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INTERAZIONI TRA LE ATTIVITA' DEL RECETTORE PER GLI ESTROGENI ALFA E LA RETE DI SEGNALI BASATI SULL'UBIQUITINA

INTERACTIONS BETWEEN ESTROGEN RECEPTOR ALPHA ACTIVITIES AND THE UBIQUITIN-BASED SIGNALLING NETWORK

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RIASSUNTO.

Il 17β-estradiolo (E2) è l'estrogeno più efficace negli esseri umani e svolge un ruolo critico nel controllo di numerosi processi cellulari che influenzano fortemente diversi aspetti della fisiologia femminile e maschile. Gli effetti cellulari di E2 (ad es. proliferazione, apoptosi, differenziamento) dipendono dalla fine regolazione dell'attività dei suoi recettori, il ERα ed il ERβ, che in seguito al legame dell'ormone mediano l'attivazione di vie extra-nucleari (ad es. attivazione di protein chinasi) e nucleari (ad es. trascrizione genica). La deregolazione degli eventi indotti dal E2 è considerato un fattore di rischio per l'insorgenza e la progressione di diverse tipologie di cancro, per cui la comprensione del meccanismo d'azione dei suoi recettori riveste una notevole importanza per l'identificazione di possibili bersagli farmacologici. In particolare, molta attenzione è rivolta al ERα, che media gli effetti proliferativi del E2 in cellule di cancro alla mammella. Gli attuali approcci terapeutici al cancro alla mammella ERα-positivo dipendono anche dall'utilizzo di farmaci che legano il ERα e modificano i suoi livelli cellulari (ad es. Tamoxifene, Faslodex); tuttavia, i possibili effetti collaterali di questi farmaci [es. resistenza, cancro in altri tessuti (es. endometriale)] spingono alla continua ricerca di nuovi bersagli farmacologici.

Tra le terapie contro il cancro, il sistema dell'ubiquitina (Ub) è oggetto di interesse in quanto è alla base di una complessa rete di interazioni proteiche che è critica per la trasduzione del segnale responsabile di molti processi cellulari. Le interazioni molecolari dipendenti dall'Ub si basano su associazioni non-covalenti tra proteine modificate con l'Ub (ubiquitinazione) e proteine (chiamate recettori per l'Ub) che possiedono un dominio di legame all'Ub (UBD). La deregolazione di questo intricato sistema è stata associata a diverse condizioni patologiche, incluse diverse tipologie di cancro. Il sistema dell'Ub controlla la segnalazione del complesso E2:ERα. Infatti, mentre la modificazione con catene di Ub (poliubiquitinazione)

controlla la degradazione del recettore e la sua attività trascrizionale, la modificazione del ERα con una singola molecola di Ub (monoubiquitinazione) è richiesta per l'attivazione dipendente dal E2 di segnali rapidi che portano alla proliferazione cellulare. Anche se non è stato chiarito il meccanismo molecolare attraverso il quale la modificazione con l'Ub modula le attività del ERα, una possibilità è che il ERα possa comportarsi come un recettore per l'Ub che lega la monoubiquitina (monoUb) su se stesso o su proteine interagenti e trasduce il segnale che porta alla proliferazione cellulare.

L'obiettivo di questo progetto di dottorato è stato valutare la capacità del ERα di legare non-covalentemente l'Ub e l'eventuale ruolo regolatorio di questo legame nei processi cellulari dipendenti dal E2.

Inizialmente, sono stati condotti esperimenti *in vitro* per comprendere l'abilità di diversi domini del ERα (A/B, C, E) di legare l'Ub derivante da lisati cellulari o ricombinante. Questi esperimenti hanno rivelato che il ERα possiede due domini che legano l'Ub (A/B ed E) contattandola direttamente. In seguito, è stata posta maggiore attenzione sul dominio E (del quale è nota la struttura) ed è stato osservato che la sua porzione *N*-terminale (aminoacidi 301-439) costituisce la minima superficie di legame all'Ub (UBS). All'interno dell'UBS sono stati poi identificati i residui critici per l'associazione non-covalente all'Ub (L429, A430).

