UNIVERSITÀ DEGLI STUDI ROMA TRE

Facoltà di Scienze Matematiche, Fisiche e Naturali
Dipartimento di Biologia
Scuola Dottorale in Biologia
Sezione di Biologia Applicata alla Salute dell'Uomo (BASU) – XXV ciclo

REGULATION OF CELL PHYSIOLOGY THROUGH THE MODULATION OF ESTROGEN RECEPTORS ACTIVITIES BY NATURAL AND SYNTHETIC COMPOUNDS.

REGOLAZIONE DELLA FISIOLOGIA CELLULARE ATTRAVERSO LA MODULAZIONE DELLE ATTIVITÀ DEI RECETTORI DEGLI ESTROGENI INDOTTA DA COMPOSTI NATURALI E SINTETICI.

PhD Student: Dr Piergiorgio La Rosa

A.A. 2011/2012

Supervisors: Dr. Filippo Acconcia
Pr. Maria Marino
Coordinatore sezione BASU: Pr. Paolo Visca

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Summary

17-β-estradiol (E2) is the most active estrogen in humans and exerts profound effects on the growth, differentiation, and functioning of many reproductive and non-reproductive tissues.

A number of synthetic substances known as xenoestrogens show estrogenic effects; among them, bisphenol A (BPA) is one of the best characterized because human exposure is a risk factor for many disease. Also naturally plant-produced molecules [*e.g.,* naringenin (Nar)] are known to display a mild estrogenic activity, which is exerted in mammals as a consequence of dietary intake. Although "dietary estrogens" are related to a lower predisposition to breast cancer development, reduced incidence in osteoporosis and cardiovascular disease, adverse effects are known. Thus, because of their ability to interfere with many aspects of natural hormones-dependent control of body homeostasis, reproduction, and developmental processes, xenoestrogens and phytoestrogens are also known as endocrine disruptors (EDs).

Two estrogens receptors (ER) are known in humans to interact with endogenous E2 and EDs: these two isoforms, the ERα and the ERβ, modify the expression of specific genes acting as ligand-activated transcription factors. Gene regulation occurs trough ERs recruitment at target gene promoting sequences and follows a "direct genomic pathway", which expects the ERs to recognize and to bind DNA at the estrogen responsive elements (ERE) sequences, or to interact with transcription factors that in turn bind to DNA in a mechanism known as "genomic indirect pathway".

In addition, rapid effects of E2 occur within seconds, are insensitive to treatment with inhibitors of transcription and depend on the presence of the ERs to the plasma membrane. Palmitoylation of the ERs determines localization to the plasma membrane, where ERs form multimolecular complexes to trigger rapid E2-activated signal transduction pathways that control proliferative and anti-apoptotic effects (e.g., ERK/MAPK and PI3K/AKT pathways) or pro-apoptotic and differentiating effects (e.g., p38/MAPK pathway), as a function of the ER isoform present.

Also EDs can bind and modulate genomic and rapid ERs activities: in many cancer cell lines Nar impairs ERα-mediated rapid activation of signaling kinases (*i.e.,* ERK/MAPK and PI3K/AKT); in the mean time, the ERα rapid pathways-dependent cyclin D1 transcription is

avoided by blocking AP-1 binding to its promoter. In parallel Nar enhances the persistent phosphorylation of p38/MAPK and, consequently, the induction of a pro-apoptotic cascade. However, Nar does not impair the ERα-mediated transcriptional activity of an ERE-containing promoter and, in addition, Nar acts as E2-mimetic in the presence of ERβ. BPA stimulation mediates the transduction of signaling pathways that culminates with ERK/MAPK and PI3K/AKT phosphorylation in ERα expressing cells, while, in an endogenous ERβ expressing colon cancer cell line (DLD1), BPA behaves as an antagonist by blocking the activation a pro-apoptotic cascade.

Different layers of regulation finely modulate the complexity and the vastness of this signaling network, correctly addressing ERs to the right place in the right moment, through the numerous ER post-translational modifications. Indeed, beside palmitoylation, in response to E2 binding ERs are also phosphorylated on serine (S) residues. Another layer of complexity is introduced by the control of ERs intracellular levels, which represents a crucial step to regulate ER-dependent transcription and hormone-dependent effects. The binding of E2 produces ERα ubiquitination, an event that leads to the 26S-proteasome-mediated receptor degradation, drastically lowering the protein half life. This mechanism is, however, not fully understood and many steps of the process are still to be cleared; there are many evidences that phosphorylation plays a major role in ERα degradation. Less is known about ERβ degradation; there are some proofs that this receptor is degraded by the proteasome, but stronger evidence shows that ERβ does not undergo the ubiquitination processes and that E2 induces the increase in ERβ intracellular content.

Because all the effects of E2 occurs through the above-mentioned ligand-dependent modulation of ERs intracellular content, the goal of the present project was to understand the mechanisms underlying the ligand-dependent modulation of ER intracellular levels to better clarify their modulation abilities in ERα- and ERβ-driven physiological processes.

Results obtained with a wide spectrum of approaches demonstrate that Nar and BPA affect ERα and ERβ protein intracellular content in this way influencing the resulting ERs-dependent effects. In particular, while BPA mimics E2 effects in inducing the 26S proteasome-dependent ERα degradation, Nar induces the receptor accumulation; importantly, this modulation seems to be connected with degradation, as

both E2 as well as EDs induce the ERα mRNA levels down-regulation. Studying ERα palmitoylation, we found that this modification is the upstream structural determinant that guarantees the physiological balance of the ERα protein levels; in the presence of E2, lack of ERα palmitoylation causes faster receptor degradation, thus demonstrating that this receptor posttranslational modification is involved in the regulation of ERα stability and ERα S118 phosphorylation. Our finding demonstrates that E2 maintains both a constant level of S118 phosphorylation, whereas it triggers a significant reduction in total ERα content and a parallel increase in ERα gene transcription. We also show that the rapid E2-dependent activation of the PI3K/AKT pathway but not of the ERK/MAPK pathway regulates ERα phosphorylation and that the effect of the lack of ERα palmitoylation on E2-evoked ERα degradation is mimicked by PI3K/AKT pathway inhibition and unaffected by ERK1/2 inhibitor. Thus, the PI3K/AKT pathway is involved in the regulation of the ERα cellular levels. Reduction in S118 phosphorylation also correlates with a faster E2-induced receptor elimination and is paralleled with an ERα transcription impairment. Indeed both palmitoylation and phosphorylation control ERα activity and stability and are linked each other in a consequent process: E2 induces ERα depalmitoylation and S118 phosphorylation and this process leads to ERα-induced transcription and then to receptor degradation; nevertheless, these two post-translational modifications stabilize the receptor, as the lack of ERα palmitoylation or phosphorylation, leads to a faster ERα degradation.

Our experiments also show that the EDs produce as E2 an increase in ERE-mediated transcription and that the ERα partial antagonist Nar determines ERα phosphorylation as well as the E2 mimetic BPA, that partially stabilizes the receptor. Analysis of the modality by which Nar and BPA affect ERα protein intracellular content reveals that BPA mimics the E2 effects in inducing the 26S proteasome-dependent ERα degradation while Nar induces the receptor accumulation by blocking ERα proteolytic degradation. This mechanism requires Nar to induce the persistent activation of the p38/MAPK, as in the presence of the pharmacological inhibition of the p38/MAPK, Nar acquired the ability to trigger ERα degradation.

More importantly, we also found that the Nar-dependent accumulation of ERα results in an increased receptor transcriptional activity and that, upon Nar stimulation, E2 looses its capacity to regulate

ERα turnover and to physiologically control ERα gene transcription and that both EDs raise ERβ cellular content alone and in co-administration with E2.

These discoveries indicate that in a cellular context exposed to Nar the absolute physiological receptor response or the one in response to E2 is changed because of dysregulated receptor expression. Thus, Nar modulation of ERα cellular content could further affect the E2-dependent regulation of specific cellular processes leading to scenarios that strongly diverge from the physiological ones.

In conclusion, the studies conducted during this PhD project demonstrate that the fine hormone-dependent modulation of ERs intracellular levels is intrinsically connected with all the aspects of the molecular mechanisms (i.e., genomic and extra-nuclear) that ERs uses to transduce the physiological E2 intracellular message and as a consequence that EDs hijack the ERs signaling pathway by deregulating ERs expression. In turn, cells exposed to EDs undergo an altered response if compared to the E2-dependent physiological one.

Riassunto

Il 17-β-estradiolo (E2), l'estrogeno più efficace negli esseri umani, esercita effetti profondi sulla crescita, il differenziamento e il funzionamento di molti tessuti riproduttivi e non.

Sostanze sintetiche, note come xenoestrogeni, presentano effetti estrogenici, tra queste, il bisfenolo A (BPA) è uno dei più caratterizzati in funzione del rischio di esposizione per gli esseri umani. Anche molecole naturali, prodotte da organismi vegetali (come la Naringenina [Nar]) esprimono una lieve attività estrogenica, esercitata, attraverso la dieta. L'assunzione di questi "dietary estrogens" comporta una minor predisposizione allo sviluppo del cancro al seno, una ridotta incidenza di osteoporosi e malattie cardiovascolari, anche se sono noti alcuni effetti collaterali. Per la loro capacità di interferire con molti aspetti del controllo sull'omeostasi ormone-dipendente, la riproduzione e i processi di sviluppo, gli xenoestrogeni ed i fitoestrogeni sono noti anche come interferenti endocrini (EDs).

Negli esseri umani, due recettori interagiscono con estrogeni endogeni e EDs: queste due isoforme, α e β, modificano l'espressione di geni specifici agendo come fattori di trascrizione attivati dal legante. La regolazione genica avviene attraverso il reclutamento degli ER su specifiche regioni promotrici, gli Elementi Responsivi agli Estrogeni (ERE) in una modalità definita "diretta", o attraverso l'interazione degli ERs con altri fattori trascrizionali in un meccanismo "genomico indiretto".

É noto, ad ogni modo, che gli ERs scatenano anche effetti rapidi (nell'arco di pochi secondi) e insensibili al trattamento con inibitori della trascrizione. La palmitoilazione dei ERs è il requisito essenziale che determina la localizzazione alla membrana plasmatica degli ERs, dove i recettori formano complessi multimolecolari che innescano eventi di trasduzione del segnale, il cui risultato comporta effetti proliferativi e anti-apoptotici, attivando le vie di trasduzione del segnale ERK/MAPK e PI3K/AKT, o effetti pro-apoptotici e differenziativi attraverso la via p38/MAPK, a seconda della isoforma attivata.

Anche gli EDs legano e modulano l'attività dei ERs: in molte linee cellulari tumorali la Nar compromette l'attivazione ERα-mediata delle chinasi (ad esempio, ERK/MAPK e PI3K/AKT), inoltre, la trascrizione della ciclina D1 ERα-dipendente è impedita, a seguito del blocco di AP-1 sul suo promotore. Negli stessi sistemi cellulari, i flavonoidi compromettono l'attivazione della via ERK/MAPK e della

via PI3K/AKT mediate dal E2, mentre comportano la fosforilazione persistente (60 min) della via p38/MAPK e, di conseguenza, l'induzione di una cascata pro-apoptotica. Tuttavia la Nar non compromettere l'attività trascrizionale mediata dal ERα sui promotori ERE e si comporta come E2-mimetica in presenza del ERβ. Il BPA media la trasduzione di segnali intracellulari che culminano con la fosforilazione delle ERK e della AKT in cellule esprimenti ERα, mentre, in linee cellulari di cancro del colon (DLD1) esprimenti ERβ, BPA si comporta come un antagonista bloccando l'attivazione della cascata pro-apoptotica.

Diversi livelli di regolazione modulano finemente la complessità e la vastità della rete di segnalazione che caratterizza i ERs e indirizzano correttamente i recettori nei distretti subcellulari adeguati, attraverso le numerose modificazioni post-traduzionali che i ER sono in grado di subire. Accanto alla palmitoilazione, in risposta al E2 i ERs sono fosforilati su residui di serina (Ser). Un altro livello di complessità è introdotto dalla modulazione del turnover dei recettori, che rappresenta un passo fondamentale per regolare la trascrizione ER-dipendente e coinvolge sia la degradazione proteica che la trascrizione dei geni dei ERs. Il legame del E2 produce, infatti, l' ubiquitinazione del ERα, un evento che porta alla degradazione mediata dal proteasoma 26S, riducendo drasticamente l'emivita della proteina. Questo meccanismo non è, tuttavia, pienamente compreso e molti passaggi del processo risultano tuttora oscuri; ci sono molte prove che la fosforilazione del recettore α giochi un ruolo importante nel processo di degradazione. Sul ERβ le informazioni sono ancora più scarse; sebbene alcune prove indichino la degradazione proteasomale di questo recettore, altre dimostrano che il ERβ non va incontro al processo di ubiquitinazione.

