolins

Both ER α and ER β physically interact with caveolins (i.e., caveolin-1, caveolin-2 and caveolin-3, Cav1, Cav2, and Cav3, respectively) in caveolae (Fig. 4B).

Caveolae are ω -shaped membrane invaginations where many signaling molecules and adaptors are grouped (Resh, 2006). The first evidence demonstrating that the ERs bind to caveolins was reported by the group of Dr. Levin in 2002 (Razandi *et al.*, 2002). In this seminal work, the authors showed the presence of the ERs in purified vesicles from endothelial cell plasma membranes. Moreover, ER α physically interacted with Cav1 and Cav2 and E2 differentially triggered the ER α :caveolin association in different cell lines. The Cav1:ER α interaction was mapped both on Cav1 and on the ER α . Indeed, Cav1 scaffolding domain is required for ER α translocation to the plasma membrane and the S residue 522 within the ER α is necessary but not sufficient for the association of the receptor to Cav1 (Razandi *et al.*, 2003; Razandi *et al.*, 2002). Association of Cav1 with the ER α was later found by our group to be dependent on receptor palmitoylation as mutation of the ER α palmitoylation site abrogated such interaction (Acconcia *et al.*, 2005a).

Also, ER β interacts with Cav1, but in this case, E2 increases the amount of the Cav1:ER β complex (Galluzzo *et al.*, 2007). Association of ER α and ER β with Cav1 and Cav2 shows a degree of tissue specificity as it has been recently shown that ER α but not ER β form a complex with Cav1 in uterine artery endothelial cells (Pastore *et al.*, 2019).

Finally, it has been also reported that the ER α can interact with Cav3 in cardiomyocytes and that this association is required for conveying extra-nuclear E2 signaling also in this cellular context (Chung *et al.*, 2009).

4.2.2.2 Striatin

The 110-kDa Cav1-binding protein striatin has been also identified as an ER α interactor that facilitates the receptor localization to the plasma membrane and allows the E2-induced activation of ERK/MAPK, PI3K/AKT, and eNOS as E2 triggers the formation of the striatin:ER α complex (Fig. 4B), which is prevented by the pure anti-estrogen ICI182,780 (Lu *et al.*, 2004). Structure-function relationships in the striatin:ER α association revealed that striatin binds to the region of the A/B domain of the ER α encompassing the amino acids 183-253 (Lu *et al.*, 2004). More detailed analysis revealed that the consensus binding site of ER α on striatin is a group of 3 amino acids [i.e., lysin (K) 231 and arginine (R) 233 and 234]. Mutation of the ER α in the KRR site abrogated striatin binding, reduced extra-nuclear E2 signaling but did not affect the nuclear transcriptional activity of the receptor (Lu *et al.*, 2016).

Overall, the ER α appears to be sandwiched between striatin through its A/B domain (i.e., N-terminal portion of the protein) (Lu *et al.*, 2016) and Cav1 through its E domain (i.e., C-terminal portion of the protein) (Razandi *et al.*, 2003). Moreover, while E2 reduced Cav1:ER α association (Acconcia *et al.*, 2005a), possibly because of intramolecular reorganization of the ER α causing de-palmitoylation of the receptor, it increases the striatin:ER α interaction (Lu *et al.*, 2016).

Therefore, E2 determines rapid proximal membrane-specific events that result in the activation of the ER α -mediated extra-nuclear signaling.

4.2.3 Cytoplasmic scaffolding proteins

Cytoplasmic ER α and ER β binding partners exist and have been implicated also in extra-nuclear E2 signaling.

4.2.3.1 Calmodulin

Calmodulin is signaling cytoplasmic adaptor protein, which upon Ca²⁺ binding changes its 3D-conformation, exposes novel protein epitopes and interacts with diverse proteins to regulate a myriad of physiological functions (Junho *et al.*, 2020; Takemoto-Kimura *et al.*, 2017).

Calmodulin has been shown to physically interact with ER α but not with ER β (Li & Sacks, 2007). The site of calmodulin binding to ER α has been mapped within the hinge domain (i.e., amino acids 248-317) (Li *et al.*, 2005) and the structural complex between the ER α interacting peptide with

calmodulin has been further solved (Zhang *et al.*, 2012). Notably, these two proteins have been shown to co-immunoprecipitate also in breast cancer cell lines independent of E2 administration (Li *et al.*, 2001). Although the subcellular localization of calmodulin:ER α interaction has not been dissected in detail, it was initially shown by both affinity chromatography and sucrose gradient sedimentation that such complex could occur in rat uterine cytosol (Bouhoute & Leclercq, 1992).

Structural and functional analyses of the effects of Ca²⁺-dependent calmodulin:ER α interaction allowed to present a model for the impact of calmodulin:ER α association in E2 signaling. Indeed, it has been proposed that upon E2-dependent Ca²⁺ liberation from intracellular stores, calmodulin associates with ER α in the cytoplasm, thus reducing the amount of E2-triggered ER α proteasome degradation. The calmodulin:ER α complex then would migrate into the nucleus where Ca²⁺-activated calmodulin is required for maximal E2-dependent induction of ERE-based gene transcription and the control of E2-dependent cell proliferation (Li *et al.*, 2005; Li & Sacks, 2007; Zhang *et al.*, 2012).

Therefore, calmodulin represents a receptor interactor both mediating ER α shuttling from the cytoplasm to the nucleus in response to E2 and integrating extra-nuclear signaling (i.e., E2-dependent liberation of Ca²⁺) with nuclear ER α function.

4.2.3.2 p130Cas/BCAR

The adaptor protein p130Cas (Crk-associated substrate) also known as breast cancer resistant 1 (BCAR1) is a scaffolding protein bridging the actin cytoskeleton to the extracellular matrix and thus plays important roles in the regulation of cell migration and cell invasion (Camacho Leal Mdel *et al.*, 2015). Many signaling molecules are organized in the cells through binding to p130Cas/BCAR at the focal adhesion sites including Src, PI3K, and FAK (Camacho Leal Mdel *et al.*, 2015).

p130Cas/BCAR interacts rapidly and transiently with the ER α (Fig. 4C) as a function of E2 (Cabodi *et al.*, 2004). The p130Cas/BCAR:ER α association requires the ability of E2 to activate Src and appears to be important for the downstream activation of the ERK/MAPK pathway leading to cyclin D1 transcription and cell cycle progression (Cabodi *et al.*, 2004).

4.2.3.3 HPIP

Another cytoskeletal-associated protein has been reported to be an ER α extra-nuclear interactor (Fig. 4C). The microtubule-binding protein HPIP (hematopoietic PBX interaction protein) was found to associate with ER α in a yeast two-hybrid screening of a mammary gland cDNA expression library (Manavathi *et al.*, 2006). HPIP binds to the ER α D and F domains in vitro (Manavathi *et al.*, 2006). In cell lines, E2 trigger the formation of the HPIP:ER α :p85 (i.e., subunit of the PI3K):Src complex (Manavathi *et al.*, 2006) and mediates the activation of the ERK/MAPK and PI3K/AKT pathway as disruption of the complex through a mutant HPIP and siRNA-mediated reduction of this cytoskeletal protein prevent such hormone-dependent effect (Manavathi *et al.*, 2006; Wang *et al.*, 2008). In addition, HPIP addresses the receptor in association with the microtubules in an E2-dependent manner and was required for E2-induced cell migration (Manavathi *et al.*, 2006).