L'introduzione della doppia mutazione L429,A430 (LAAG), nel dominio E *in vitro* e nell'intero (wt) ERα trasfettato nelle cellule, riduceva significativamente il legame all'Ub. Successivamente è stato valutato il ruolo della ERα-UBS negli effetti cellulari indotti dal E2. Per fare questo, sono state selezionate le cellule HEK293 che dopo la trasfezione esprimessero stabilmente il ERα wt e LAAG. In queste cellule è stata analizzata sia la proliferazione cellulare che l'approvigionamento di colesterolo indotte dal E2 ed è stato trovato che la mutazione dell'UBS sul ERα le previene. In seguito, poichè l'induzione della proliferazione da parte del E2 dipende fortemente dall'attivazione extra-

nucleare di chinasi di segnale, è stata valutata la fosforilazione attivatoria di alcune vie di segnale attivate del E2 in maniera conservata in diversi tipi cellulari. I risultati ottenuti indicano che la mutazione della UBS impedisce l'attivazione mediata dal E2 della via PI3K/AKT ma non della via ERK/MAPK. Questi dati indicano che la UBS sul dominio E del ERα gioca un ruolo critico per gli effetti mitogenici del E2. In particolare, l'incapacità del ERα LAAG di attivare la via PI3K/AKT sembra essere il principale responsabile di questo effetto. L'attivazione della via PI3K/AKT dipende dal fatto che il E2 induce una maggiore associazione del ERα con recettori per i fattori di crescita (ad es. IGF1-R) e richiama proteine di segnale alla membrana. Poichè i dati ottenuti mostrano anche una deregolata associazione del ERα LAAG con il IGF1-R è possibile che questo possa contribuire a prevenire la corretta attivazione della via di segnale a valle. Inoltre, la via PI3K/AKT controlla le attività del ERα anche fosforilando il recettore in risposta al E2. In particolare, la fosforilazione del recettore sul residuo di Ser118 dipende dalla via PI3K/AKT e gioca un ruolo critico per l'attività trascrizionale del E2:ERα. In accordo con i dati precedenti, il ERα LAAG non risultava fosforilato in Ser118 ne' era in grado di mediare l'incremento dell'mRNA dei livelli della Ciclina D1 dopo il trattamento con E2.

In seguito, poichè i dati ottenuti suggerivano una ridotta abilità del ERα LAAG nel mediare l'espressione genica dei geni bersaglio del E2, sono stati effettuati esperimenti di DNA Micro Arrays che lo hanno definitivamente dimostrato. Inoltre, l'Ingenuity Pathway Analysis applicata ai dati ottenuti ha permesso di identificare CREB1 come altro fattore di trascrizione attivato dal E2 attraverso il wt ma non LAAG ERα. CREB1 controlla direttamente la trascrizione di geni coinvolti nella proliferazione cellulare (ad es. Ciclina D1), sopravvivenza cellulare (ad es. Bcl-2) e metastasi (ad es. VEGF), la cui deregolazione favorisce la progressione tumorale. I dati ottenuti mostrano che il E2 non è in grado di indurre l'attivazione di CREB1 ne' la conseguente trascrizione di

geni target (ad es. Bcl-2, Cyclin D1) in cellule esprimenti ERα LAAG. Quindi l'UBS sul ERα è importante per l'attivazione trascrizionale di CREB1 indotta dal E2. Per comprendere se questo effetto fosse dipeso dall'incapacità del ERα LAAG di attivare la via PI3K/AKT è stata inibita questa via di segnale. I dati ottenuti indicano che l'attivazione della PI3K/AKT contribuiva alla fosforilazione di CREB1 indotta dal E2 in cellule HEK293 esprimenti il ERα wt e in cellule MCF-7. Infine, bloccando, attraverso un approccio farmacologico, la fosforilazione di CREB1 a monte o la sua attività trascrizionale a valle, è stato trovato che queste linee cellulari non sono in grado di proliferare in seguito alla stimolazione con il E2. Questi dati permettono di ipotizzare che un complesso di membrana dipendente dall'UBS sul ERα, e responsabile dell'attivazione della via di segnale PI3K/AKT indotta dal E2, controlli la proliferazione cellulare attraverso la modulazione dell'attivazione del ERα e di CREB1, richiesti per la trascrizione genica. Nel complesso, i dati riportati in questo progetto di dottorato indicano che il ERα possiede una UBS sul suo dominio E che gioca un ruolo chiave per garantire le attività nucleari ed extra-nucleari indotte dal E2 necessarie per la proliferazione cellulare.