Questo progetto dimostra che Nar e il BPA regolano il contenuto intracellulare di ERα rivelando che, mentre il BPA mima gli effetti del E2 nell'indurre la degradazione del recettore, la Nar induce l'accumulo dei livelli proteici del ERα; è importante notare che questa modulazione sembra essere collegata con l'attività del proteasoma, visto che sia il E2 sia gli EDs riducono i livelli di mRNA di ERα. Studiando la palmitoilazione del ERα, è statu ulteriormente scoperto che questa modificazione è il determinante strutturale che garantisce l'equilibrio fisiologico dei livelli proteici del ERα; in presenza del E2, la mancanza di palmitoilazione provoca una rapida degradazione del recettore, dimostrando che questa modificazione post-traslazionale è coinvolta

nella regolazione della stabilità del ERα e della fosforilazione in Ser (S) 118 del recettore. I nostri dati dimostrano che il E2 mantiene un livello costante di fosforilazione in S118, che innesca una riduzione significativa del contenuto totale del ERα ed un parallelo aumento della trascrizione ERα-mediata. Abbiamo inoltre dimostrato che è la rapida attivazione E2-dipendente della via di trasduzione del segnale PI3K/AKT piuttosto che quella delle ERK/MAPK a regolare la fosforilazione di ERα e che l'effetto di aumento della degradazione in seguito alla mancata palmitoilazione del ERα è riprodotto dall'inibizione della via PI3K/AKT e non influenzata da quella delle ERK/MAPK. Anche la riduzione della fosforilazione in S118 è correlata con una più rapida eliminazione E2-indotta del recettore e, di pari passo, con una minore trascrizione ERα-mediata. Dunque palmitolazione e fosforilazione sono reciprocamente correlate nel regolare il processo di controllo della stabilità e dell'attività del recettore α; il E2 induce la depalmitolazione del ERα e la sua fosforilazione, portando alla trascrizione genica e alla successive degradazione. Queste due modificazioni post-traduzionali, tuttavia, stabilizzano il recettore, visto che sia la mancanza di palmitolazione che di fosforilazione producono una più rapida degradazione del recettoere. I nostri esperimenti dimostrano anche che, al pari del E2, i EDs producono un aumento della trascrizione ERE-mediata e che sia la Nar (antagonista parziale del ERα) sia il BPA (mimetico del recettore), determinano la fosforilazione del ERα, stabilizzando così parzialmente il recettore. L'analisi della modalità con cui la Nar ed il BPA regolano i contenuti proteici del ERα rivelano che il BPA imita gli effetti del E2 nell'indurre la degradazione proteasoma-dipendente mentre la Nar induce l'accumulo del recettore bloccandone la degradazione proteolitica. In questo meccanismo sembra fondamentale l'attivazione persistente della via p38/MAPK, dato che, inibendo farmacologicamente la sua attivazione, la Nar acquisita la capacità di attivare la degradazione del ERα. Inoltre, abbiamo ulteriormente dimostrato che l'accumulo Nar-dipendente dei livelli del ERα si esprime in un' aumentata attività trascrizionale del recettore e che, in seguito a stimolazione con la Nar, il E2 perde la sua capacità di regolare il turnover del ERα e la sua attività fisiologica

Il nostro lavoro indica, pertanto, che in un contesto cellulare esposto alla Nar, la normale risposta fisiologica E2-indotta cambia in seguito alla de-regolazione dei livelli di recettore; questa differente

modulazione porta ad una specifica risposta, che diverge da quella fisiologica.

1 Introduction.

1.1 Estrogens.

Estrogens are a class of steroidal hormones synthesized in all vertebrates [1] primarily produced in follicles, corpus luteum and placenta and in smaller amount in the liver, breasts, adrenal gland and fat tissue. The three major endogenous compounds that belong to this class, estrone (E1), 17-β-estradiol (E2) and estriol (E3), share the typical steroid structure (three ciclohexane rings and one ciclopentane ring) and are synthesized from cholesterol, in an enzymatic cascade of reaction in which the last step is driven by the enzyme aromatase [2]; nevertheless, E2 is the most active estrogen in humans and it exerts profound effects on the growth, differentiation, and functioning of many reproductive and non-reproductive tissues, including bone, liver, muscle, cardiovascular system, and brain [3, 4]. E2 plays a pivotal role not only in female, but also in male reproductive development and physiology, influences metabolism, maintenance of lipids, bone tissues and cardiovascular and neuronal systems[1, 5].

1.2 Estrogen receptors (ERs).

Two estrogens receptors are known in humans to interact with endogenous and esogenous ER ligands: these two isoforms, α and β, are encoded by different genes located on different chromosomes (chromosome 6 locus 6q25.1 and chromosome 14 locus 14q23-24.1 respectively) [6]. In both cases, a mRNA with 8 esonic sequences and 7 introns is spliced and translated in a conserved structure: the A/B region, encoded by exon 1, establishes a protein-protein interaction domain [7] and modulates gene expression of target genes [8]. The AF-1 domain belongs to this region [9] and is directly, or *via* co-activators/co-repressors, responsible for the binding to the primary transcription machinery. It reacts to the conformational changes that occur in the ligand binding domain (LBD) and the DNA binding domain (DBD) influencing its functional state. Moreover, along with the DBD or beyond its influence, it induces the constitutive modulation of the receptor's target genes in a ligand-independent fashion, targeting genes that contain, in the promoter region, the right receptor's consensus sequences [10]; nevertheless, complete receptor transcription activation needs the AF-2 domain cooperation. One of the major difference between the ERα and ERβ results in the divergences in this domain: in

particular, the distinctive response to estrogen-like ligands, such as 4-hydroxytamoxifen, raloxifene and ICI 164,384 (which are partial ERα agonist and ERβ antagonist) could depend on missing parts of the ERβ AF-1 domain which are, instead, present in the ERα receptor sequence [9].

The region that corresponds to the DNA binding domain (DBD) (*i.e.,* the C region) is encoded by exon 2, 3 and a part of exon 4. It is functionally divided into 2 sub-domains [2] and plays a pivotal role regulating the receptor dimerization processes and recognition of specific DNA sequences, known as estrogen responsive elements (ERE) [10]. The minimal consensus ERE sequence, recognized by both receptors on the DNA strand, is a palindromic inverted repetition: 50-GGTCAnnnTGACC-30 (where n corresponds to any nucleotide); nevertheless, immediately flanking sequences are crucial in determining the affinity which the receptor binds to the ERE sequences with [11]. The DBD domain importance is emphasized by the fact that its protein sequence is the most conserved in all ERs from different animal species; for this reason both receptors bind DNA strand with almost the same specificity and affinity [12].

The hinge, or D region, encoded by exon 4, is one of the most variable sequence within ERs. Even if little is known about its function, this domain is the target of many post-translational modifications and is the region that contains the nuclear localization signal (NLS) [13].

The LBD, the AF-2 domain and a part of the nuclear localization region are encompassed in the *C*-terminal region of the receptor; the last portion of exon 4 and the exons 5-8 encode for it. The E/F region is also responsible for the receptor binding to chaperone proteins (*i.e.,* heat shock proteins), which complex the receptor in the absence of ligands and unmask the steroid binding cleft upon ligand binding. This machinery also facilitates activated-ERs translocation to the nucleus [14]. The LBD folding shows two layers of α-helices (H1-4, H7, H8 and H11) encasing the central core of the structure (α-helices H5-6, H9 and H10); a two-stranded β-sheet (S1 and S2) and H12 complete the ligand-binding portion [15].

1.3 Genomic signaling.

The ERs modify the expression of specific genes acting as ligand-activated transcription factors [16]. Gene regulation occurs trough ERs recruitment at target gene promoters and follows two

different pathways. The direct genomic pathway expects the ERs to recognize and to bind the ERE sequences, that show typical enhancer properties [17]. The sequence itself determines the receptor affinity for the DNA strand and the tightness of the binding, as many E2-regulated genes miss a perfectly corresponding ERE sequence [18]. Another class of genes is modulated by ERs even if ERE sequences are completely missing. The lack of the classic responsive elements imposes the necessity of a second transcription factor to mediate the binding to the DNA strand. Proteins like the stimulating protein 1 (Sp1) or the fos/jun transcription factor complex on the activator protein 1 (AP-1) are involved to stimulate gene expression [19] in a mechanism known as "genomic indirect pathway".

Nevertheless, ERs gene modulation requires, in both cases, the receptor to interact with a complex of protein with a co-activator or co-repressor behaviour, exercised trough AF-1 and AF-2 domains; these motifs work as scaffold for other proteins, which have a direct interaction with the DNA strand [20]. Around 50 different proteins form the co-activators group. Among these, the most important family comprises 3 polypeptides: Steroid Receptor Coactivator (SRC) 1, 2 and 3, that, by interacting with ERs via the AF-2 domain, lead to the recruitment of chromatin modification proteins [21]. Co-activators, gathered to gene promoters, enhance the transcription activity through mechanisms that include the recruitment of transcription factors or other proteins with crucial enzymatic activity needed for an efficient transcription, like the ATP-coupled chromatin remodelling the SNF complex, HATs, metiltransferases e ligases [22]. Such interactions lead to chromatin remodelling and to nucleosomal complexes dissociation. It is also possible that co-repressors interact with AF-2 domain of ERs; even if the number of these proteins is relatively low, they exert an important role by negatively regulating the expression of the ERs-regulated genes [23].

ERs interaction with co-activators seems to deeply rely on H12 position to generate a competent AF-2 region [15, 24]. Upon the binding with a natural or a synthetic ligand, the occupancy of the binding cavity produces a H12 displacement that may vary depending on the compound that binds the receptor. In particular, even if partial-agonists (*e.g.,* genistein, 4-hydroxytamoxifen, and raloxifene) and antagonists (*e.g.,* ICI 164,384) can efficiently fill the binding cavity, they produce a H12 non-allosteric displacement [25, 26] due to their chemical structure, that

does not allow a proper accommodation in the confines of the binding site; this is the contrary of what happen when natural and synthetic ERs agonists (*i.e.*, E2 and diethylstilbestrol, respectively) bind ERs. It is also possible to observe a different H12 positioning between the two ERs: some ERs ligands (*e.g.* the5,11,cis-diethyl-5,6,11,12-tetra-hydrochrysene-2,8-dyol [THC]) act as ERα agonist, producing the same E2-induced H12 positioning, while others do not allow the ERβ LBD to assume the right conformation, thus acting as antagonist [27]. A proper H12 positioning is needed for a transcriptionally competent AF-2 conformation; thus E2-like compounds that bind the ERs lead to co-activators recruitment or impairment depending on whether they act as agonist or as antagonist, giving rise to a different expression patterns depending on the ligand.

1.4 Rapid extra-nuclear signaling.

In addition to genomic effects of E2, that needs at least a couple of hours to be observed at cellular level, rapid effects also take place. These effects occur within seconds or minutes, are insensitive to treatment with inhibitors of transcription (actinomycin D) and translation (cycloheximide) [28]; they are also activated following stimulation with the complex E2-BSA (17β-estradiol conjugated to bovine serum albumin), unable to cross the plasma membrane [30]. These effects require ERs to be localized at the plasma membrane, where receptors are concentrated in caveolae micro-domains, making contact with the scaffolding protein caveolin-1 [31]. At this level, ERs can interact with several proteins involved in signal transduction of hormones and growth factors (*e.g.,* G-proteins, receptor tyrosine kinases, serine/threonine kinases and adapter proteins) and form multimolecular complexes that are required to trigger the rapid signal transduction events [30, 32]. It is well established that E2 is able to induce proliferative and anti-apoptotic effects in different cell lines activating ERK/MAPK and PI3K/AKT pathways, or pro-apoptotic and differentiating effects *via* p38/MAPK pathway depending on the ER isoform present [33]. Indeed, the E2:ERα complex rapidly (15 min) activates the proliferative ERK/MAPK and PI3K/AKT and the pro-apoptotic p38/MAPK pathways; after 30 min of E2 stimulation the expression of the anti-apoptotic protein Bcl-2 is enhanced and the activation of the p38/MAPK is blocked in an ERK/MAPK-dependent manner, thus enabling the cell cycle progression [34, 35]. Conversely,

the E2:ERβ complex induces the rapid (15 min) and persistent (24 h) phosphorylation of p38/MAPK pathway, driving cells to the apoptotic route [36].

1.5 ERs post-translational modification.

Different layers of regulation finely modulate the complexity and the vastness of the ERs signaling network, as correctly addressing ERs to the right place in the right moment is a critical issue for cells. This problem is elegantly solved through the numerous post-translational modifications that ERs are able to undergo to: palmitoylation is required to promote membrane localization and ERs:caveolin-1 association. Both ERα and ERβ are palmitoylated on cysteine (C) residues with ERα being palmitoylated on C447 and ERβ on C399. The sequences encompassing these two residues are highly homologues with each other [37]. Palmitoylation occurs through the action of two palmitoyl-acyl-transferases (PAT) [38] and the mutation of the C447 to A or the chemical inhibition of PAT activity with 2-bromo-hexadecanoic acid (2-Br) prevent plasma membrane localization and the E2-mediated extra-nuclear signaling [33, 37, 39].

In response to E2 binding, ERs are also phosphorylated. ERα is phosphorylated on many serine (S) residues with S118 and S167 being the main ones; these two residues, belonging to the A/B region (*i.e.* the AF-1 domain) regulate co-factors recruitment and enhance ERα transcriptional activity [40]. The homologue residue on ERβ, despite the poor homology between ERs in the A/B region, is S87, part of a motif shared with other steroid receptors. ERβ phosphorylation on S87 enhances ERβ interaction with SRC-1 [41].

1.6 ERs intracellular content.

Regulation of ERs intracellular levels is a fundamental cell property to tightly regulate all the effects of E2 and all the effects triggered by the hormone occur in parallel with the concomitant modulation of receptors intracellular levels. ERs level modulation represents a crucial step in order to regulate ER-dependent gene transcription and involves both receptors degradation and ERs gene transcription; however, ligand modulation of receptor intracellular levels is different for ERα or ERβ.