ER β was also found to associate with HPIP (Wang *et al.*, 2008) (Fig. 4C) but the impact of HPIP in ER β -mediated extra-nuclear E2 signaling was not evaluated. However, it has been proposed that ER β could compete with ER α for binding to HPIP (Wang *et al.*, 2008). Therefore, these data demonstrate that in cell context co-expressing both ERs extra-nuclear interactors could modulate the extra-nuclear E2 signaling.

4.2.3.4 PELP1/MNAR

Proline, Glutamate, and Leucine-Rich Protein 1 (PELP1) also known as Modulator of Non-Genomic Activity of Estrogen Receptor (MNAR) is a cytoplasmic scaffolding protein that binds the ERs (Fig. 4C) and works as an integrator of both extra-nuclear and nuclear E2 actions. PELP1/MNAR is an SH2 domain-binding protein that is expressed in several tissues (Vadlamudi *et al.*, 2001; Wong *et al.*, 2002, 2009). PELP1/MNAR is an E2 target gene as its promoter contains both classic ERE sequences as well as AP-1 enhancers. E2 can increase PELP1/MNAR expression through both ER α

and ER β that are recruited to PELP1/MNAR promoter upon E2 binding (Mishra *et al.*, 2004). PELP1/MNAR primary subcellular localization is the nucleus but it is also located in the cytosol (Nair *et al.*, 2004; Vadlamudi *et al.*, 2001; Wong *et al.*, 2002, 2009). In the nucleus, besides being an E2 target gene PELP1/MNAR is also an ER α transcriptional coactivator coordinating receptor transcriptional activity (Nair *et al.*, 2004; Vadlamudi *et al.*, 2001). PELP1/MNAR also stimulates E2-induced cell proliferation and its expression is deregulated in breast cancer (Balasenthil & Vadlamudi, 2003).

PELP1/MNAR binds ER α and ER β in the cytosol (Fig. 4C) (Nair *et al.*, 2004; Vadlamudi *et al.*, 2001; Wong *et al.*, 2002, 2009). The molecular interactions between PELP1/MNAR and ER α have been characterized. The association of the ER α to PELP1/MNAR is E2-dependent and requires the receptor to be phosphorylated on the Y537 residue and to be physically bound to Src. The ER α :Src complex then binds to PELP1/MNAR. Such mechanism appears to be conserved as also the ER β associates to PELP1/MNAR in the same manner (Wong *et al.*, 2002, 2009). Remarkably, besides the phosphorylated Y537 also the A/B domain seems to be required for the formation of the complex (Barletta *et al.*, 2004; Wong *et al.*, 2002, 2009).

As an extra-nuclear binding protein for both the ER α and the ER β , PELP1/MNAR has been known as a modulator of the E2-dependent activation of the ERK/MAPK pathway (Wong *et al.*, 2002, 2009). However, this concept has been questioned since the initial discoveries were retracted (Wong *et al.*, 2002, 2009). Nonetheless, solid evidence also demonstrated this protein controls extra-nuclear E2 signaling via ER β (Vadlamudi *et al.*, 2004). Thus, a careful analysis of the literature is required to understand the specific role of this protein in the control of extra-nuclear E2 actions.

Nonetheless, PELP1/MNAR represents a proof-of-function protein coordinating both nuclear and extra-nuclear E2 actions.

4.2.3.5 MTA1s

The metastasis-associated protein 1 splice variant (i.e., MTA1s) (Kumar *et al.*, 2002) is an endogenous variant of the MTA1, which was initially discovered in highly metastatic breast cancer cells (Toh *et al.*, 1994) and later recognized as a regulator of ER α transcriptional activity in the nucleus (Mazumdar *et al.*, 2001; Molli *et al.*, 2008; Talukder *et al.*, 2003).

In an attempt to identify MTA1 binding partners in breast cancer cells, Kumar and co-workers (Kumar *et al.*, 2002) discovered a series of possible MTA1 variants, one of which displayed an unexpected molecular weight when translated in vitro. This MTA1 variant was later identified as MTA1s. Because the sequence of this protein contained a nuclear receptor consensus binding domain, lacked a nuclear localization signal, and was a splice variant of an ER α nuclear interactor, the function of MTA1s was studied in the context of the ER α . MTA1s binds to the receptor A/B and E domains. The association of the two proteins occurred preferentially in the extra-nuclear compartment. E2 triggers the formation of the endogenous MTA1s:ER α complex and enhances the extra-nuclear activation of the ERK/MAPK pathway while it reduces nuclear ER α -dependent transcriptional activity because it sequesters the receptor outside of the nucleus (Kumar *et al.*, 2002). Interestingly, although a direct interaction of MTA1s with the ER β has never been reported, MTA1s has been implicated also in the extra-nuclear ER β -mediated functions (Al-Bader *et al.*, 2015; Zhang *et al.*, 2009).

Therefore, MTA1s represents a splice variant of a nuclear ERs interactor working in the extra-nuclear compartment.

5. High-throughput analyses of the extra-nuclear ERs interactome.

To our knowledge, only the group of Dr. Weisz attempted to identify cytoplasmic interaction partners of unliganded ER α and ER β to characterize the molecular basis of the extra-nuclear mechanism of action of these receptors (Stellato *et al.*, 2015). This high-throughput mass spectrometry approach on cytoplasmic extracts from human breast cancer cells overexpressing ER α and ER β allowed the identification of 84 and 142 proteins associated with unliganded ER α and ER β ,

respectively. Their subsequent functional annotation gave the proof-of-principle that also in the extra-nuclear compartments the ERs associate with different and specific interactors and can affect different cytoplasmic pathways (Stellato *et al.*, 2015).

However, the number of the ERs specific binding partners is surprisingly low (i.e., around 100 per each receptor) considering that the ERs are high allosteric proteins, and that the extra-nuclear environment is extremely crowded by different proteins.

5.1 Bioinformatic identification of the extra-nuclear ERs interactomes.

To gain further insights regarding the extra-nuclear ERs interactomes, here we have bioinformatically interrogated the freely accessible BioGrid database (<https://thebiogrid.org/>) where all the different physical interactions for both the ER α (i.e., 2155 protein interactors) and the ER β (i.e., 2239 protein interactors) are annotated¹ and filtered out from these two lists all the *nuclear* proteins annotated in the human protein atlas (<https://www.proteinatlas.org/>). The resulting lists corresponded to the annotated ER α (i.e., 898 protein) or ER β (i.e., 1029 protein) interacting partners in the extra-nuclear compartments (Supplementary Table 1).

Because each of the identified proteins can be included in different subcellular compartments, to understand the functional impact of the extra-nuclear ERs interactors, we annotated each protein in each subcellular compartment (Supplementary Table 2). As shown in figure 5A and 5B, most of the ERs extra-nuclear interacting partners are cytosolic proteins. The second most represented group includes proteins with mitochondrial localization. Plasma membrane and membrane-based organelles proteins are the third most abundant category of physical interactors of the ERs. Finally, although numerically less prominent, cytoskeletal proteins have also been found to be associated with both ERs (Fig. 5A and 5B).