Concludendo, le nostre scoperte indicano che il ruolo regolatorio del legame non-covalente all'Ub sul ERα deve essere aggiunto ai meccanismi molecolari noti ed attivati dal E2 per l'induzione della proliferazione cellulare. Poichè il ERα riveste un ruolo chiave nella progressione del cancro alla mammella, questo progetto mette in luce nuovi possibili bersagli farmacologici. A tal proposito, l'interferenza nel legame UBD:Ub tramite specifiche piccole molecole è stato già proposto come futuro bersaglio farmacologico contro alcuni tipi di cancro.

SUMMARY.

The E2:ERα signalling controls a plethora of physiological processes but plays also a critical role in breast cancer progression, thus the deep understanding of the mechanisms that control E2-induced cell proliferation would help to identify new putative druggable targets for the treatment of breast cancer. The ubiquitin (Ub)-system is gaining much attention for cancer therapies because it allows to build complex interactions network that is critical for signal transduction to many cellular processes. The Ub-based network depends on non-covalent binding between ubiquitinated proteins and proteins that possess an ubiquitin binding domain (UBD), called Ub-receptors. The deregulation of this intricate system has been associated with several pathological conditions including several types of cancers. Recent papers reported that the Ub-system deeply impacts the E2:ERα signalling; indeed, while ERα modification with polyUb chains controls the receptor turnover and transcriptional activity, ERα modification with single Ub molecule (*i.e.,* monoubiquitination) is required for the E2-dependent activation of rapid signalling to cell proliferation. Even if it has not been clarified how the Ub modification on ERα modulates the receptor activities, one possibility, which has never been considered is that ERα could recognize and transduce the Ub modification on itself or on interacting proteins through an UBD.

The main goal of the present PhD project was to understand the non-covalent Ub-binding abilities of ERα and their regulatory role in E2-dependent cellular processes.

To this purpose, initial experiments were performed *in vitro* by using purified ERα domains and recombinant or cell lysates-derived Ub molecules. We found that ERα has two different Ub-binding surfaces (UBSs) (in A/B and E domains) that have different binding abilities towards specific Ub-based chains. By focusing on the E domain Ub-binding ability we mapped an UBS on its *N*-terminal portion (*i.e.,* 301-439) and identified the structural determinants

required for ERα to non-covalently associate to Ub (*i.e.,* L429, A430). Remarkably, the introduction of L429,A430 double mutations (*i.e.,* LAAG) in the context of the full lenght ERα reduced Ub-binding also in cells. However, the ERα-UBS mutant basal intracellular localization as well as E2 binding affinity was not affected by the introduction of LAAG mutations.

Next, we found that the LAAG mutation blocks the E2-induced cell proliferation. Since proliferating cells have an increased cholesterol requirement, E2-dependent modulation of the cellular levels of the master regulators [*i.e.,* the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) and the low-density lipoprotein receptor (LDLr)] of cholesterol sourcing was next evaluated. We found that, differently from the wt, the LAAG ERα was not able to increase the cellular levels of the HMGR as well as LDLr after E2 treatment. Because the E2-dependent cell proliferation depends on the activation of extra-nuclear signalling kinases we evaluated the activating phosphorylation of some signalling pathways activated by E2 in several cell lines. Our results indicate that the ERα-UBS mutation impairs the E2-induced activation of the PI3K/AKT but not ERK/MAPK signalling. Moreover, the PI3K inhibitor blocked the E2-dependent increase of the HMGR and LDLr, as well as of cell proliferation also in breast cancer MCF-7 cells, endogenously expressing ERα. These data indicate that the UBS on ERα E domain plays a critical role for E2 mitogenic effects. In particular, the LAAG ERα inability to activate the PI3K/AKT pathway seems to be the principal responsible for this effect in MCF-7 cells as well as in HEK293 transfected cells because this mutant is unable to direct the physiological association of the ERα with the IGF1-R. The PI3K/AKT pathway also controls the ERα activities by phosphorylating the receptor in response to E2. In particular, the receptor phosphorylation on Ser118 residue depends on the PI3K/AKT pathway and plays a critical role in E2:ERα transcriptional activity. In accordance with previous data, we found no phosphorylation on this residue as well as no

induction of the Cyclin D1 mRNA levels after E2 treatment in LAAG ERα cells. These data suggest an impairment of the ERα-UBS mutant ability to mediate E2 target genes expression.