The E2 binding produces ERα ubiquitination an event that leads to the 26S-proteasome-mediated receptor degradation [42]. This event

drastically lowers the protein half life from at least 24 hours, in the unliganded state, to less than 2 hours [43]. ERα is marked for degradation by the proteasome trough the action of protein ligases, which covalently attach ubiquitin to a target lysine [44]. The mechanism that leads to this modification contemplates ubiquitin-activating enzymes (UBA) to activate ubiquitin, before transferring the protein to ubiquitin-conjugating enzymes. Finally the receptor ubiquitination is achieved trough the action of the ligases [45]. In order to proceed with the degradation process, additional ubiquitin moieties are attached to the receptor, thus forming the ubiquitin chain [46]. The polyubiquitinated receptor is finally recognized and degraded by the 26S-proteasome complex. Proofs that upon E2 binding ERα undergoes this kind of regulation are given by treating cells with proteasome inhibitors (*e.g.,* MG-132): thus the major route of clearance of liganded ERα follows this mechanism [44]. Even if in the absence of the hormone ERα undergoes a basal amount of degradation, upon E2 binding the degradation process fastens the protein turnover, which produces a switch in the steady state of the receptor. ERα gene transcription is also regulated by many factors, which include several different transcription factors as well as ERα, which regulates its own expression. ERα mRNA synthesis can be also regulated by its promoter methylation and, more recently, a number of subtype-specific microRNAs (miRNAs) have been found to affect ERα expression [Thomas et al., 2011]. The final goal of this mechanism is the reduction of the total ERα content and in turn the reduction in the cell response to the hormone-induced stimuli, in a typical negative feedback regulation [47]. This mechanism is however not fully understood and many steps of the process are still to be cleared; there are many evidences that receptor phosphorylation plays a major role in its degradation [48]; although this receptor posttranslational modification is required for receptor activation, it is not known if it could be a signal for receptor ubiquitination, following the ERα DNA binding and modulation of target genes [49].

Less is known about ERβ regulation of intracellular content; even if there are some proofs that this receptor is degraded by the proteasome [50], stronger evidence show that ERβ does not undergo the ubiquitination processes [51]. Moreover, following E2 stimulation, ERβ induces its transcription [52] instead of down-regulate it. Thus, the intracellular concentration of the ERs results from a dynamic balance between ER synthesis and ER breakdown [53]; moreover the hormone-

induced stimuli is the result of the balance between ERα and ERβ expression, especially in cells that express both ERs isoform, like muscle myoblasts and myotubes. Nonetheless, even if the fine regulation of the ERs expression comes along with the hormone-induced rapid effects, the knowledge on the contribution of the E2-induced extra-nuclear signaling is still poor.

1.7 Endocrine disruptors (EDs).

A number of synthetic substances known as xenoestrogens show estrogenic effects. This highly heterogeneous group consists of synthetic chemicals used as drugs (*e.g.,* tamoxifen, raloxifene), in industry and agriculture (*e.g.,* pesticides) and acting as food contaminants (*e.g.,* bisphenols). Probably, the best-characterized exponent of this class is bisphenol A (BPA) a molecule widely used in plastic manufacturing and found as environmental contaminant. BPA is one of the most worldwide-produced chemicals, with more than 6 billion pounds/year produced and over 100 tons released into the atmospheare [54]. High temperature and high or low pH make BPA to be released from tin cans and polycarbonate plastic containers allowing the chemical to leach into food and water. Thus, it is a risk factor for human exposed to BPA [55]. Its simple structure, consisting in two benzene rings and two (4, 4')-OH substituents allows the molecule to bind both ERs binding pocket, with a 10-fold higher affinity to ERβ [56]; nevertheless, E2 affinity to ERs is 10.000-fold higher with respect to BPA [57]. Several reports proved that BPA is capable to induce severe adverse effects (*e.g.,* normal reproductive tract development disruption in rodents [58], inhibition of testosterone synthesis in adult rats BPA-exposed during perinatal periods [59]). Although risk of BPA exposure is not completely assessed, it is clear that human exposure easily occurs: BPA has been detected in pregnant women and also in their foetuses (in maternal and foetal plasma and amniotic fluid, BPA concentration ranges from 1 to 9 ng/ml) [60] and in the 95% of urine samples tested in U.S. at concentrations ≥0.1 μg/L [61].

Not only human-derived compounds are able to mimic estrogenic responses, a large number of naturally plant-produced molecules are known to display a mild estrogenic activity, which is exerted, in mammals, through dietary intake [62]. "Dietary estrogens" or phytoestrogens belong to a large class of compounds that include about 5000 molecules, known as flavonoids and further divided into six

subclasses according to their chemical structure: flavonols, flavones, flavanols, flavanonols, flavanones, and isoflavones [63]. Such vastness and heterogeneity make the characterization of the estrogenic effects of these compounds quite hard: due to their structure divergences, flavonoids can act as estrogen-mimetics, display an anti-estrogenic effect, or even do not affect E2 signaling at all [64]. Moreover, impact on human physiology strongly relies on the nature and the distribution of these compounds in the diet. In particular, citrus fruit and soy are the sole sources of flavonones and isoflavonones, respectively [65]; polyphenols like quercetin are found in almost all vegetables, fruit, wine and tea [66]; others, like naringenin (Nar), are more exclusively distributed and found in orange, grapefruits and tomatoes skin. In most cases, however, food contains complex and poorly characterized mixtures of polyphenols, which level can change depending on food storage, processing and environmental factors [65]. Obviously, habits, and availability is crucial to determine the differences in polyphenol human intake. In particular, flavanones are relevant only in countries where citrus fruit is available, particularly in Southern Europe [65], while isoflavon intake in Asian countries is definitely higher than Europe or America's (25-40 mg/day versus less than 1 mg/day) [67]. These differences allowed the understanding of important properties of these compounds, such as the lower predisposition of Asian women to breast cancer developing [68]. Moreover, a reduced incidence in osteoporosis [69] and cardiovascular disease [70] is related to a diet rich in flavonoids. These evidence and flavonoids antioxidant properties [66] led to a neat increase in flavonoid usage as dietary components although adverse effects are known [71] and the E2-like and E2 antagonistic effects are not yet fully clarified. Flavonoids are considered potentially able to exert a protective role against the development of E2-dependent pathologies (*e.g.,* endocrine tumors) through the binding to ERs [72]; among others, nutritionally relevant concentrations of Nar are known to induce apoptosis in different cancer cell lines ERs-containing (*e.g.,* colon, breast, and uterus cancer cell lines) [73, 36]. Although flavonoids, as in the case of BPA, show a higher affinity towards ERβ than ERα, these phytochemicals are able to activate both receptors [74]. However, for their ability to interfere with many aspects of natural hormones-dependent control of body homeostasis, reproduction, and developmental processes, xenoestrogens and phytoestrogens are also known as endocrine disruptors (EDs) [74]. EDs act directly *via* steroid

hormone receptors or indirectly through non-steroid receptors (*e.g.,* neurotransmitter receptors such as the serotonin receptor, dopamine receptor, norepinephrine receptor), orphan receptors [*e.g.,* aryl hydrocarbon receptor (AhR)], and on enzymatic pathways involved in steroid biosynthesis and/or metabolism [75].

1.8 EDs influence on ERs signaling.

Several research groups have demonstrated that Nar triggers the activation of ERE containing genes via both ERα and ERβ [72], in the mean time, it impairs ERα interaction with the transcriptional factors Sp1 and AP-1 [36]. Interestingly, since Sp1 and AP-1-dependent gene expression is strictly dependent on the extra-nuclear pathway activation, xenoestrogen could affect non-ERE containing genes transcription (*e.g.,* cyclin D1) through the modulation of extra-nuclear ERs activities [72]. These evidence, observed in human hepatoma cells (HepG2), which express only endogenous ERα*,* and in cervix carcinoma cells (HeLa), devoid of any ER isoforms but transiently transfected with the human ERα expression vector, indicate that flavonoids hinder the ERα-dependent proliferative effects.

Data from our laboratory also show that in the same cell systems, flavonoids impair the E2-mediated activation of ERK/MAPK and PI3K/AKT pathway, while they enhance the persistent (60 min) phosphorylation of p38/MAPK and, consequently, the induction of a pro-apoptotic cascade (*i.e.*, caspase-3 activation and PARP cleavage). Thus, Nar decouples the ERα action mechanisms by preventing the activation of proliferative pathways and instead by driving cells to apoptosis [36]. The mechanisms through which flavonoids impair the rapid signals activation involve the decrease in ERα localization at the plasma membrane and the consequent reduction of receptor association to caveolae. In particular, Nar induces ERα de-palmitoylation faster than E2, which results in a rapid ERα dissociation from membrane caveolin-1, thus impairing the receptor association with adaptors and/or signaling proteins (*i.e.,* MNAR and c-Src). This event precludes the activation of mitogenic signaling cascades, while the activation of p38/MAPK is independent from ERα palmitoylation, as demonstrated in HeLa cells transfected with the ERα unpalmitoylable mutant. All these events lead to the activation of the apoptotic cascade [36]. However, Nar does not impair the ERα-mediated transcriptional activity of an ERE-containing promoter [72]. Thus, flavonoids modulate specific ERα mechanisms and

they can be considered as mechanism-specific ligands for ERs [76]. In addition, Nar acts as E2-mimetic in the presence of ERβ by rapidly activating p38/MAPK and the apoptotic cascade in HeLa cells transiently transfected with ERβ expression vector or in endogenous ERβ expressing colon cancer cell line (DLD1) colon adenocarcinoma cells [36]

BPA is also known to display estrogenic activity: it is a weak ligand for both ERα and ERβ [77] even if it principally acts through the nuclear activation of the ERα [78, 79] and does not affect ERβ-based ERE containing gene transcription; this ED is able to prevent the E2:ERβ-mediated activation of ERE-based transcriptional activity [80]. Through ERα binding, it stimulates cell proliferation in ERα-containing breast cancer cells and in ERα-overexpressing HeLa cells, and mimics E2 in enhancing ERα-mediated transcriptional activity of ERE-containing promoters [72]. It has been demonstrated by our group that BPA-dependent proliferative response requires the ERα-mediated extra-nuclear signaling activation. In fact, upon BPA stimulation ERα mediates the transduction of signaling pathways that culminates with ERK/MAPK and PI3K/AKT activation. These effects are not present in empty vector-transfected cells and are completely prevented by the anti-estrogen ICI 182,780. As a whole, these data indicate that BPA acts as an E2-mimetic by binding to ERα leading to the activation of rapid extra-nuclear pathways that drive cells to proliferation [78].

Conversely, in DLD1 cells, BPA behaves as an antagonist by blocking the activation a pro-apoptotic cascade [80]. Our group has reported that the physical association between ERβ, caveolin-1, and the unphosphorylated form of p38/MAPK are present in DLD-1 cell line even in the absence of E2 [33]; after E2 stimulation, ERβ rapidly interacts with p38, leading to the phosphorylation of the kinase and the activation of apoptotic cascades [33]. Intriguingly, both in the absence and in the presence of E2, BPA stimulation specifically prevents the ERβ:p38 association without affecting the ERβ:caveolin-1 complex formation. Thus, BPA decouples ERβ from the downstream signals important for the E2-induced pro-apoptotic cascade. The BPA-induced inhibition of the ERβ:p38/MAPK interaction suggests that different ligands could modulate ER extra-nuclear signals changing in this way the final cellular outcomes by inducing distinct receptor conformational changes. Moreover, E2 induces the ERβ-dependent increase of the pC3 ERE-containing promoter transcription, while BPA does not exert any

transcriptional effect. When added together with E2, BPA prevents the E2-induced ERβ transcriptional activity. Furthermore, BPA prevents the E2 ability to increase ERβ levels, which requires both the genomic and the extra-nuclear ERβ activities. All these data suggests a complete antagonistic role of BPA on ERβ signal transduction pathways [80].

Beside their modulation of ERs rapid and nuclear activities, it is also possible that EDs could also influence ERα and ERβ cellular content like the endogenous ligands regulate receptors intracellular levels, transcription and subsequent ERs-dependent cellular effects. Nonetheless, nothing is known about the regulation that occurs following EDs binding to ERα or ERβ produced by EDs on the ERs intracellular content and expression.

2. Aim.

On this basis, the main goal of the present project was to understand the mechanisms underlying the ligand-dependent modulation of ER intracellular levels to better clarify ligand effects in ERα- and ERβ-driven physiological processes.

The highly regulated cellular mechanisms, which control the ERs intracellular levels, point to a critical role of ERs stability for E2 signaling. Indeed, numerous clinical and *in vitro* studies suggest that the alteration of ERs expression is an important step in the development and progression of E2-related diseases including different type of cancers [81]. Thus, deregulation of the balance between ERα and ERβ expression could be a critical step in several E2-dependent diseases. Signaling modulation of the ERα and ERβ intracellular levels also occur. Indeed, receptor phosphorylation appears to be required for ERα degradation [82] and the extra-nuclear E2-activated signal transduction cascades have been implicated in the modulation of ERα and ERβ cellular content. In particular, ERK/MAPK and PI3K/AKT pathways are involved in the E2-dependent regulation of the ERα degradation [83] and the activation of the p38/MAPK controls the E2-mediated increase of ERβ mRNA and protein levels [52]. This evidence together with the fact that EDs (*e.g.,* Nar and BPA) affect ERα and ERβ activities, by modulating their extra-nuclear signaling, led to the hypothesis that they could also modulate ERα and ERβ cellular content by modulating ERs mRNA and protein levels. Thus, EDs modulation of ERs cellular content could further affect the E2-dependent regulation of specific

cellular processes (*e.g.,* proliferation, differentiation and apoptosis) suggesting scenarios that strongly diverge from the physiological ones.