Next, we studied the pathways in which the ERs interacting partners in the extra-nuclear compartments enriched by using the <https://reactome.org/> database and stratified the results according to the highest ratio among the identified proteins and the number of protein in the Reactome database (Supplementary Table 3 and 4). Analysis of these two latter lists of pathways revealed that the ER α and ER β interactors enriched in 28 common pathways while 10 and 11 pathways were specifically enriched for either ER α or ER β interactors, respectively (Fig. 5C and Supplementary Table 5).

Inspection of the extra-nuclear enriched pathways revealed that ER α specific interactors belonged to proliferative pathways while ER β specific ones are included in the anti-proliferative signaling (Fig. 5C and Supplementary Table 5). Remarkably, it has previously shown that membrane-initiated ER α signaling mediates the proliferative effects of E2, whereas membrane-initiated ER β signaling directs the anti-proliferative effects of E2 (Acconcia & Marino, 2011). On the contrary, common pathways in which both ER α and ER β extra-nuclear interactors are enriched correspond to signaling by Rho GTPases and membrane trafficking of vesicles (Fig. 5C).

While the cross-talk between Rho GTPases and ERs have been already reported in different cell lines (El Marzouk *et al.*, 2007; Sanchez *et al.*, 2010; Sanchez *et al.*, 2009; Sanchez *et al.*; Simoncini *et al.*, 2006; Su *et al.*, 2001), the role of ERs in intracellular trafficking systems are less well studied and deserve further analysis.

Notably, it must be pointed out here, however, that extra-nuclear localized ERs appear to function in association with physical structures within the cytoplasm (i.e., the cytoskeleton and the membrane-based organelles).

5.2 The relationships among endocytic proteins and the ERs

Although historically endocytic (and exocytic) trafficking of proteins has been considered as a simple mechanism to internalize (and to extrude) specific external (and internal) molecules, the actual paradigm dictates that vesicle-based membrane-trafficking of proteins represents a conserved function in all living organisms allowing the spatial control of cell signaling and the resulting

¹ in the BioGrid database only 3 annotated interactors are available for GPER1.

regulation of a myriad of physiological effects (Sigismund *et al.*, 2012). This view implies that all the physiological functions occur in the context of an ‘endocytic matrix’, which shapes the connections among signal transduction, gene expression, and cellular responses in a spatial-temporal dimension (Scita & Di Fiore, 2010; Sigismund *et al.*, 2012).

In such a context, it is not surprising that the identified common extra-nuclear ERs interactors belong to the membrane trafficking and vesicle-mediated transport systems. Members of the epidermal growth factor receptor (EGFR) trafficking machinery (i.e., eps15, eps8, intersectins) (Sigismund *et al.*, 2012), proteins that work as coating of intracellular vesicles (e.g., clathrin light and heavy chains, caveolin-1) (Sigismund *et al.*, 2012), and structural proteins of endocytic compartments (e.g., LAMP2 for lysosomes, giantin for Golgi, HRS for endosomes and SQSTM1 for autophagosomes) (Eskelinen, 2006; Eskelinen *et al.*, 2021; Raiborg & Stenmark, 2002; Satoh *et al.*, 2019) appear to be ERs binding partners (Supplementary Table 1 and 2).

5.3 Endocytic proteins and E2 extra-nuclear signaling

Our work has demonstrated a direct connection among the ER α , extra-nuclear E2 signaling, and endocytic proteins (Totta *et al.*, 2015b). We have indeed observed that the receptor is present in the endosomes as it co-localizes together with the early endosome antigen (EEA) and with the late endosome marker HRS (Pesiri *et al.*, 2014; Totta *et al.*, 2014). Topological localization of the ER α with LAMP-2 at the lysosomes (Sampayo *et al.*, 2018; Totta *et al.*, 2014) and with SQSTM1 at autophagolysosomes (Totta *et al.*, 2016) was also evidenced by immunofluorescence analyses.

Direct physical association of the ER α with clathrin heavy chain (CHC) was also observed (Totta *et al.*, 2015b). The binding of ER α to CHC occurs in the cytoplasm and a series of *in vitro* analyses demonstrated that the ER α D and E domains are the sites of CHC interaction (Totta *et al.*, 2015b). On the contrary, the receptor binds the *N*-terminal portion of CHC (Totta *et al.*, 2015b), which is the main CHC cargo interaction module (Lemmon & Traub, 2012). A functional association among endocytic proteins and ER α has been further reported (Segala *et al.*, 2019; Totta *et al.*, 2016).

The interference with membrane trafficking proteins influences the E2-dependent control of both extra-nuclear signaling (e.g., ERK/MAPK; PI3K/AKT) (Totta *et al.*, 2016; Totta *et al.*, 2015a; Totta *et al.*, 2015b; Totta *et al.*, 2014) and ER α intracellular levels (Totta *et al.*, 2015b). Particularly, out of the 101 endocytic proteins identified to regulate receptor stability (Supplementary Table 6) (Totta *et al.*, 2015b), 19 (i.e., ACTR2, AP2A2, ARPC5, CAV1, CDC42, CFL1, CLTC, EPN2, EPS15, HIP1, HIP1R, ITSN2, PACSIN3, PAK1, RAB5C, RAC1, VAPA, VCP, WASF2) are found in the list of ER α extra-nuclear interactors (Supplementary Table 1).

Therefore, it is possible to state that ER α physically belongs to the endocytic compartments and that proteins of the endocytic compartments regulate receptor functions. Notably, direct evaluation of the interaction of the ER β with endocytic proteins remains to be done.

5.4 E2 internalization via membrane-located ERs

From a functional point of view, it is difficult to reconcile the presence of the ER α in the endocytic compartments unless one assumes that the E2 does not freely diffuse in target cells and the plasma membrane-localized receptor traffics through endocytic routes.

Data are available to support both statements. Indeed, it has been shown that E2 is not freely up-taken by E2 target cells but rather it is internalized through the endocytic receptor for LDL megalin, which binds E2 in complex with its steroid hormone-binding globulin (SHBG) (Hammes *et al.*, 2005). Remarkably, mice models lacking the megalin gene showed a phenotype resembling the one observed in ER α antagonists-treated animals (Hammes *et al.*, 2005).

Internalization of E2 has been reported already in 1984 when Pietras and Szego showed that radioactive E2 can be retrieved in lysosome-enriched sub-cellular compartments in rat uterine cells (Pietras & Szego, 1984). Subsequently, E2:BSA was observed to be internalized in endosomes and lysosomes (Kisler *et al.*, 2013; Moats & Ramirez, 2000), and E2:dendrimers reached a non-specified final destination in the cytoplasm of treated cells (Bondar *et al.*, 2009; Micevych & Christensen, 2012;

Wong *et al.*, 2015). Moreover, vesicles containing ER α were observed to be internalized following E2 administration (Sampayo *et al.*, 2018). Finally, it has to be pointed out that reports are available to show that the biophysical composition of the cell membrane includes a high proportion of E2 that is stuck in the phospholipid bilayer and cannot flip between the two membrane leaflets (Scheidt *et al.*, 2010).