DNA Micro Arrays experiments definitively demonstrated that the LAAG mutant ERα was less transcriptionally active than the wt receptor. Interestingly, the Ingenuity Pathway Analysis on the obtained data helped us identifying CREB1 as another transcription factor activated by E2 through wt but not LAAG ERα. CREB1 directly controls the transcription of genes involved in cell proliferation (*i.e.,* Cyclin D1), cell survival (*i.e.,* Bcl-2) and metastasis (*i.e.,* VEGF), whose deregulation promotes the tumoral progression. Interestingly, we found that CREB1 activating phosphorylation on Ser133 residue as well as target genes expression (*e.g.,* Bcl-2, Cyclin D1) were not induced by E2 in LAAG ERα HEK293 cells. Thus, the ERα-UBS is important for the E2-triggered CREB1 transcriptional activation. To understand if the lack of CREB1 activation after E2 treatment could be ascribed to the LAAG ERα inability to mediate the activation of the PI3K/AKT, we inhibited this pathway. The obtained data indicate that the PI3K/AKT contributed to E2-induced CREB1 phosphorylation in wt ERα HEK293 as well as MCF-7 cells. Finally, by using specific inhibitors we blocked E2-activated CREB1 activities upstream (*i.e.,* block of the activating phosphorylation) or downstream (*i.e.,* block of the transcriptional activity) and found an inability of these cell lines to proliferate. It is tempting to speculate that an ERα-UBS-dependent membrane complex, responsible for the E2-triggered PI3K/AKT signalling activation, controls cell proliferation through the regulation of ERα and CREB1 activation required for gene transcription.

Overall, the data reported in this PhD project indicate that the ERα possesses an UBS on its E domain that plays a critical role for E2-induced nuclear and extra-nuclear signalling to cell proliferation.

In conclusion, our findings open new avenues in the field of E2-activated molecular mechanisms to physiological effects that now have to include also the non-covalent Ub-binding abilities of ERα. Given the key role played by ERα in breast cancer progression, the comprehension of the regulatory role of the ERα-UBS on E2 mitogenic effects reveals new putative druggable targets. In this respect, the interference of the UBD:Ub interaction by using specific small molecules has just been proposed as a future pharmacological target against cancer.

1. BACKGROUND.

1.1 Estrogens.

Estrogens are steroid hormones synthesized from cholesterol in a series of enzymatic reactions in which the last step is catalyzed by the aromatase enzyme. The three physiologically occurring estrogens are estrone (E1), 17β-estradiol (E2) and estriol (E3), that share a common four-ring structure (Fig. 1). E2 is the most active and the major estrogen in pre-menopausal women; its production is under the control of the hypothalamic-pituitary-ovarian (HPO) axis and predominantly depends on ovaries. Smaller amount of E2 is synthesized by peripheral tissues (*e.g.,* adipose tissue, adrenal gland). Estrogens are transported in the blood bound to the sex hormone binding globulin and diffuse in target tissues (*e.g.,* breast, endometrium, bone, brain, liver) where they control a plethora of physiological processes including reproduction, bone density, brain functions and cholesterol metabolism. Despite the physiological actions of endogenous E2, abnormally high levels of E2 or deregulated E2 signalling are associated with the increased incidence of certain types of cancer (*e.g.,* breast and endometrial) [1-3]. The biological actions of E2 are mediated by the estrogen receptors (ERs), the ERα and ERβ that exert opposite effects on cellular processes including proliferation and apoptosis. ERs possess a subtype-specific expression and differentially influence the development and progression of E2-dependent cancer. Indeed, the oncogenic effects of E2 are mainly dependent on the ERα-mediated activities, which promote an increase of the cell proliferation and a reduction of the apoptosis [4, 5]. Indeed, the therapeutic protocol for ERα-positive breast cancer relies, among others, on drugs that bind ERα and change the breast cancer cell intracellular content (*e.g.,* 4-OH-tamoxifen- TAM; ICI 182,780-Faslodex); however, these drugs display serious side effects (*e.g.,* endometrial cancer for TAM) and determine tumor resistance. Moreover, when a breast cancer sample needs to be classified to select a therapy, only the nuclear pool of

ERα is considered while it has been reported that the membrane pool of the receptor plays a critical role for E2-induced cell proliferation [4].

Thus, it is required to find alternative approaches to fight breast cancer; in this respect, a deep understanding of the mechanisms of ERα-mediated E2-induced cell proliferation would help to identify new putative pharmacological targets.