To this purpose, in this PhD project we used ductal carcinoma cells (MCF-7) and the colon cancer cell line DLD-1 expressing only one isoform of the receptor (ERα and ERβ, respectively) to avoid the influence that the second isoform could produce on the modulation of ERs signaling network. We also used the Human Embrionic Kidney (HEK293) cell line and the cervix adenocarcinoma HeLa cells that we endowed with wild type ERα or with ERα mutated in the C447 residue to Alanine (A) (*i.e.,* un-palmitoylable mutant) or in the S118 residue to A (*i.e.,* un-phosphorylable mutant). Where necessary, we also used a set of specific inhibitors to block cellular mechanism and to verify their involvement in the specific measured parameter.

3. Results.

3.1 Impact of EDs on cellular physiology.

In order to evaluate differences in the modulation of ERα activities induced by EDs, we have undertaken a high-throughput approach, which allowed us to assess the "signature" that endogenous, natural and synthetic compounds leave on the cell by activating the ERα receptor. Lysates from ductal carcinoma cells (MCF-7 cells), treated with E2, Nar and BPA for 24 hrs were processed in a two-dimensional gel electrophoresis and differences in the resulting cellular proteomes were analysed. More than 575 spots on the gels show differences in their quantification values. More importantly, at least 24 proteins show a significant diversity in their pattern of expression, depending on the substance that binds ERα (data not shown). These data implicate that EDs influence the physiology of cells expressing the ERα and further suggest that the observed differences in Nar- and BPA-induced protein expression pattern could be a consequence of a Nar and BPA direct influence on ERα intracellular levels.

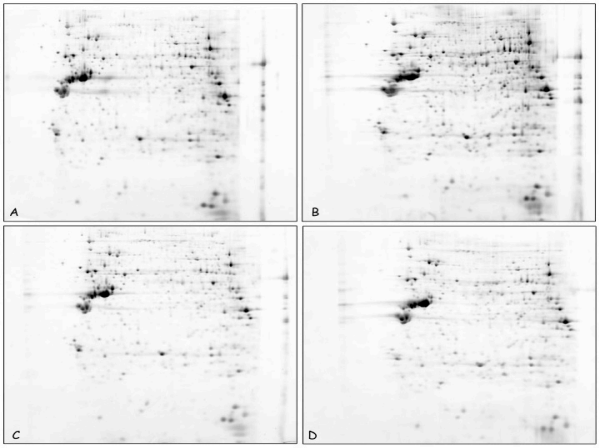

Figure 1: EDs effect on cellular proteome.
Two-dimensional electrophoresys of MCF-7 cells were treated for 24 hrs with vehicle (A), E2 10^{-8}M (B), Nar 10^{-6}M (C) or BPA 10^{-5}M (D). Results were then analyzed to point out qualitative and quantitative differences in protein expression. Figure shows representative blots.

3.2 Effect of EDs on ERα expression.

Because EDs could control ERα cellular levels and this control could result in a different protein expression, we evaluated the effect of BPA and Nar on the regulation of ERα protein expression. MCF-7 cells were treated for 24 hrs with BPA or Nar in a concentration range known to engage ERα [78, 84]. As expected, 24 hrs E2 administration reduced ERα protein levels (Fig. 2A and A'). A dose-dependent reduction in ERα intracellular levels were observed in cells exposed to BPA while none of the Nar concentrations significantly affected ERα content (Fig. 2A and A').

It is widely known that E2 decreases ERα protein half-life from 24 to 2 hrs [53] but ligand-dependent ERα degradation could occur faster than 24 hrs; for this reason, a more detailed time course analysis of the effect of BPA and Nar in MCF-7 cells showed that E2 rapidly (2 hrs) induces ERα degradation while BPA-induced ERα breakdown requires 4 hrs treatment to be apparent. Conversely, no significant changes in ERα protein content were detected when MCF-7 cells were treated with Nar at all the tested time points (Fig. 2B and B'). Because ERα regulates also the mRNA transcription of its own gene, we also evaluated the impact of all ERα ligands on receptor mRNA levels. RT-qPCR analysis showed that in MCF-7 cells 24 hrs treatment E2, Nar and BPA reduces ERα mRNA content (Fig. 2C).

This evidence indicates that BPA mimics E2 in determining ERα down-regulation while Nar reduces ERα mRNA content without affecting receptor protein level.

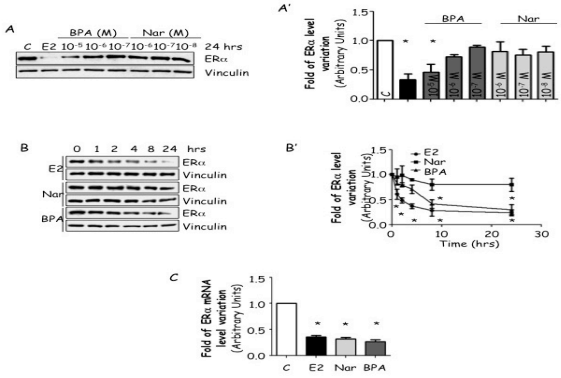

Figure 2: EDs effect on ERα.
Western blot analysis of ERα levels (A and B) and relative densitometric analysis (A' and B') of MCF-7 cells treated for 24 hrs or at indicated time points with E2 10^{-8}M, Nar 10^{-6}M or BPA 10^{-5}M. (A). RTq-PCR analysis of ERα mRNA levels treated with E2 10^{-8}M, Nar 10^{-6}M or BPA 10^{-5}M for 24 hrs. Loading control was done by evaluating vinculin expression in the same filter.*indicates significant differences with respect to the relative control sample (P<0.001).Figure shows representative blots.

3.3 Effect of ERα palmitoylation on receptor degradation.

Because Nar and BPA differentially affects ERα expression (Fig. 2) and mainly influence ERα activities by targeting ERα-triggered extra-nuclear effects [36, 72, 77], it is possible that rapid signaling could play a role in controlling ERα intracellular content.

To test this hypothesis, we evaluated the role of ERα membrane localization on receptor intracellular levels by taking advantage of the fact that palmitoylation is required to trigger rapid ERα signaling and that genetic (*i.e.,* mutation of the ERα palmitoylation site C447 to A) or pharmacological (*i.e.,* inhibition of PAT activity) interference of ERα palmitoylation both disrupt E2:ERα extra-nuclear signaling activation. Thus, we stably transfected HEK293 cells with wt ERα and the un-palmitoylable C447A ERα mutant and used the PAT inhibitor 2-Br in MCF-7 cells. In ERα wt HEK293 cells, E2 is capable of inducing a significant reduction in ERα cellular content within the first 4 hours (Fig. 3A and A'). Longer E2 treatment (8 hours) did not further enhance receptor degradation (Fig. 3A). Notably, the difference in the time-dependent E2-mediated receptor degradation between MCF-7 cells and

HEK293 stable cell lines could be ascribed to ERα overexpression. On the contrary, in HEK293 cells stably expressing the C447A mutant ERα, 2 hours of E2 administration were sufficient to determine a significant reduction in ERα levels (Fig. 3A and A'). In order to further demonstrate the impact of ERα palmitoylation on receptor degradation, we analyzed the time-course of E2-dependent ERα breakdown in HEK293 stably expressing the wt ERα both in the presence and in the absence of the PAT inhibitor 2-Br. Under 2-Br pre-treatment, E2 induced an higher reduction of ERα cellular levels than the one observed in the absence of the PAT inhibitor (Fig. 3B and B') while 2-Br alone did not modify the basal ERα cellular content of stable HEK293 cells (data not shown). These data demonstrate that inhibition of PAT activity as well as mutation of the ERα palmitoylation site determine a receptor pool that undergoes to a faster elimination in response to E2 in stable expressing ERα cells, thus indicating that ERα palmitoylation protects the receptor from E2-dependent degradation.

Accumulating evidence identifies the ERK/MAPK and PI3K/AKT pathways as the principal transduction cascades activated by E2 in many different cell contexts [85]. In line with these notions, time-course analysis revealed that E2 induces a rapid increase in ERK1/2 and AKT phosphorylation in the wt ERα expressing HEK293 cells while the hormone fails to trigger it in the C447A mutant receptor expressing cells (Fig. 3C). Notably, the basal ERK1/2 activation was increased and the basal AKT phosphorylation was reduced when the cells were transfected with the C447A mutant receptor with respect to the wt ERα (Fig. 3C), possibly because of compensatory mechanisms due to the introduction of the exogenous mutated receptor. 2-Br treatment also dampened E2-induced ERK1/2 and AKT phosphorylation in MCF-7 cells (Fig. 3D). These data confirm that ERα palmitoylation is required for the activation of the rapid E2 extra-nuclear signaling [33, 37] and further suggest the notion that a direct link between E2-induced extra-nuclear signaling and ERα degradation could occur.

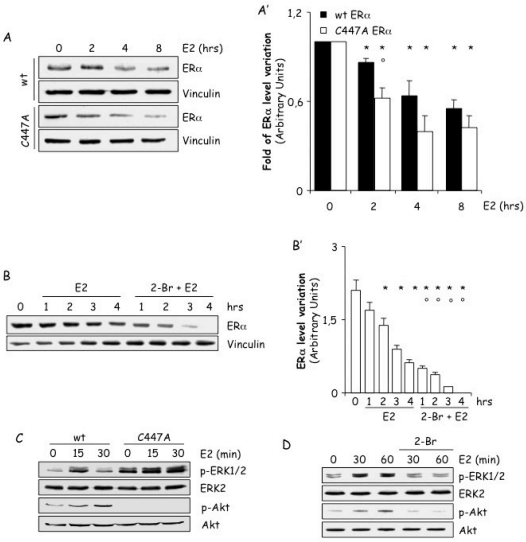

Figure 3: Effect of palmitoylation on ERα stability.
Western blot analysis of ERα cellular levels, ERK1/2 and AKT phosphorylation in HEK293 cells stably expressing the pcDNA flag-ERα (wt) and the pcDNA flag-ERα C447A (C447A) (A and C) and MCF-7 cells (B and D) treated with E2 10^{-8}M at indicated time points. Where indicated, cells were treated for 30 min with the palmitoyl-acyl-transferase inhibitor (2-bromopalmitate, 2-Br, 10 μM) before E2 administration. Loading control was done by evaluating vinculin expression in the same filter. * indicates significant differences with respect to the relative control sample; ° indicates significant differences with respect to the corresponding E2 sample ($P < 0.05$). Figure shows representative blots.

3.4 Effect of phosphorylation on ERα receptor degradation.

Beside palmitoylation, ERα S118 phosphorylation also plays a role in E2-activated ERα intracellular signaling [49]. Because ERα palmitoylation is involved in the process of E2-evoked ERα elimination (Fig. 3), we sought to determine the impact of ERα palmitoylation on the E2-evoked S118 phosphorylation. MCF-7 cells were pre-treated with the PAT inhibitor 2-Br and than time-course analysis of S118 phosphorylation was performed under E2 stimulation. However, as E2 determines a reduction in ERα cellular content both in the presence and in the absence of 2-Br (Fig. 3B), the receptor S118 phosphorylation was analyzed by quantifying the fraction of the modified ERα with respect to the total observed receptor quantity. Figure 4A and 4C' shows that E2

treatment induced a rapid increase in the amount of the S118 phosphorylated pool of the ERα within the first 30 min of hormone administration. Although total receptor cellular levels were reduced by E2, the amount of the S118 phosphorylated ERα remained constant for the next 2 hours of E2 administration. 2-Br incubation reduced the amount of the S118 phosphorylated ERα in response to E2 without changing either the overall S118 phosphorylation kinetic or the basal ERα S118 phosphorylation levels (Fig. 4A and 4C'). Accordingly, E2 increased in a time-dependent manner the phosphorylation of the receptor in the S118 residue also in stable wt ERα expressing HEK293 cells but not in HEK293 cells stably expressing the C447A mutated receptor (Fig. 4B). These data indicate that ERα palmitoylation is also required for the E2-dependent phosphorylation of the ERα on the S118 residue.

Next, we evaluated the impact of the E2 extra-nuclear signaling cascades on the ERα S118 phosphorylation status and on E2-induced receptor degradation. In MCF-7 cells, the AKT pathway inhibitor Ai but not the ERK pathway inhibitor PD pre-treatment resulted in a reduction in the amount of the S118 phosphorylated ERα in response to E2 with respect to cells that were treated with the hormone alone (Fig. 4C and C'), without affecting the basal ERα S118 phosphorylation levels (data not shown). Also in this case, the overall E2-dependent ERα S118 phosphorylation kinetic was not changed under either inhibitor treatments (Fig. 4C and C'). Remarkably, as shown in figure 4D, incubation of MCF-7 cells with Ai, induced an increase in the time-dependent E2-evoked reduction of ERα cellular amount with a statistically significant maximum effect (70%) occurring after 30 min of E2 stimulation. On the contrary, PD administration did not change the ability of E2 to induce the reduction of ERα cellular levels (Fig. 4E).

These data indicate that ERα palmitoylation and E2 extra-nuclear-activated PI3K/AKT pathway control S118 phosphorylation and further indicate that inhibition of the PI3K/AKT pathway sensitizes ERα to E2-dependent removal, thus demonstrating that the E2-dependent membrane-ERα-activated PI3K axis activation defends the receptor from E2-mediated degradation.