The mechanism through which E2 is internalized appears to involve the trafficking of the membrane ER α in association with transmembrane receptors (i.e., IGF1-R and β 1-integrin) (Fig. 6) (Sampayo *et al.*, 2018; Totta *et al.*, 2015b).

We have indeed shown that upon E2 administration the membrane-localized receptor becomes de-palmitoylated and dissociates by Cav1 (Acconcia *et al.*, 2005a). The E2:ER α then binds to CHC and associate with the IGF1-R, where it recruits the above-described signaling intermediates. Such molecular complex then traffics to the endosomes and then to the lysosomes where the ER α is partially degraded (Totta *et al.*, 2015b) (Fig. 6 left part). Whether a fraction of the ER α is also recycled back to the plasma membrane remains to be evaluated (Totta *et al.*, 2015b).

Sampayo and co-workers (Sampayo *et al.*, 2018) instead observed that membrane ER α is internalized upon E2 administration through a Cav1-dependent pathway in association with β 1-integrin. Also, in this case, the protein complex can be trafficked to the lysosomes for degradation or back to the plasma membrane in a switch that requires the presence of fibronectin and some Rab proteins in the complex (Fig. 6 right part) (Sampayo *et al.*, 2018). The internalization of the membrane located ER α through the Cav1-dependent pathways appears also to require the activity of dynamin II (Marczell *et al.*, 2018).

Altogether these data demonstrate that the E2:ER α membrane complex can be internalized through different endocytic pathways leading the receptor to different functional fates (e.g., degradation, recycling, signaling) and suggest that the extra-nuclear E2 effects could be spatially organized in the cytoplasm (Fig. 6).

6. Discussion.

Research over the last decades has led to the definition that E2 triggers an intertwined intracellular mechanism in which at least two different receptors (i.e., ER α and ER β) work both as transcription factors and extra-nuclear plasma membrane-localized receptors, which mediate the activation of a plethora of signal transduction cascades that ultimately coordinate gene expression. In this way, the pleiotropic effects of E2 can occur.

While intense investigations were initially pointed to understand the molecular mechanisms through which the same nuclear ERs are localized to the plasma membrane, the recognition of palmitoylation as the pre-requisite for cell surface receptor tethering, for extra-nuclear E2 signaling, and for the consequent nuclear activation of ERs functions (Acconcia *et al.*, 2005a; Pedram *et al.*, 2007) (La Rosa *et al.*, 2012) redirected the interest to try understanding how a single hormone can originate so many different extra-nuclear effects and thus regulate a variety of physiological effects in different tissues.

The introduction of mice models in which the ER α palmitoylation site was mutated to impede not only receptor plasma membrane localization but also the extra-nuclear E2 signaling eventually demonstrated that the rapid effects of E2 are operational in vivo (Adlanmerini *et al.*, 2014; Pedram *et al.*, 2014). More interestingly, the use of these animal models is facilitating the accumulation of evidence firmly demonstrating that the extra-nuclear E2 effects are tissue-dependent and activated in a tissue-specific manner (Adlanmerini *et al.*, 2014; Farman *et al.*, 2018; Fontaine *et al.*, 2020; Gustafsson *et al.*, 2016; Nanjappa *et al.*, 2016; Pedram *et al.*, 2014; Vinel *et al.*, 2016; Yu *et al.*, 2020).

The question is now to understand the tissue specificity of the extra-nuclear E2 effects. The evidence reviewed here strongly points towards the ability of the ERs to differentially associate with different extra-nuclear binding partners also as a function of E2.

Based on the available literature data, it is possible to conceptualize that in the extra-nuclear compartments the ERs hitchhike the already present proteins to fine-tune their extra-nuclear activities with the nuclear ones. At least three groups of proteins can be recognized as classes of extra-nuclear interactors. Binding partners of the ERs belong to specific signaling kinase pathways (e.g., Src, p85, p38, Shc), cytoskeletal proteins (e.g., p130Cas/BCAR, HPIP), and receptors (e.g., IGF1-R, ER α , and ER β). Additionally, some proteins are dual ERs interacting partners as they associate with the ERs in the nucleus but also work in the cytoplasm (e.g., calmodulin, PELP1/MNAR, MTA1s).

Another level of extra-nuclear functioning of the ERs is their possibility to bind to endocytic proteins. While it is well-known that both ERs interact with Cav1, the discovery that the ER α can bind also to CHC is recent. CHC and Cav1 are vesicle coating proteins that specify and discriminate different kinds of internalization routes for the endocytic trafficking of RTKs (Sigismund *et al.*, 2012). Our bioinformatic analysis together with reported evidence clearly shows that the ERs are intrinsic proteins of the endocytic and intracellular trafficking compartment of the cells.

Structural reasons can be adducted to explain such diversity of ERs:protein interactions. On one hand, because ERs can have a topology-dependent structural organization (Dan *et al.*, 2003; Welsh *et al.*, 2012), it is also conceivable that their 3D organization could be the cause (or the consequence) for the association of the ERs with different interacting partners in different subcellular environments and that the extra-nuclear differential response of different cell types (or tissues) to E2 could be dependent on the repertoire of the different extra-nuclear receptor conformations in the apo- or ligand-bound state. On the other hand, one can also assume that the physical structures (i.e., organelles) of the cytoplasm can play a role in the spatial-temporal control of extra-nuclear E2 signaling.

In conclusion, context-dependent diversity of the ERs-mediated extra-nuclear E2 actions could be specified by the great flexibility of the physical structures of ERs, which originate different conformations that, in turn, can be regulated by different PTMs (Le Romancer *et al.*, 2011), each with its characteristic functional property in the context of the spatial-temporal organization of the logistics of the cells [i.e., the endocytic matrix (Scita & Di Fiore, 2010; Sigismund *et al.*, 2012)].

In turn, the extra-nuclear ER α and ER β interactomes would direct cell and tissue specificity of the extra-nuclear E2 signaling.

7. Future perspectives.

This outlined concept can be immediately challenged because there is not a systematic experimental characterization of the extra-nuclear ERs interactomes. This caveat has to be considered as the recognition of a novel starting point from which to derive 'objective' knowledge (Khun, 1962).

We are aware that great technical limitations can underly the substantial lack of evidence about the specific interactions of the ERs in the cytoplasm. Indeed, the amount of the ERs localized in the extra-nuclear compartment is much less (approximately 10-15%) than the amount of the receptors in the nuclei of ERs expressing cells (Acconcia & Marino, 2011).