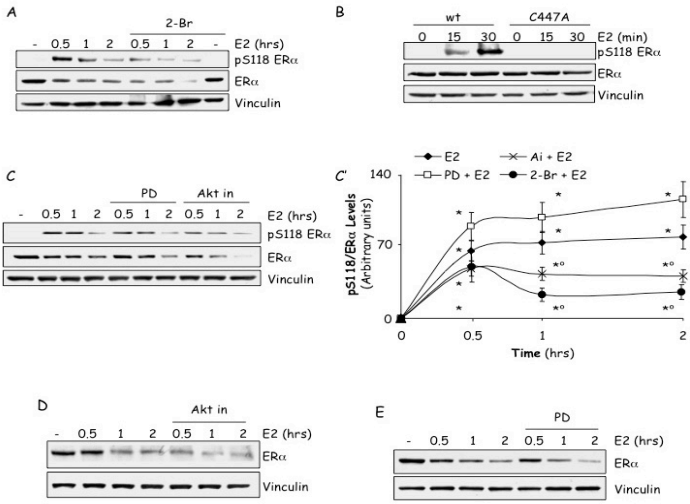

Figure 4: Effect of phosphorylation on ERα
Western blot analysis of ERα S118 phosphorylation (A,B and C), relative densitometric analysis (C') and ERα cellular levels (D and E) in MCF-7 cells (A, C, D and E) and in HEK293 cells stably expressing the pcDNA flag-ERα (wt) and the pcDNA flag-ERα C447A (C447A) (B) treated with E2 10^{-8}M at different time points. Where indicated, cells were treated for 30 min with the palmitoyl-acyl-transferase inhibitor (2-bromopalmitate, 2-Br, (10 μM), or for 1 h, AKT inhibitor (Akt in, 5μM) or with the ERK1/2 inhibitor PD 98059 (PD, 10μM) before E2 administration. Loading control was done by evaluating vinculin expression in the same filter. * indicates significant differences with respect to the relative control sample; ° indicates significant differences with respect to the corresponding E2 sample ($P < 0.05$). Figure shows representative blots.

3.5 Effect of palmitoylation and phosphorylation on ERα mediated transcription.

It is well known that ERα S118 phosphorylation is required for full ERα transcription of the ERE-containing genes [40, 86]. Because the lack of ERα palmitoylation prevents ERα S118 phosphorylation, we next studied its impact on E2-dependent ERα transcriptional activity. Real-time qPCR analysis revealed that in MCF-7 cells pre-treatment with the PAT inhibitor 2-Br prevents the increase in the amount of the E2-responsive ERE-containing gene presenelin 2 (pS2/TIFF) mRNA levels observed after 2 hours of E2 administration (Fig. 5A). The cell pre-treatment with either the AKT inhibitor Ai or the ERK1/2 inhibitor

PD also dampened the E2-induced increase in the pS2 mRNA cellular content (Fig. 5A), thus sustaining the notion that rapid E2 extra-nuclear signaling is required for ERα transcriptional activity [87]. Notably, incubation of MCF-7 cells with the inhibitors alone did not affect the total content of pS2/TIFF.

As a transcription factor, ERα cycles on and off its ERE-containing promoters with a frequency of about 30 minutes. E2 rapidly enhances the amount of the ERα associated to its responsive promoters and prolongs the frequency of the ERα:promoter association to about 60 minutes [88]. The data presented above suggest that ERα palmitoylation could be a pre-requisite for E2-activated ERα ERE-containing gene expression. Therefore, it is possible that lack of ERα palmitoylation may impair E2-activated ERα:promoter association. To test this hypothesis, we coupled chromatin immunoprecipitation (ChIP) assays with real-time qPCR analysis in MCF-7 cells to analyze the E2-dependent recruitment of ERα to the pS2/TIFF promoter region both in the presence and in the absence of the PAT inhibitor 2-Br. 2-Br administration completely prevented the E2-induced ERα recruitment to pS2/TIFF promoter without affecting the basal ERα:promoter association (Fig. 5B). Notably, the specificity of the binding of ERα to the pS2/TIFF promoter was determined by using a primer set 1 kb upstream of the ERE in pS2/TIFF, which served as a negative control (data not shown).

These data suggest that ERα palmitoylation rather than S118 phosphorylation is required for ERα-regulated ERE-containing gene expression. Therefore, in order to dissect the relative contribution of ERα palmitoylation and S118 phosphorylation on the E2-dependent ERα-mediated transcriptional activity, mutation of the S118 residue to A was first introduced both in the wild type and in the un-palmitoylable C447A mutant ERα and than the ability of the wt and mutant receptors to modulate E2-dependent ERE-based transcriptional activation was assayed in transiently transfected HeLa cells. As shown in figure 5C, 24 hours of E2 treatment was able to trigger the activation of the artificial promoter containing three repetitions of the ERE sequence (*i.e.,* 3×ERE-TATA, pERE) in the presence of both wt ERα and all the mutant receptors. Although the E2:ERα-mediated activation of the pERE promoter was significantly reduced (40%) in the presence of the S118A ERα mutant than in the presence of the wt receptor, when HeLa cells were transfected with either the C447A mutant ERα or with the S118A,

C447A double mutant receptor, the E2-induced pERE promoter activity was 70% and 50% less stimulated that the one in wt or S118A ERα containing HeLa cells, respectively (Fig. 5C).

Therefore, these data demonstrate a prevalent role of ERα palmitoylation with respect to ERα S118 phosphorylation for receptor transcriptional activity.

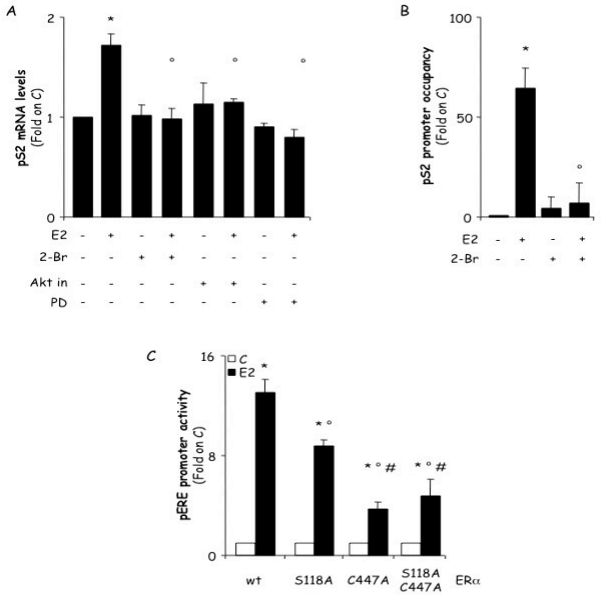

Figure 5: Effect of membrane localization on ERα transcription.
RT-qPCR analysis of pS2 mRNA expression normalized on the GAPDH mRNA expressionin MCF-7 cells treated with E2 10^{-8}M for 2 hours (A). Where indicated, cells were treated for 30 min with the palmitoyl-acyl-transferase inhibitor (2-bromopalmitate, 2-Br, 10 μM), or for 1 h either with the AKT inhibitor (Akt in, 5μM) or with the ERK1/2 inhibitor PD 98059 (PD, 10μM) before E2 administration. (B) Chromatin Immunoprecipitation analysis of ERα pS2 promoter occupancy normalized on input DNA in MCF-7 cells treated with E2 (10^{-8}M) for 1 hours. (C) Luciferase assay detection on HeLa cells transiently co-transfected with the reporter plasmid 3xERE TATA and either with the pcDNA flag-ERα (wt), pcDNA flag-ERα S118A (S118A), pcDNA flag-ERα C447A (C447A) or the pcDNA flag-ERα S118A C447A (S118A C447A) expression vectors and than treated 24 hours with E2 (10^{-8}M). * indicates significant differences with respect to the relative C sample ($p < 0.01$). ° indicates significant differences with respect to the E2 or wt E2 sample ($p < 0.01$). # indicates significant differences with respect to the S118A E2 sample ($p < 0.01$)

3.6 Effect of EDs on ERα-mediated transcription.

Although the evidence reported above demonstrates that E2-induced ERα-mediated extra-nuclear signaling finely controls the amount of the receptor intracellular content, phosphorylation and ERα transcriptional activity (Fig. 3-5), our findings on effect of Nar, which fails to trigger ERα degradation (Fig. 2) but it is known to activate ERα-dependent ERE-containing gene transcription [74], immediately challenge this concept suggesting a more complicated mechanism that link all these ERα-dependent activities. Thus, we decided tackle this problem by better evaluating the EDs ability to trigger ERα mediated transcription of a panel of E2:ERα target genes as pS2/TIFF, progesterone receptor (PR) and cathepsin D (CatD) in MCF-7 cells in comparison with E2. RT-qPCR analysis confirmed that 24 hrs of BPA or Nar treatment increase the pS2/TIFF, PR and CatD mRNA levels as the cognate E2 did (Fig. 6A-C). Because Nar-dependent lack of ERα degradation and induction of gene transcription could be related to its ability to trigger the stabilizing and transcription-inducing receptor S118 phosphorylation, we evaluated the impact of BPA and Nar to trigger ERα S118 phosphorylation in comparison to E2. Surprisingly, figure 6D shows that 2 hrs E2, BPA or Nar treatment increased the amount of the Ser118 phosphorylated ERα. Thus, BPA and more importantly Nar regulate ERα-dependent gene transcription, ERα Ser118 phosphorylation and receptor stability.

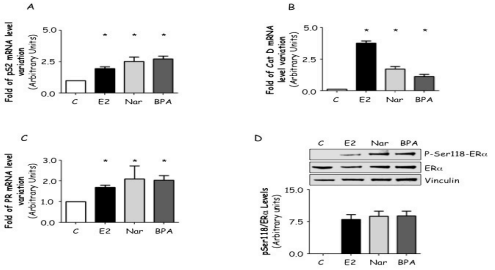

Figure 6: Effect of EDs on ERα transcription and stability.
RT-qPCR analysis of presenelin 2 (pS2/TIFF) (A), progesterone receptor (PR) (B) and cathepsin D (CatD) (C) mRNA expression, normalized on the GAPDH mRNA expression in MCF-7 cells treated with E2 10^{-8}M, Nar 10^{-6}M or BPA 10^{-5}M for 24 hours. (D) Western blot analysis of ERα S118 phosphorylation in MCF-7 cells treated with E2, Nar or BPA for 2 hours. The same filter was re-probed with anti-ERα antibody. Loading control was done by evaluating vinculin expression in the same filter. * indicates significant differences with respect to the relative control sample. ($P < 0.01$)

3.7 Effect of BPA and Nar on ERα degradation.

The data presented above indicate that BPA and Nar both induce ERE-containing gene transcription and ERα S118 phosphorylation while BPA induces reduction in ERα protein and mRNA levels and Nar triggers reduction in ERα mRNA levels (Fig. 2 and 6). Thus, it is possible that these EDs could modulate ERα expression through two different post-transcriptional mechanisms. In order to test this hypothesis, we used the protein-biosynthesis inhibitor cycloheximide (CHX) as a tool to study the effect of E2, BPA and Nar on the receptor degradation without the contribution of the neo-synthesized ERα pool (*i.e.,* pre-formend ERα) [89]. As expected, 24 hrs treatment of MCF-7 cells with E2, BPA or CHX induced a significant reduction in total ERα cellular content while Nar did not affect it (Fig. 7A). On the contrary, while E2 and BPA increased the effect of CHX on ERα breakdown, Nar treatment prevented the CHX-induced ERα degradation (Fig. 7A), thus suggesting that BPA, as E2, triggers proteolytic ERα degradation while Nar could induce an ERα intracellular accumulation.

Prompted by these results, we further investigated the Nar effect on the pre-formed ERα cellular pool [89]. Time-course analysis confirmed that prolonged (24-48 hrs) Nar treatment was able to prevent the CHX-dependent reduction in ERα cellular content while E2 further increased it (Fig. 7B). In parallel, we also evaluated if BPA-dependent ERα degradation was ascribable, as in the case of E2, to the action of the 26S proteasome [53]. Pre-treatment of MCF-7 cells with the 26S proteasome inhibitor MG-132 blocked the 24 hrs E2- and BPA-induced ERα reduction in intracellular levels (Fig 7C). As expected, the inhibition of the 26S proteasome induced an increase in the total amount of ubiquitinated proteins (Fig. 7C).

These data demonstrate that BPA mimics the effect of E2 in inducing ERα degradation and that Nar affects ERα intracellular content in a different manner than E2 and BPA.

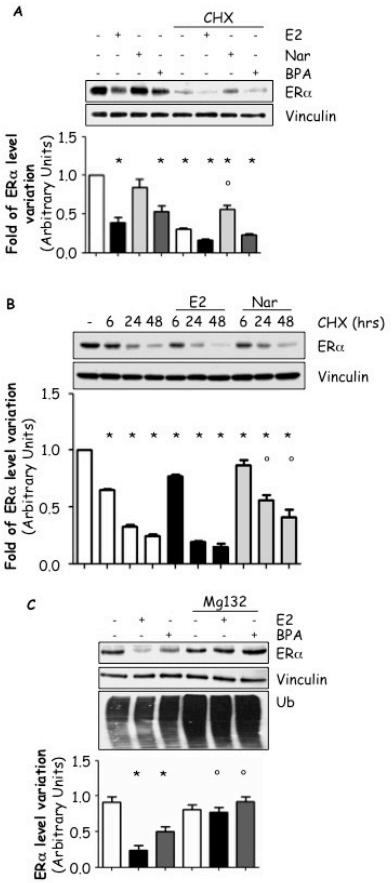

Figure 7: Effect of EDs on ERα degradation.
Western blot analysis of ERα (A, B and C) or ubiquitin (C) cellular levels in MCF-7 cells treated with E2 10^{-8}M, Nar 10^{-6}M or BPA 10^{-5}M for 24 hours (A and C) or at the indicated time points (B); Where indicated, cells were treatedfor 60 min with the 26S proteasome inhibitor MG-132 (1 μg/ml) or cycloheximide (CHX) 1 μg/ml before ligand administration (C). Loading control was done by evaluating vinculin expression in the same filter.* indicates significant differences with respect to the relative control sample; ° indicates significant differences with respect to the corresponding CHX (A and B) and E2 or BPA (C) sample.

3.8 Role of p38/MAPK pathway on the E2- and Nar-mediated control of ERα intracellular levels.

As we demonstrated, the E2-activated ERα extra-nuclear signaling protects the receptor from E2-induced degradation. Thus, one explanation for the observed Nar-dependent effect on ERα degradation (Fig. 7A and B) could be the Nar ability to selectively activate specific ERα extra-nuclear signaling pathways that shield ERα from breakdown. One possible candidate is the p38/MAPK pathway, which is activated by Nar in the presence of ERα [90]

To test this hypothesis, we first studied if the E2-induced p38/MAPK [90] could influence E2-induced ERα degradation. Thus, the E2 ability to trigger the p38/MAPK activation was evaluated in MCF-7 cells. As expected, E2 induced a rapid (15-30 min) increase in p38/MAPK phosphorylation, which was reduced after 2 hrs of E2 administration (Fig. 8A). Furthermore, incubation of MCF-7 cells with the p38/MAPK inhibitor SB 203,580 (SB) increased in the time-dependent E2-triggered reduction in the ERα cellular amount with a statistically significant effect occurring after 30 min of E2 stimulation (Fig. 8B). This evidence indicates that the activation of the p38/MAPK pathway defends ERα from E2-mediated degradation.

Therefore, the role of this signaling kinase in the Nar-dependent modulation of ERα intracellular levels was next studied. As previously reported in ERα-transfected cells [90], Nar evoked a rapid (15 min) and persistent (3 hrs) increase in p38/MAPK phosphorylation also in MCF-7 cells (Fig. 8C). Remarkably, differently than E2, 2 hrs Nar stimulation of MCF-7 cells induced the reduction of ERα cellular levels only when the p38/MAPK was inhibited. Notably, no effect of SB on basal receptor levels was detected (Fig 8C).

These data indicate that the inability of Nar to trigger receptor degradation is due to a Nar-activated p38/MAPK-dependent ERα protection.

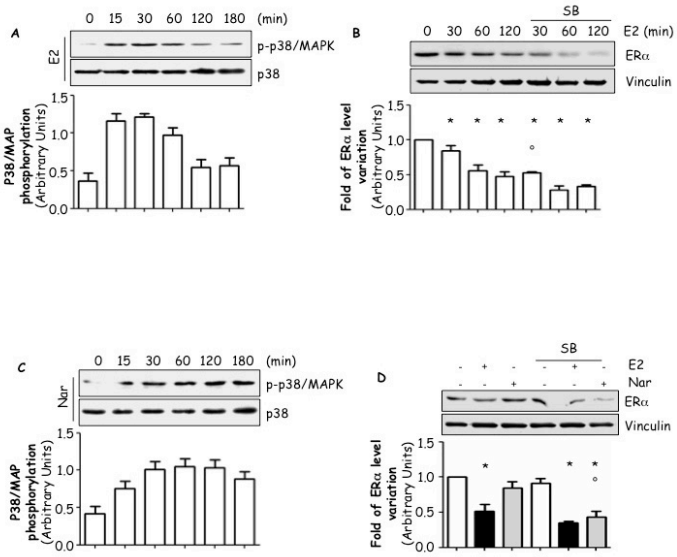

Figure 8: Role of p38/MAPK pathway on modulation of ERα intracellular levels. Western blot analysis of p38/MAPK phosphorylation (A and C) and ERα levels (B and D) in MCF-7 cells treated with E2 10^{-8}M or Nar 10^{-6}M at different time points. The filter was re-probed with anti-p38. Where indicated, cells were treated for 60 min with the p38/MAPK inhibitor SB 203,580 (SB) (1 μM). Inhibitor alone was administrated for 3 hours. Loading control was done by evaluating vinculin expression in the same filter. * indicates significant differences with respect to the relative control sample; ° indicates significant differences with respect to the corresponding E2 (B) and Nar (D) sample ($p < 0.01$).

3.9 Nar impact on ERα expression and ERE-containing gene transcription alone or in combination with E2.

The Nar-induced persistent p38 phosphorylation, the p38-dependent regulation of ERα intracellular content (Fig. 8) together with the Nar accumulation effect in the pre-formed ERα cellular pool (Fig 7B) suggest that Nar could produce ERα accumulation over long-term cell exposition. For this reason, we next evaluated the Nar ability to control ERα expression by treating MCF-7 cells chronically (48 hrs). Figure 9A and A' show that the reduction in ERα cellular levels could be detected both after 24 and 48 hrs of E2 administration to MCF-7 cells. On the contrary, 48 hrs Nar treatment increased ERα cellular content with respect to control-treated cells (Fig. 9A and A'). Moreover,

RT-qPCR analysis revealed that in MCF-7 cells 48 hrs of E2 or Nar treatment reduces ERα mRNA levels (Fig. 9B) and induces the increase in the ERE-containing pS2/TIFF gene mRNA content (Fig 9C). Remarkably, a significantly higher level of total pS2/TIFF mRNA was detected when MCF-7 cells were stimulated 48 hrs with Nar than the one found under E2 stimulation (Fig. 9C).

Because these data demonstrate that Nar induces a cellular accumulation of ERα, which results in an increased ERα transcriptional activity, we next asked whether this interference effect of Nar on ERα intracellular content could lead to alterations in the physiological E2-dependent control of ERα degradation and receptor transcriptional activity. To this purpose, we set up an experimental protocol where ERα intracellular levels and either ERα, pS2/TIFF, CatD or PR mRNA content were assayed in MCF-7 cells pre-treated with 24 hrs with Nar before additional 24 hrs E2 co-administration. Under these conditions, we observed not only that Nar prevented the E2-induced reduction in ERα intracellular levels (Fig. 10A) but also that the pS2/TIFF and CatD mRNA content was significantly higher in Nar-treated cells than in those where E2 was administrated (Fig. 10B). Interestingly, Nar pre-treatment in MCF-7 cells did not significantly affect ERα and PR mRNA levels.

This evidence strongly demonstrates that Nar alters ERα cellular content and consequently ERα gene transcription.

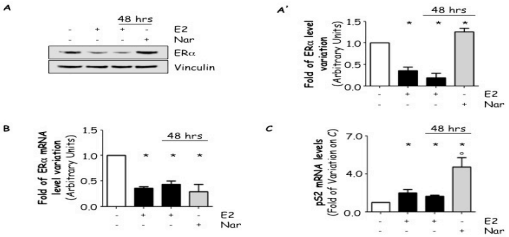

Figure 9: Impact of chronic Nar treatment on ERα expression and gene transcription.

Western blot and relative densitometric analysis of ERα cellular levels (A and A') in MCF-7 cells treated with E2 10^{-8}M or Nar 10^{-6}M, 24 or 48 hours. RT-qPCR analysis of ERα (B) and presenelin 2 (pS2/TIFF) (C) mRNA expression in MCF-7 cells treated with E2 10^{-8}M or Nar 10^{-6}M for 24 hrs or indicated time points. Loading control was done by evaluating vinculin expression in the same filter. * indicates significant differences with respect to the relative control sample; ° indicates significant differences with respect to the corresponding E2 samples. ($p < 0.01$)

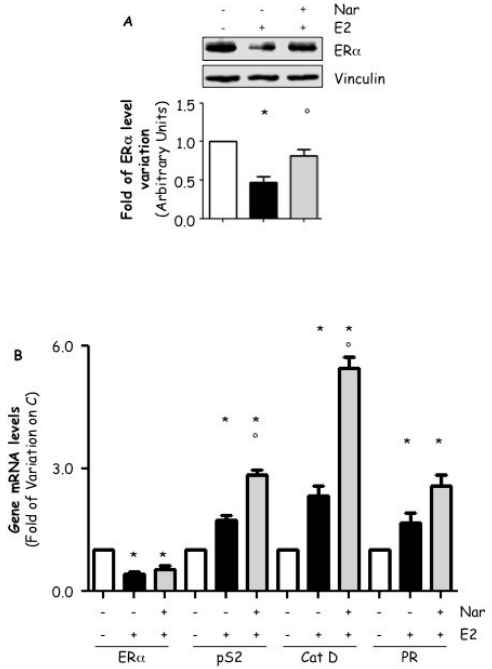

Figure 10: Role of Nar interference on E2-induced ERα control.
(A) Western blot analysis of ERα cellular levels and (B) RT-qPCR analysis of ERα, presenelin 2 (pS2/TIFF), progesterone receptor (PR) and cathepsin D (CatD) (C) mRNA expression in MCF-7 cells treated with E2 for 24 hours both in the presence and in the absence of 24 hours pre-treatment with Nar. Loading control was done by evaluating vinculin expression in the same filter. * indicates significant differences with respect to the relative control sample; ° indicates significant differences with respect to the corresponding E2 samples ($p < 0.01$).

3.10 EDs impact on ERβ cellular levels

As the p38/MAPK pathway is also the principal extra-nuclear signaling effect triggered by ERβ activation, we next analyzed the effect produced by EDs on the regulation of ERβ intracellular levels. The treatment of DLD-1 cells, endogenously expressing ERβ, with different doses of both Nar and BPA for 24 hrs show an increase in ERβ intracellular content with respect to control and E2 exposed cells (Fig 11A). Co-administration of Nar and BPA together with E2 in an experimental setting identical to the one described above (Fig 10A and

11B) revealed that ERb intracellular levels are significantly increased with respect to control and E2 treated cells (Fig. 10B), thus indicating that these EDs display an additive effect with E2 in modulating ERβ intracellular content. Therefore, Nar and BPA alter the physiological E2 control of ERβ expression.

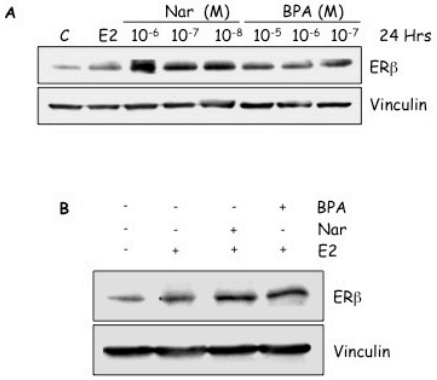

Figure 11: Effect of EDs on ERβ levels regulation.
Western blot analysis of ERβ cellular levels in DLD-1 cells treated with E2 (10^{-8}M) or with different concentrations of, Nar (10^{-6}M) and BPA (10^{-5}M) (A) and in DLD-1 cells treated for 24 hours with E2 10^{-8}M, both in the presence and absence of 24 hours pre-treatment with Nar (10^{-6}M) and BPA (10^{-5}M) for 24 hours. Loading control was done by evaluating vinculin expression in the same filter

4. Discussion and Conclusions.

The main goal of this work was to understand the mechanisms underlying endogenous and exogenous ligand-dependent regulation of ERs cellular levels and activities. To this purpose we have undertaken several different approaches that allowed us to analyze both the modulation of ERs signaling and the regulation of ERs level in cell lines expressing only one isoform of the receptor.

Because the ERα regulation of E2-induced nuclear and rapid events are widely known while the knowledge on the processes that regulate the receptor cellular content are still unclear, we started to analyze the effect of endogenous, natural and synthetic ERs ligands on MCF-7 cells, which endogenously express ERα isoform of the receptor.

Modulation of ERα intracellular content happens with the appearance of the E2 physiological effects and it is connected with E2:ERα-mediated gene transcription. Moreover, ERα degradation is also under the control of the extra-nuclear E2-activated ERα signaling [48] Interestingly, BPA and Nar, two prototype EDs, modulate ERα signaling to physiological functions by acting respectively as E2 mimetic and as an anti-E2 through the targeting of ERα extra-nuclear signaling. In particular, BPA induces cell proliferation through the activation of the ERα-mediated ERK/MAPK and PI3K/AKT pathways while Nar behaves as an anti-proliferative molecule by evoking the ERα-dependent p38/MAPK pathway activation [74, 90]. Thus, this evidence together with the critical role of ERα expression for E2 signaling [84, 91] led to the hypothesis that BPA and Nar could also modulate ERα cellular content and that this modulation could be the principal mediator of the differences observed in MCF-7 ERα-induced proteome (Fig. 1).

ERα protein levels are regulated by a dynamic balance between ERα synthesis and ERα breakdown [53]. Particularly, the native ERα protein levels are under the control of the 26S proteasome and ERα polyubiquitination is the signal to activate receptor degradation. Exposure to E2 results in a ligand-dependent reduction in the total ERα content trough the 26S proteasome-dependent degradation of the neo-synthesized ERα and of the E2-activated ERα (*i.e.,* pre-formed receptor) [53]. Analysis of the modality by which Nar and BPA affect ERα protein intracellular content reveals that BPA mimics the E2 effects in inducing the 26S proteasome-dependent ERα degradation; on the contrary, Nar does not affect ERα cellular levels at all (Fig. 2-7-10). Importantly, these differences on Nar and BPA modulation of ERα

cellular content control seems to rely on 26S proteasome mediated degradation, as both E2 as well as EDs induce the ERα mRNA levels down-regulation (fig 2C), while only the endogenous hormone and the EDs reduce ERα protein level (Fig. 2A and B) and the incubation with the proteasome inhibitor, MG-132, reverts the observed effect (Fig. 7C). As E2 and EDs produced the same regulation of ERα mRNA levels, we suspected that rapid E2-induced ERα signaling, and therefore ERα membrane localization, could also participate in ERα protein regulation.