However, we propose different strategies to discover and characterize the extra-nuclear ERs interactome.

i) The most direct approach would be the overexpression of the ERs. However, this cellular manipulation would artificially increase the amount of the ERs in all subcellular compartments, increasing the possibility of artifacts.

ii) It would be possible to evaluate the extra-nuclear ERs interactome by directly targeting the ERs in the extra-nuclear compartments. This solution would require either a modification of the structure of the ERs or their labeling with specific extra-nuclear targeting protein tags. However, such modifications could themselves affect the physiological associations of the ERs with the specific interactors. In this respect, an ER α variant in which all the nuclear localization signals have been mutated has been characterized for an-increased-to-exclusive extra-nuclear localization (Burns *et al.*, 2011; Totta *et al.*, 2014) but these reagents have not been used to study the extra-nuclear ER α interactome.

iii) Perhaps the most physiological approach (although expensive and time-consuming) to study the context-dependent extra-nuclear ERs interactome could be the use of wild type and palmitoylation defective mice models (Adlanmerini *et al.*, 2014; Pedram *et al.*, 2014) where the extra-nuclear interactome could be derived in each tissue as the difference between the identified ER α interacting partners in intact and mutated mice.

Other strategies tackling this problem (e.g., the development of specific inhibitors of membrane ERs signaling; the evaluation of rapid E2 actions underpinning disease and sexual dimorphism) could be developed as a function of specific technical advancements (e.g., SILAC mass spectrometry), but, whatever the case, the solution of this challenging issue will eventually clarify the picture of the ERs (and possibly also of other steroid receptors) functioning, thus allowing on one hand the definitive proofs for a physiological role of extra-nuclear ERs actions in health and disease and on the other hand the identification of novel druggable targets for the innovative treatment of diseases where the ERs play fundamental roles (e.g., breast cancer).

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9. Figure Captions

Figure 1. History of the ERs.

(A) Timeline of the ages in which the main discoveries/main concepts (colored boxes) of the estrogen receptor (ER) field have been accumulated. (B) Panel derived from a PubMed research performed on 12/07/2021 using GPER as keyword. For details, please see the text.

Figure 2. The integration between the extra-nuclear and nuclear E2:ERs complex molecular mechanisms.

The same nuclear estrogen receptors (ERs) associate to the plasma membrane at the level of caveolae by receptor palmitoylation and association with the integral caveolar protein caveolin-1 (Cav1). Upon 17 β -estradiol (E2) binding to the membrane ERs a variety of rapid extra-nuclear signaling cascade activate and integrate with the E2:ERs complex-dependent regulation of gene transcription (i.e., nuclear mechanism) for the regulation of diverse cellular processes. Caveolin-1 (Cav1); Phosphatidylinositol-4,5-Bisphosphate 3-Kinase/ AKT Serine/Threonine Kinase 1 (PI3K/AKT); Protein Kinase C/Phospholipase C (PLC/PKC); Ataxia Telangiectasia Mutated/Checkpoint Kinase 2 (ATM/CHK2); Ataxia Telangiectasia And Rad3-Related Protein/Checkpoint Kinase 1 (ATR/CHK1); Avian Sarcoma (Schmidt-Ruppin A-2) Viral Oncogene Homolog/Extracellular regulated kinases (Src/ERK); protein 38/Mitogen-activated protein kinases (p38/MAPK); Focal Adhesion Kinase (FAK); Signal Transducer And Activator Of Transcription (STAT); Ras Homolog Family Member A (RhoA) and WASP family Verprolin-homologous protein (WAVE). For details, please see the text.

Figure 3. Structure and epitope-dependent localization of the ERs.

(A) Biochemical anatomy of the conserved structure of the ER α and the ER β . The domains of the ERs (i.e., A to F) are also visualized in terms of their 3D-conformations. Blu (i.e., C domain) and pink (i.e., E domain) domains represent the unique parts of the ERs, which assume a structured conformation (i.e., Zinc-finger motif for C domain and 12 α -helices for E domain). The other domains

(i.e., A/B, D, and F) are depicted in green and correspond to the intrinsically disordered regions of the ERs. (B) Structural-dependent intracellular distribution of the ERs. Antibodies directed against epitopes located within the C-terminal end of the ERs (red antibodies) recognize the protein both inside and outside of the nucleus while antibodies directed against epitopes located within the N-terminal part of the ERs (green antibodies) recognize the protein only into the nucleus (Dan *et al.*, 2003; Welsh *et al.*, 2012). For details, please see the text.

Figure 4. Reported extra-nuclear interactors of the ERs.

The extra-nuclear proteins binding to ER α and ER β are reported and grouped as a function of their cellular location or intracellular function. (A) Signaling and adaptor molecules interacting with the ERs. (B) Plasma membrane and (C) cytoplasmic scaffolding proteins interacting with the ERs. Caveolin-1 (Cav1), Caveolin-2 (Cav2) Caveolin-3 (Cav3); Avian Sarcoma (Schmidt-Ruppin A-2) Viral Oncogene Homolog/Extracellular regulated kinases (Src); protein 85 (p85); Integrin-linked kinase (ILK), Glycogen Synthase Kinase 3 β (GSK3 β); G-protein coupled receptor (GPCR); Insulin-like Growth factor 1 Receptor (IGF1-R); Src Homology 2 Domain-Containing) Transforming Protein (Shc); Spliced Form of Metastasis Associated Protein 1 (MTA1s); Proline, Glutamate, And Leucine-Rich Protein 1 (PELP1); Cas Scaffolding Protein Family Member 1 (BCAR) and Hematopoietic PBX Interaction Protein (HPIP). For details, please see the text.

Figure 5. Bioinformatic analysis of the extra-nuclear ERs interactome.

Subcellular localization of the ER α (A) and the ER β (B) interacting partners. (C) Meta-analysis of the pathways in which the ER α and the ER β interacting partners are enriched. For details, please see the text.

Figure 6. The reported intracellular trafficking routes in which the ER α is internalized.

The E2:ER α complex internalization follows both clathrin (CHC)-mediated and caveolin-1 (Cav1)-mediated pathway. In the CHC-mediated pathway (red and left part of the figure), the E2-activated ER α traffics in association with IGF-R and other signaling intermediates by binding to CHC. The internalized complex goes in the early and late endosomes and the receptor can be degraded in the lysosomes. Recycling back to the plasma membrane has been also hypothesized (Totta *et al.*, 2015b). In the Cav1-mediated pathway (red and right part of the figure), the E2-activated ER α traffics in association with β 1-Integrin the internalized complex goes in the early and late endosomes and the receptor can be degraded in the lysosomes or recycled back to the plasma membrane as a function of the association with specific Rab proteins (Sampayo *et al.*, 2018). Caveolin-1 (Cav1); Clathrin Heavy Chain (CHC); Avian Sarcoma (Schmidt-Ruppin A-2) Viral Oncogene Homolog/Extracellular regulated kinases (Src); protein 85 (p85); Insulin-like Growth factor 1 Receptor (IGF1-R); Src Homology 2 Domain-Containing) Transforming Protein (Shc); Early Endosome Antigen (EEA); Hepatocyte Growth Factor-Regulated Tyrosine Kinase Substrate (HRS); Lysosomal Associated Membrane Protein 1 and 2 (LAMP1, LAMP2); Ras-related Protein Rab 7 and 11 (Rab7, Rab11). For details, please see the text and the published papers (Sampayo *et al.*, 2018; Totta *et al.*, 2015b).

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Table 1.

‘Social’ perception	‘Objective’ knowledge
Estrogens are female hormones.	Estrogens are found both in females and in males.
Tissues expressing the ERs (<i>i.e.</i> , the target tissues of estrogens) are the ones of the female reproductive tract.	ERs are expressed in non-reproductive tissues (<i>e.g.</i> , nervous system) and in males.
ERs are nuclear receptors that solely act as transcription factors modulating gene transcription.	ERs subcellular localization includes plasma membrane, endo-membranes, and organelles (<i>i.e.</i> , mitochondria). Non-transcriptional signaling also occurs. Unconventional ERs exist.

Estrogens are more than sex steroid hormones. For details, please see the text.

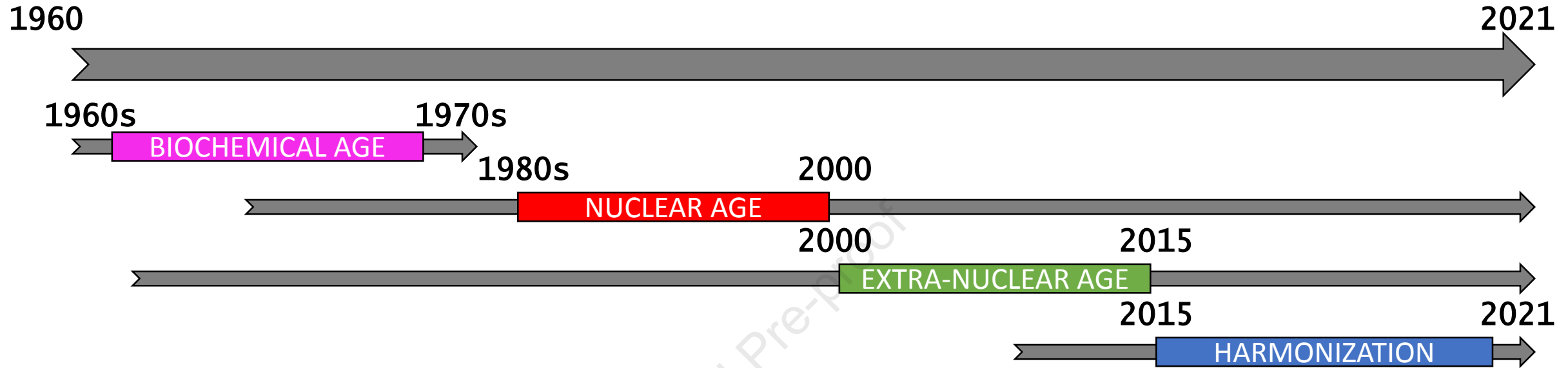
Table 2.

Physiological systems	Effect
Female and Male Gonads	Regulation of reproductive functions
Skeleton	Regulation of bone homeostasis
Cardiovascular System	Regulation of vascular physiology
Respiratory apparatus	Regulation of epithelial ion transport
Metabolic Organs	Regulation of cholesterol homeostasis, liver, and adipose tissue functions
Gastrointestinal apparatus	Regulation of gastric emptying and intestine motility
Skeletal Muscle	Regulation of cell differentiation
Nervous System	Regulation of synapse formation, cell survival, neurotransmitter function, hypothalamic regulation of hunger center, regulation of pain circuits, regulation of the autonomous nervous system
Kidney System	Regulation of epithelial ion transport
Immune System	Regulation of lymphocytes homeostasis
Microbiota	Regulation of microbiota genome stability and expression

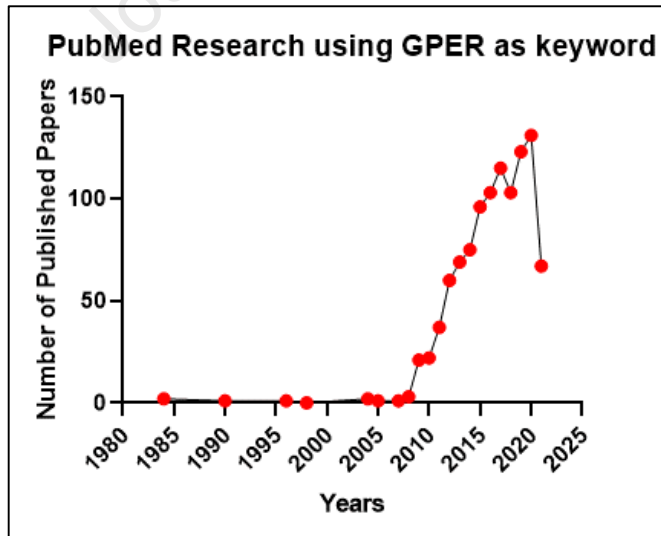
The pleiotropic effects of E2. For details and reference, please see the text.

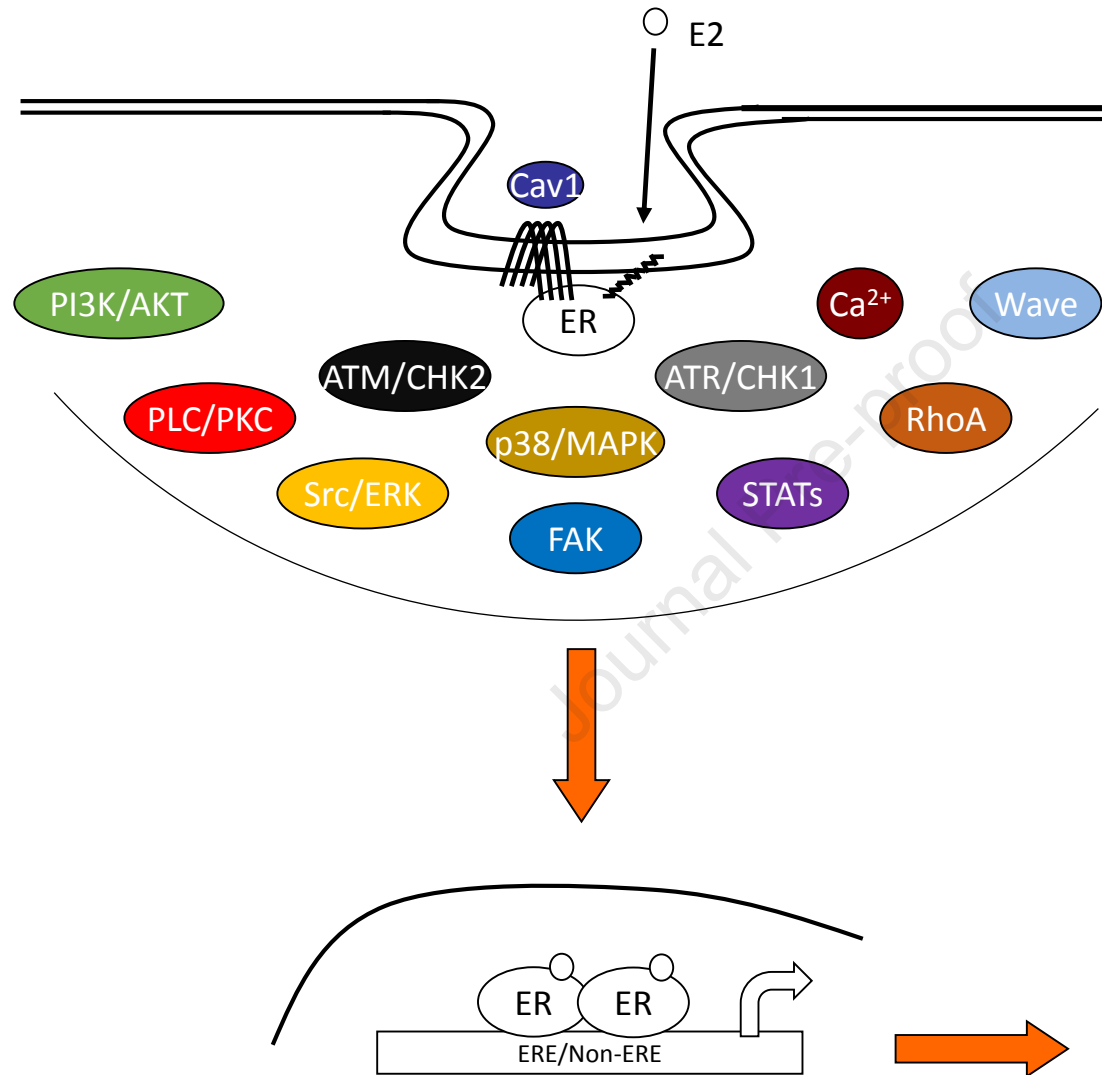
Figure 1
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A