ERα is palmitoylated on the C447 by the action of two PAT and that the PAT-dependent enzymatic palmitoylation is required for ERα to associate with caveolin-1 and to mediate E2 extra-nuclear signaling [33, 38]. Our research group has also indicated that E2 binding determines ERα depalmitoylation and dissociation from caveolin-1, a series of mechanistic events that facilitate receptor movements within membrane subdomains [33]. As a consequence, E2 activation of the extra-nuclear signaling kinase cascades (*e.g,*. ERK/MAPK and PI3K/AKT pathways) occurs and regulates several different physiological processes (*i.e.,* proliferation, apoptosis, and differentiation) [85]. Therefore, we used a genetic (*i.e.,* mutation of the ERα palmitoylation site C447 to A) and a pharmacological (*i.e.,* inhibition of PAT activity) approach to evaluate the impact of E2:ERα extra-nuclear signaling activation on E2-induced receptor degradation.

In particular, these approaches have allowed us to discover a previously unrecognized pathway in which ERα palmitoylation is the upstream structural determinant that guarantees the physiological balance of the ERα protein levels (Fig. 3). We found that, in the absence of E2, lack of ERα palmitoylation does not affect total ERα protein content, whereas, in the presence of E2, it causes faster receptor degradation (Fig. 3A and 3B). In parallel, because we also observed that inhibition of PAT activity does not change basal and E2-regulated ERα mRNA content (data not shown), we conclude that in the absence of E2, the native ERα pool requires palmitoylation for stabilization, thus demonstrating additional functions of ERα palmitoylation and showing that this receptor posttranslational modification is involved in the regulation of ERα stability.

Under the same experimental settings, we were also able to demonstrate that ERα rapid signaling and (Fig 3C and 3D) ERα S118 phosphorylation were strongly dampened (4A and 4B). Although the role of ERα S118 phosphorylation in regulating E2-induced receptor

breakdown is not clear, previous works suggested that S118 phosphorylation could be essential for ERα entry into the ubiquitin-proteasome pathway [82] and for a full ERα transcriptional activity [92], as S118-phosphorylated ERα translocates to E2-responsive promoters [93] and recruits transcriptional cofactors [86]. Our finding demonstrates that E2 maintains both a constant level of S118 phosphorylation, whereas it triggers a significant reduction in total ERα content and a parallel increase in ERα gene transcription (Fig. 2B, Fig. 4A and B, Fig. 5A).

Interestingly, the rapid E2-dependent activation of the PI3K/AKT pathway but not of the ERK/MAPK pathway regulates ERα S118 phosphorylation and the effect of the lack of ERα palmitoylation on E2-evoked ERα degradation is mimicked by PI3K/AKT pathway inhibition and unaffected by ERK1/2 inhibitor. Thus, the PI3K/AKT pathways is involved in the regulation of the ERα cellular levels. Regarding the role of ERK/MAPK pathway, while some evidences show a faster ERα degradation upon MAPK activation [94], other supports our observations [95]. The lack of the E2-dependent AKT activation prevents ERα S118 phosphorylation, whereas the blockade of the E2-induced ERK/MAPK pathway does not affect the receptor phosphorylation on this S118 residue; thus E2-induced ERα S118 phosphorylation is ERK/MAPK independent in breast cancer cells [40].

Thus, a situation can be envisioned in which after E2-induced de-palmitoylation, ERα becomes phosphorylated on S118 residue by the E2-dependent activation of the PI3K/AKT pathway. In turn, S118 phosphorylation stabilizes the receptor that in this way becomes transcriptionally activated.

In line with this concept, reduction in S118 phosphorylation correlates with a faster E2-induced receptor elimination (Fig. 3 and 4), and is paralleled with ERα transcription impairment (Fig. 5). Thus, both palmitoylation and phosphorylation control ERα activity and stability and are each other linked in a process that suggests the first post-translational modification to be the cause of the second.

Curiously, even if ERK/MAPK pathway does not intervene in ERα phosphorylation and degradation (Fig. 4C and E), the reduction in E2:ERα-dependent gene regulation is observed (Fig. 5A). This apparent contradiction can be explained by the recent discoveries that demonstrate that the ERK protein physically transport the receptor on the gene promoters induced by ERα [96]. It is logical, therefore, that the

inhibition of this pathway involves the reduction of ERα transcription as well.

The use of different ERα ligands and the analysis of their behavior has allowed us to highlight how, on the basis of different effects induced by different substances, something was missing in our findings. In particular, the fact that Nar fails to trigger ERα degradation (Fig. 2) but produces an increase in ERE-mediated gene transcription (Fig 6A, B and C) immediately challenges the concept that the E2-induced ERα-mediated extra-nuclear signaling finely controls the amount of the receptor intracellular content, phosphorylation and ERα transcriptional activity (Fig. 3-5). Nonetheless, ERα S118 phosphorylation explains these discrepancies, as both the ERα partial antagonist Nar as well as the E2 mimetic BPA determine ERα phosphorylation, thus partially stabilizing the receptor (Fig. 6D).

However, EDs control ERα expression through highly sophisticated mechanisms. Indeed, exposure to E2 results in a ligand-dependent reduction in the total ERα content trough the 26S proteasome-dependent degradation of the neo-synthesized ERα and of the E2-activated ERα (*i.e.,* pre-formed receptor) [53]. Analysis of the modality by which Nar and BPA affect ERα protein intracellular content reveals that BPA mimics the E2 effects in inducing the 26S proteasome-dependent ERα degradation (Fig. 7A and 7C). On the contrary, Nar induces the receptor accumulation by blocking ERα proteolytic degradation of the pre-formed receptor as demonstrated by the Nar effect in the presence of the protein-biosynthesis inhibitor cycloheximide or by chronic treatment of MCF-7 cells with Nar (Fig. 7A, 7B and 9A). Although we did not evaluate the role of Nar and BPA on the neo-synthesized ERα, the study of the effects of these EDs on ERα mRNA levels show that EDs mimic E2 in reducing the ERα mRNA levels (Fig. 2C, 9B and 10B), thus mimicking E2-induced ERα degradation that occurs with a parallel reduction in the ERα mRNA levels [53]. This behavior on ERα synthesis reduces the importance of the newly synthesized fraction in the evaluation of the EDs induced effects on ERα protein, making this negligible and comparable to that pre-formed one. It is also important to note that epigenetic mechanisms could participate in the regulation of ERα levels by EDs; promoter methylation and miRNAs transcription have been found to affect ERα mRNA synthesis [81]. Thus, differences in the ligand-induced allosteric regulation of the protein, which leads to a different repositioning of

helix 12 and that are responsible for the different ERα-mediated protein expression and the alternative activation of rapid signaling pathways, may also generate a different miRNAs expression, both at quantitative and qualitative level. Thus, is tempting to speculate that differences observed in the time-dependent regulation of ERα protein induced by E2, BPA and Nar could in part rely on these processes. Although neither BPA nor Nar have been found to regulate miRNA expression, recent data indicate that flavonoids (*e.g.*, genistein, daidzedin) could function as miRNA regulators [97], further inditating this hypothesis to be investigated

The mechanism by which Nar induces the accumulation in ERα intracellular levels requires the persistent activation of the p38/MAPK (Fig. 8). As demonstrated above, signaling modulation of ERα intracellular levels is dependent on the activation of the E2-evoked ERα extra-nuclear kinase cascades; E2-dependent activation of the PI3K/AKT but not of the ERK/MAPK pathway protects ERα from the E2-induced proteolytic breakdown. Moreover, p38/MAPK has been implicated in the regulation of ERα turnover [98]. Our results confirm that E2 determines the rapid and transient activation of the p38/MAPK pathway in ERα-containing cells (Fig. 8A) [90] and also demonstrate that the E2-activated p38/MAPK pathway is involved in the regulation of ERα intracellular levels (Fig. 8B). Thus, in addition to the PI3K/AKT pathway, the p38/MAPK pathway further contributes to the E2-dependent control of ERα cellular levels. Accordingly, Nar does not affect PI3K/AKT and ERK/MAPK pathway activation in the presence of ERα while it induces *via* ERα the persistent activation of the p38/MAPK cascade (Fig. 8C) [74]. In turn, we hypothesized the involvement of this kinase cascade in Nar-dependent control of ERα intracellular levels. Remarkably, in the presence of the pharmacological inhibition of the p38/MAPK (*i.e.,* SB treatment), Nar acquired the ability to trigger ERα degradation (Fig. 8D). It is known that there is an interplay between PI3K/AKT and p38/MAPK; the activation of the first pathway leads to the de-phosphorylation of the second [99]. Thus, it is tempting to speculate that the delay in ERα protein degradation observed under BPA treatment in respect to E2 (Fig. 2A and B) could be related with difference in the time-dependent activation of these two pathways; thus even if BPA mimics E2 behavior by modulating the extra-nuclear activities and the receptor gene regulation, differences in the chemical structure of these compounds could produce subtle changes in the

modulation of ERα signaling network, that lead to the observed differences in the protein expression (Fig. 1) and in the regulation of ERα cellular content (Fig. 2). Nevertheless, E2 and BPA activation of p38/MAPK is soon extinguished by the concomitant phopshorylation of PI3K/AKT pathway and both kinases work together in activating and stabilizing ERα. In MCF-7 cells upon Nar stimulation, the persistent p38/MAPK activation leads to ERα accumulation. Thus, on this basis, because BPA triggers the activation of both PI3K/AKT and p38/MAPK pathways in the presence of ERα [74, 100], we can also speculate that the reduced rate of BPA-induced ERα breakdown could be due to a different, time dependent activation of the p38/AKT pathway.

ERα S118 phosphorylation is necessary for the extra-nuclear signaling-dependent protection of ERα from E2-induced degradation. However, we found that p38/MAPK inhibition does not prevent E2-induced ERα S118 phosphorylation (data not shown) and that Nar or BPA treatment still induces this receptor phosphorylation event (Fig. 6D). Interestingly, other residues (*i.e.,* serine 294 and threonine 311) are the reported targets ERα p38/MAPK-dependent phosphorylation [98, 101]. Thus, additional phosphorylation sites may be required for ERα protection from proteolytic degradation. Nonetheless, in agreement with the concept that ERα S118 phosphorylation is required for full ERα transcriptional activity [102], BPA- and Nar-dependent ERα Ser118 phosphorylation correlates with ERE-containing ERα target gene transcription (*i.e.,* pS2/TIFF, CatD and PR) (Fig. 6A, 6B and 6C).

More importantly, we also found that the Nar-dependent accumulation of ERα results in an increased receptor transcriptional activity (Fig. 9A and 9C) and that, upon Nar stimulation, E2 looses its capacity to regulate ERα turnover and to physiologically control ERα gene transcription. Indeed, the Nar-dependent blockade of E2-induced ERα down-modulation (Fig. 10A) has the consequence to enhance pS2/TIFF and CatD transcriptional activity (10B). These discoveries indicate that in a cellular context exposed to Nar the absolute physiological receptor response or the one in response to E2 is changed because of dysregulated receptor expression. Thus, Nar modulation of ERα cellular content could further affect the E2-dependent regulation of specific cellular processes (*e.g.,* proliferation, differentiation and apoptosis).

The interfering influence of EDs on ERs is also confirmed for ERβ as both Nar as well as BPA were able to produce the increase of

ERβ protein content alone and under E2 co-stimulation protocol. This increase was reported in E2 treatment, as the hormone binding lead to ERβ gene up-regulation [52] and could be expected in Nar exposure, as this flavonoid is a known ERβ agonist. However, the notion that p38/MAPK pathway is the principal rapid signaling effect triggered by the activation of ERβ further confirms our hypothesis on the stabilizing effect of this mechanism on ERs level.

Remarkably an unexpected increase in ERβ cellular content was also produced by BPA, which is known to impair the activation of p38/MAPK pathway and the ERβ-mediated transcription [80, 33]. These discrepancies could be explained by speculating on the activation of alternative pathways modulated by this ED that could lead to the introduction of new players in the game of nuclear receptors signaling network modulation. This hypothesis, however, has to be completely explored. Nevertheless, figure 11B underline that EDs perturb the physiological E2-induced regulation of ERβ receptor levels and lead to an increased receptor cellular content, thus creating scenarios that could strongly diverge from the physiological ones.

Overall, the studies conducted during this PhD project demonstrated that the exposure to EDs drastically modifies ERα expression, which is at the basis of the differential 'signature' induced by Nar and BPA that, in turn, conveys an altered response if compared to the E2-dependent physiological one.

Because alteration (*i.e.,* reduction) of ERα expression is an important step in the development and progression of E2-related diseases including breast cancer, present findings indicate that exposure to BPA or Nar, by hijacking the physiological controls of ERα down-modulation, could induce and/or promote cancer cell proliferation, as in the case of BPA, or could be preventive, as in the case of Nar.