B



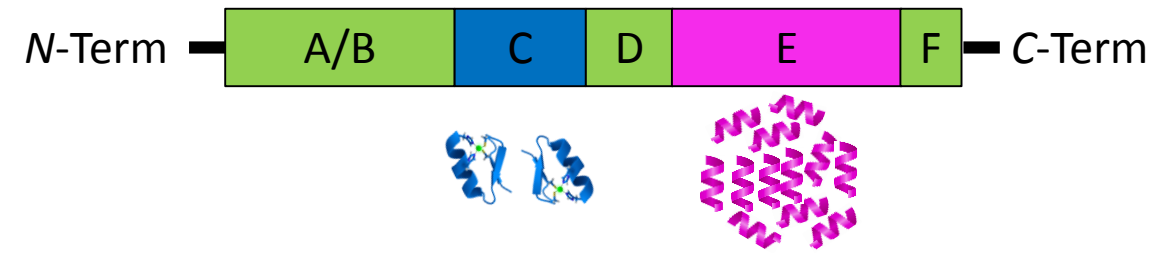


- Cell proliferation/Apoptosis
- Cell migration
- Bone physiology
- Lipid metabolism
- Muscle tissue differentiation
- Vascular physiology
- Genome integrity

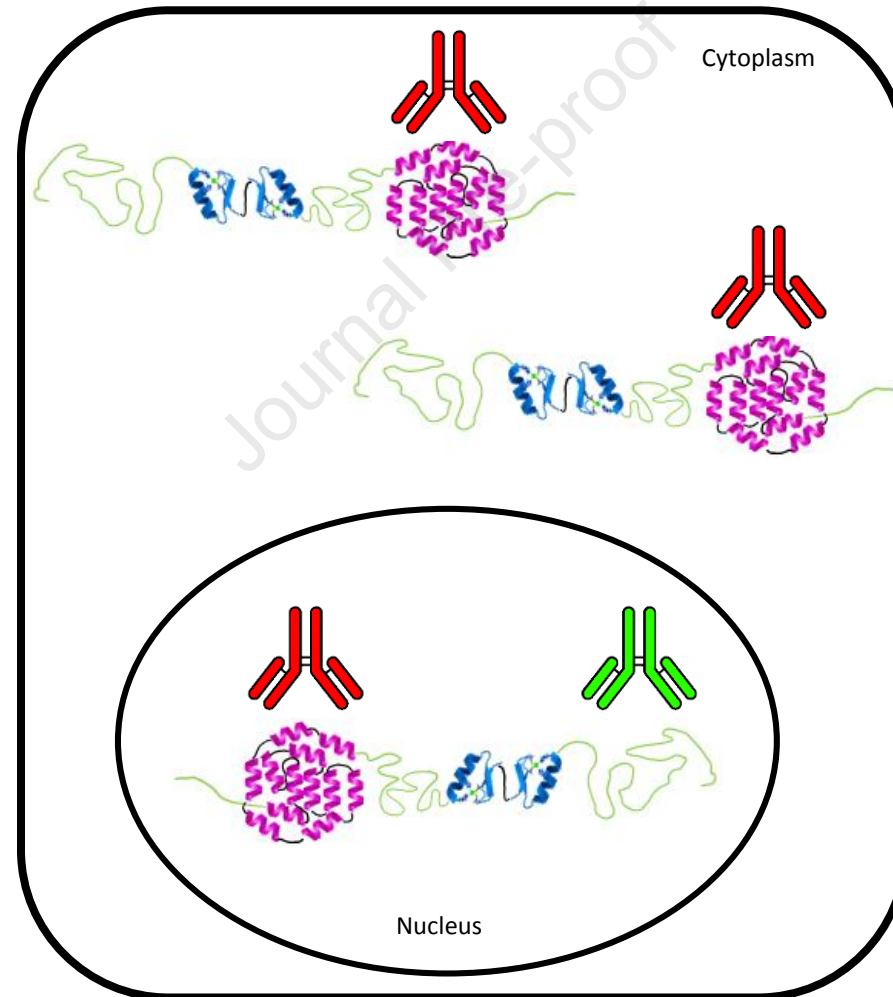
Figure 2

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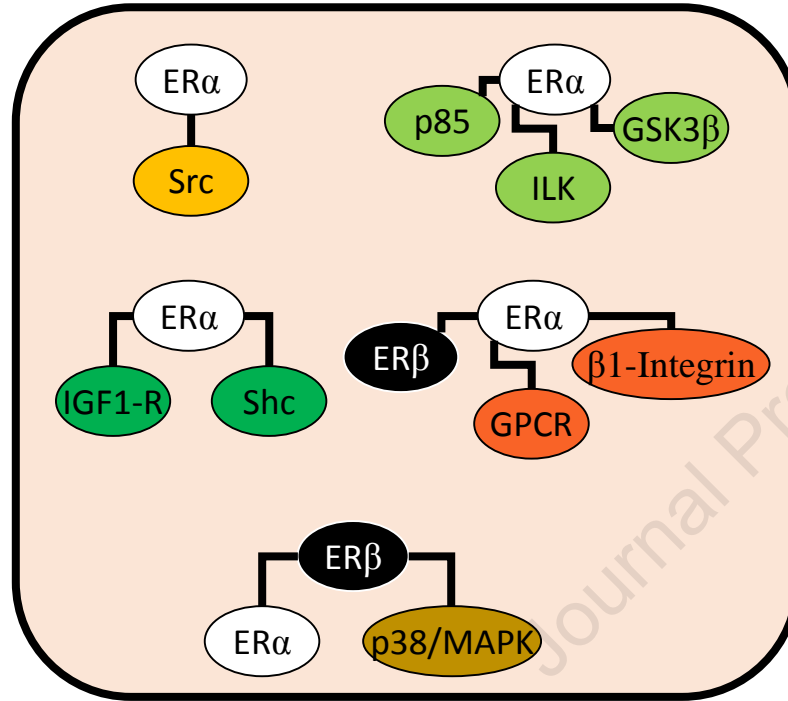
A



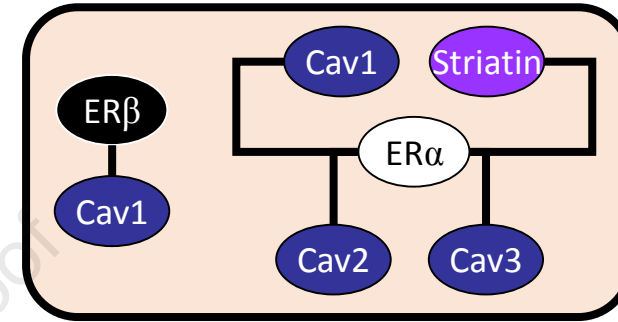
B



A



B



C

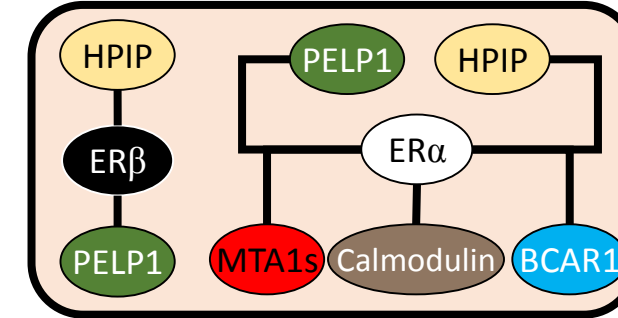
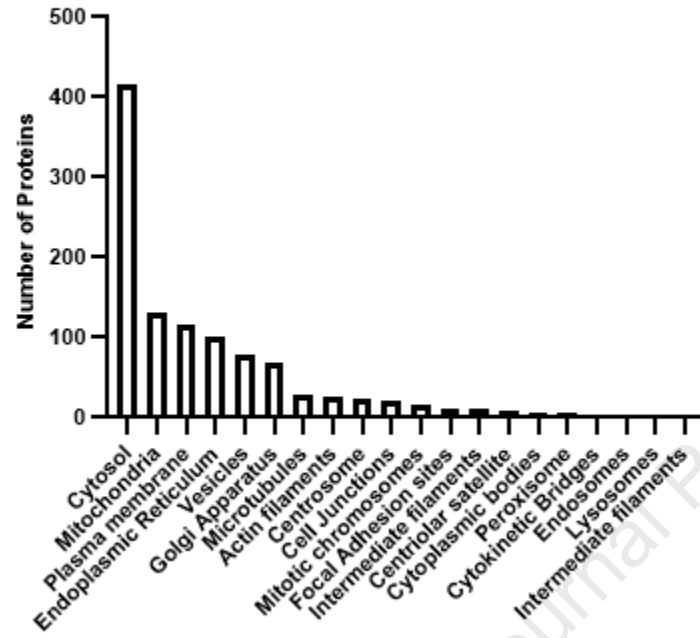


Figure 5

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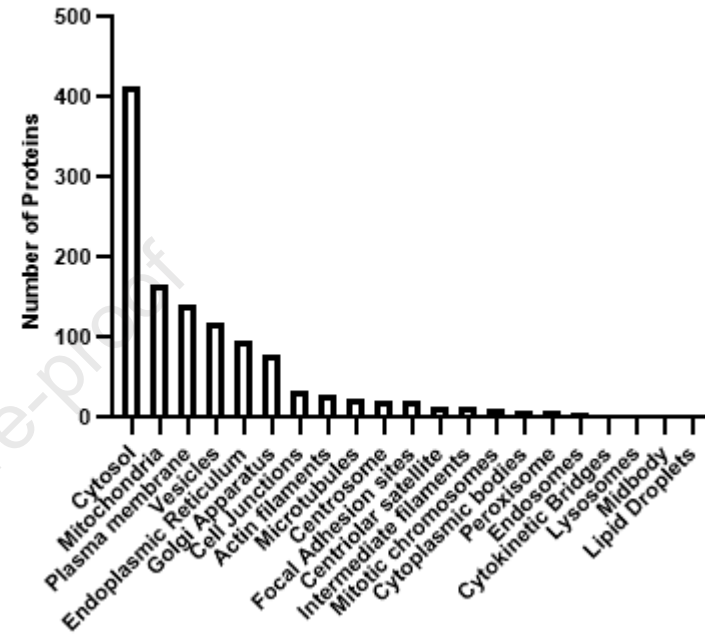
A

ER α Interactors



B

ER β Interactors



C

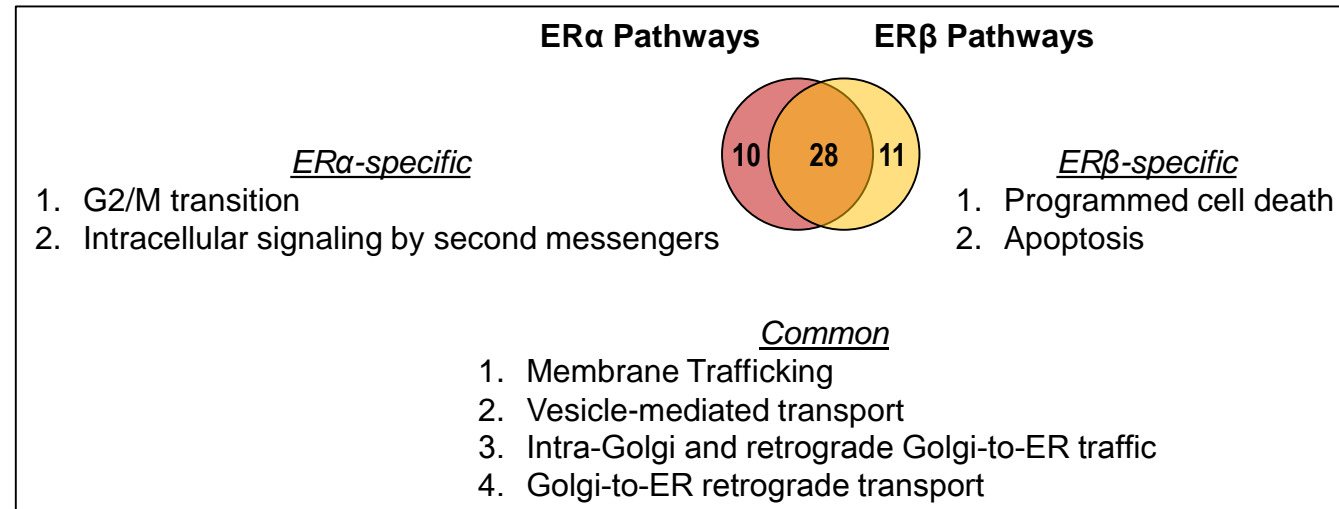


Figure 6

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