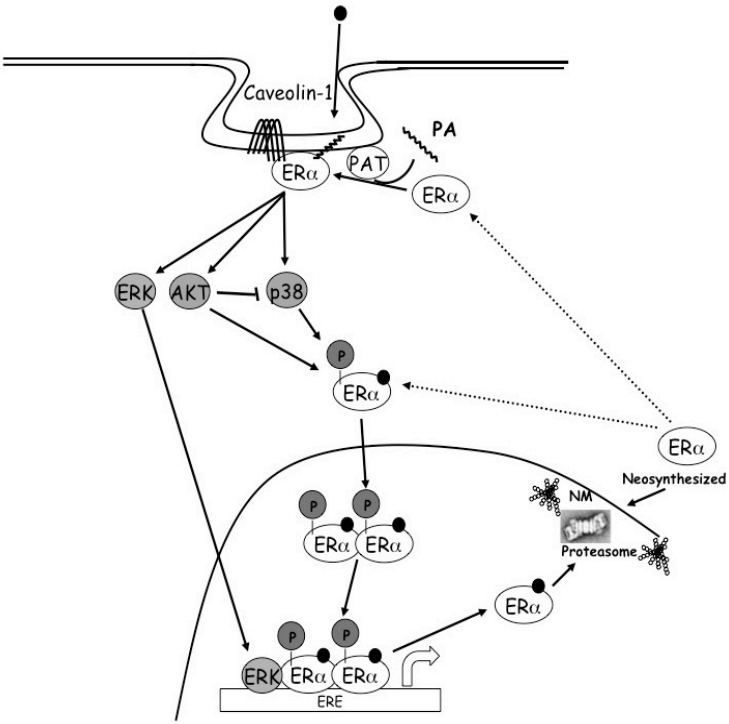

Figure 12: E2-induced extra-nuclear activities regulate ERα content transcription and degradation.
Final model that summarize our findings in the regulation of E2 modulation of ERα activities and levels

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Material and Methods

Cell culture and reagents.

Human ductal carcinoma cells (MCF-7), human cervix carcinoma cells (HeLa) and adenocarcinoma colon cells (DLD-1), as well as stably transfected human embryonic kidney 293 cells (HEK293) were grown reported (La Rosa et al. 2012). 17β-estradiol (E2), naringenin (Nar), bisphenol-a (BPA) gentamicin, penicillin and other antibiotics, Dulbecco Modified Eagle Medium (DMEM) (with and without phenol red), charcoal stripped fetal calf serum (DCC), and the palmitoyl-acyl-transferase (PAT) inhibitor 2-bromohexadecanoic acid (2-bromo-palmitate; 2-Br) [IC_{50} of ~4 μM; (20)], cycloheximide (CHX) were purchased from Sigma-Aldrich (St. Louis, MO). Bradford protein assay was obtained from Bio-Rad (Hercules, CA). Specific antibodies against anti-ERα (D12 and MC-20), phospho-ERK1/2, anti-ERK2, ubiquitin (P4D1 mouse) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); vinculin antibody was purchased from Sigma-Aldrich (St. Louis, MO). Anti-phospho-p38, anti-p38, anti-ERα phospho-Ser118, anti-phospho-AKT, anti-AKT antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA).. CDP-Star, chemiluminescence reagent for Western blot was obtained from PerkinElmer. The MAP kinase cascade inhibitor PD 98059 (PD), the Akt inhibitor (AI), p38/MAPK inhibitor, SB 203,580 (SB) and the 26S proteasome inhibitor MG132 were purchased by Calbiochem (San Diego, CA). Lipofectamine reagent was obtained from Invitrogen (Carlsbad, CA). The luciferase kit was obtained from Promega (Madison, WI). All the other products were from Sigma-Aldrich. Analytical- or reagent-grade products, without further purification, were used.

Plasmids

The reporter plasmid 3xERE TATA, the pcDNA flag 3.1 C as well as the pcDNA flag-ERα were previously described (La Rosa et al., 2011). The pcDNA flag-ERα C447A was obtained by subcloning the ERα C447A open reading frame (ORF) from the pSG5-HE0 C447A (Acconcia et al., 2005) into the pcDNA flag 3.1 C. The pcDNA flag-ERα S118A and the pcDNA flag-ERα S118A C447A were obtained by

site-directed mutagenesis of the relative templates by using the QuikChange kit (Stratagene, La Jolla, CA) and the following oligonucleotide: 5'-CACCCGCCGCCGCAGCTGGCGCCTTTCCTGCAGCCCCAC-3' (bold underlined nucleotides differ from the ERα ORF). Plasmids were than sequenced to verify the introduction of the desired mutations.

Biochemical Assays.

Cells were grown in 1% charcoal-stripped fetal calf serum medium for 24 h and then stimulated with E2 at the indicated time points; where indicated, inhibitors (SB, AI, PD, MG132 and CHX) were added 1 h before E2 administration. Unless otherwise indicated, cells were treated with E2 (10^{-8} M), Nar (10^{-6} M) or BPA (10^{-5} M). Cells were lysed in YY buffer [50 mM HEPES (pH 7.5), 10% glycerol, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA] plus protease and phosphatase inhibitors. Proteins were transferred onto a nitrocellulose membrane (GE Healthcare, Fairfield, CT). After blocking [1 h at room temperature in 5% nonfat dry milk Tris-buffered saline with Tween 20 (TBS-T) solution or in 5% BSA dissolved in TBS-T solution], filters were incubated with the appropriate primary antibody overnight at 4° C, followed by three washes of 10 min each in TBS-T and then incubated with the anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody diluted in TBS-T for 60 min at room temperature. After incubation with the secondary antibody, the filter was washed three times in TBS-T (5 min each), and the bound secondary antibody was revealed using the enhanced chemiluminescence method (GE Healthcare).

Stable transfection.

HEK293 cells were transfected using calcium chloride. Briefly, a total amount of 10 μg of DNA was mixed together with $CaCl_2$ (0.25 M) in Hepes buffer (HBS, Hepes 25 mM, KCl 10 mM, dextrose 12 mM, NaCl 280 mM Na_2HPO_4 x $7H_2O$ 1.5 mM). Sixteen hours after transfection medium was changed and the selection antibiotic was added. In particular, HEK293 cells stably expressing ERα were generated by using G418 (400 μg/ml). For the pcDNA-flag expressing cells three clones, which display the same growth rate, were selected on the basis of flag expression.

Transient transfection and luciferase Assay

HeLa cells were grown to 70% confluence and then transfected using lipofectamine reagent according to the manufacturer's instructions. Three hours after transfection, the medium was changed, and 24 hours after, the cells were serum starved for 24 hours and then stimulated with E2 for 24 hours. The cell lysis procedure as well as the subsequent measurement of luciferase gene expression was performed using the luciferase kit according to the manufacturer's instructions with a PerkinElmer Life and Analytical Sciences (Bad Wildbad, Germany) luminometer.

RNA isolation and quantitative RT-PCR analysis (qRT-PCR).

The sequences for gene-specific forward and reverse primers were designed using the OligoPerfect Designer software program (Invitrogen, Carlsbad, CA, USA). The following primers were used: for human pS2 5'-CATCGACGTCCCTCCAGAAGAG-3' (forward) and 5'-CTCTGGGACTAATCACCGTGCTG-3' (reverse), primers for human estrogen receptor α (*ERα*) 5'-GTGCCTGGCTAGAGATCCTG-3' (forward) and 5'-AGAGACTTCAGGGTGCTGGA-3' (reverse), primers for human cathepsin D (CatD), 5'-GTACATGATCCCCTGTGAGAAGGT-3' (forward) and 5' GGGACAGCTTGTAGCCTTTGC-3' (reverse), primers for human progesterone receptor (*PR*) 5'-AAATCATTGCCAGGTTTTCG-3' (forward) and 5'-TGCCACATGGTAAGGCATAA-3' (reverse), primers for human GAPDH 5'-CGAGATCCCTCCAAAATCAA-3' (forward) and 5'-TGTGGTCATGAGTCCTTCCA-3' (reverse) . Total RNA was extracted from cells using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To determine *pS2, CatD, ERα* and *PR* gene expression levels, cDNA synthesis and qPCR were performed using the GoTaq two-step RT-qPCR system (Promega) in ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Each sample was tested in triplicate and the experiment repeated three times. All primers used were optimized for real-time amplification in a standard curve amplification (>98% for each pair of primers) and verifying the production of a single amplicon in a melting curve assay. Results were normalized to the expression of *GAPDH* mRNA. The relative level for each gene was calculated using the $2^{-\Delta\Delta Ct}$ method and reported in arbitrary units.

Chromatin immunoprecipitation.

ChIP assays were performed essentially as previously described (Barnett et al., 2008). After starvation and ligand treatment, MCF-7 cells were cross-linked using 1% formaldehyde at 37° C for 10 min. Glycine (0.125 M) was than added for 5 min at RT. Cells were next washed twice with PBS and harvested in ice-cold PBS. Cell pellets were first re-suspended in nuclei isolation buffer [50 mM Tris (pH 8.0), 60 mM KCl, 0.5% NP40, protease inhibitor, and 10 mM DTT], centrifuged at 3,000 × g for 5 min, and resuspended in 200 µl lysis buffer [0.5% SDS, 10 mM EDTA, 0.5 mM EGTA, 50 mM Tris (pH 8.0), protease inhibitor, and 10 mM DTT]. Nuclei were sonicated (Fisher Scientific, Sonic Dismembrator Model 100) three times at 80% maximum power for 5 s and the sonicate was centrifuged at 14,000 × g for 10 min. The supernatant was diluted up to 500 µl with dilution buffer [1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris (pH 8), protease inhibitor, and 10 mM DTT] and 1/10 was taken aside as input for qPRC analysis. The samples were than pre-cleared with 50 µl of protein G beads for 1 h rotating at 4° C. Following protein G beads removal, lysates were incubated at 4° C rotating o.n. with 5 µg of anti-ERα antibody (MC-20, Santa Cruz Biotechnology), then pulled down at 4° C for 1 h with 50 µl of protein G beads. After brief centrifugation, precipitates were sequentially washed twice with 1 ml of washing buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), and 150 mM NaCl], once with 1 ml washing buffer II [1% NP-40, 1 mM EDTA, 20 mM Tris-HCl (pH 8.0), 250 mM LiCl], and twice with 1 ml of TE buffer [1 mM EDTA and 10 mM Tris-HCl (pH 8.0)]. Chromatin complexes were centrifuged and than eluted by incubating at R.T. for 30 min the beads with 50 µl 1% SDS e 0,1 M $NaHCO_3$. Following centrifugation, this step was repeated for 10 min at R.T. The cross-linking was reversed by incubating at 65° C overnight with 200 mM of NaCl and 200 mg/ml of proteinase K (Invitrogen Corp.). RNase A (1 mg/ml) was also added for 30 min at 37° C. DNA was next purified with QIAquick columns (Qiagen). Real-time qPCR analysis was done with primers for the *pS2* gene (*pS2* promoter primers) -463 to -159 or 1 kb upstream of this element to serve as a negative control (*pS2* upstream primers) -1,953 to -1,651. The sequences of the *pS2* promoter primers were 5' GAATTAGCTTAGGCCTAGACGGAATG 3' and 5' AGGATTTGCTGATAGACAGAGACGAC 3'. For the *pS2* upstream

primers, the sequences were 5’ CTCCCTCTTCAGGCCTCTCT 3’ and 5’ TTCCCTGGTGTTGTCAAGTG 3’ (35).

2-D electrophoresis and quantitative analysis

MCF-7 cells treated with E2, Nar and BPA for 24 hrs were collected by centrifugation, lysed with 200 ml lysis solution (7 M urea, 2 M thiourea, 4% w/v CHAPS, 0.5 ml protease inhibitor mix) and centrifuged (13,000 g, 30 min, 10 °C). Proteins were collected in the supernatant and their concentration was determined using the Bio-Rad Protein Assay (Bio-Rad, USA). 2-D was performed according to (Görg et al., 2000), with minor modifications. Samples (about 500 mg) were diluted to 250 ml with a buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 0.5% IPG buffer 3e10 NL, 2 mM tributylphosphine and traces of bromophenol blue, and loaded on 13 cm IPG DryStrips with a non-linear 3e10 pH gradient by in-gel rehydration (1 h at 0 V, 10 h at 50 V). Isoelectrofocusing (IEF) was performed at 20 °C on IPGphor (GE Healthcare, UK) according to the following schedule: 2 hrs at 200 V, 2 hrs linear gradient to 2000 V, 2 hrs at 2000 V, 1 hrs of linear gradient to 5000 V, 2 hrs at 5000 V, 2 hrs linear gradient to 8000 V and 2 hrs and 30 min at 8000 V. Immobilized pH gradient (IPG) strips were then equilibrated for 2 × 30 min in 50 mM TriseHCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS and traces of bromophenol blue containing 1% DTT for the first equilibration step and 2.5% iodoacetamide for the second one. SDS-PAGE was performed using 11% 1.5 mm thick separating polyacrylamide gels without stacking gel, using SE 600 system (Hoefer, USA). The second dimension was carried out at 45 mA/gel at 18 °C. Molecular weight marker proteins (11e170 kDa from Fermentas, Canada) were used for calibration. Gels were stained with Coomassie Brilliant Blue R-350, scanned with an Epson Perfection V750 Pro transmission scanner (Epson, Japan) and analyzed with ImageMaster 2D Platinum V5.0 software package (GE Healthcare). Spots were automatically detected by the software and manually refined afterward; gels were matched and the resulting clusters of spots confirmed manually. Spots were quantified on the basis of their relative volume (spot volume normalized to the sum of the volumes of all the representative spots in the same gel) and those that consistently and significantly varied between controls and treated samples were identified.

Statistical analysis.

A statistical analysis was performed using the ANOVA test with the InStat.3 software system (GraphPad Software Inc., San Diego, CA). Densitometric analyses were performed using the freeware software Image J by quantifying the band intensity of the protein of interest respect to the relative loading control band intensity. In all analyses p values less than 0.01 were considered significant but for densitometric analyses p was < 0.05. Data are means of three independent experiments ± S.D.

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