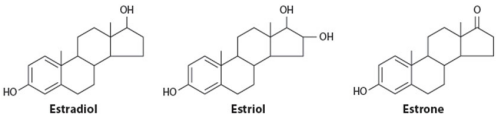

Figure 1. Chemical structures of estrogens. 17β-Estradiol, Estriol and Estrone share the typical steroid structure: three ciclohexane rings and one ciclopentane ring. [2]

1.2 Estrogen Receptor α (ERα).

E2 is a pleiotropic hormone that regulates human physiology far behind the control of reproductive tissues by binding to its cognate ERs. ERs are homologous members of the nuclear receptors super-family that act as a ligand-activated transcription factors; they are encoded by different genes, are expressed in many different tissues and regulate the expression of different target genes, thus transducing the E2 signalling in opposite ways (*i.e.,* mitogenic for ERα and pro-apoptotic for ERβ) [4].

The ERα signalling is strictly dependent on the receptor structural characteristics, which allow it to localize to the plasma membrane as well as in the nucleus. These biochemical features permit the receptor to activate both E2-induced rapid (*i.e.,* activation of signalling kinases) and delayed (*i.e.,* gene transcription) effects. The ERα-mediated E2-triggered signals starting from plasma membrane, cytosolic, and nuclear compartments integrate to control the E2-dependent physiological effects [6].

1.2.1 ERα structure.

The pleiotropic action of E2 depends also on the highly allosteric plasticity of the ERs, which is due to their six modular domains biochemical architecture (Fig. 2). The *N*-terminal A/B domains of ERα is flexible, un-structured and allows intra- and inter-molecular protein interactions necessary for gene transcription activation; within this protein portion, a ligand-independent transcriptional activation function (AF-1) is present and can be activated by growth factor-evoked signalling cascades [6]. The C and D domains allow ERα docking to DNA and/or trafficking. In particular, the C domain (*i.e.,* DNA binding domain- DBD) consists in the repetition of two zinc-finger motifs, which bind estrogen response elements (EREs) located in the DNA sequence of the target gene promoters [6]. The C domain also contributes to ERα dimerization. The flexible hinge, or D domain, contains the nuclear localization signals (NLS) required for the receptor trafficking to this compartment. Although the D domain structure-function relationship is not very well understood, the length of the D domain has been found to affect, together with the AF-1 and AF-2 regions, the E2-driven ERα transactivation [7]. Moreover, the D domain is a target of extensive post-translational modifications that affect the stability and/or activity of the receptor. The E domain contains the cavity where E2 binds (*i.e.,* ligand binding domain- LBD). This protein segment has a hydrophobic structure based on α-helices and is also able to lodge agonists and antagonists [6].

A ligand-dependent activation function (AF-2) is present also within the *C*-terminal part of this domain. The AF-2 region, together with the AF-1, guarantees receptors association with co-activators and co-repressors (*i.e.,* co-factors) that bridge the activated ERs with basal transcriptional apparatus. Remarkably, specific receptor:co-factors association selectively occur in different tissues thus contributing to the diversification of the E2-dependent effects. Finally the F domain is located at the very end of the *C*-terminus and its functions are at the present poorly

understood. Nonetheless, a role for this domain in the E2-induced ERα proteasomal degradation has been reported [8].

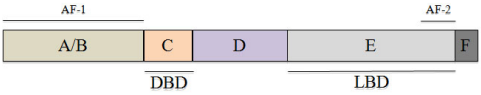

Figure 2. Estrogen Receptors (ERs) structure. The ERs possess six functional domains (A-F): the *N*-terminal A/B domain, the C domain DNA binding domain (DBD), the D domain hinge region, the E domain ligand binding domain (LBD) and the *C*-terminal F region. Regions with transcriptional activation functions, AF-1 and AF-2, are located on A/B and E domain.

1.2.2 ERα nuclear signalling.

The E2-dependent ERα activities are usually classified in nuclear, when the nuclear receptor pool is involved, or extra-nuclear, when the plasma membrane pool of ERα is involved. The ERα nuclear activities have been extensively studied and the nuclear one is the unique pool of ERα considered when a breast cancer sample needs to be categorized to select a pharmacological treatment [4]. Into the nucleus, the E2-activated dimeric ERα directly contacts ERE sequences in the promoter of related genes (*e.g.,* presenilin2- pS2/TIFF); remarkably, ERα can also indirectly regulate gene transcription (*e.g.,* Cyclin D1) through transcription factors such as the stimulating protein 1 (Sp-1), the activator protein 1 (AP-1) and the cAMP response element-binding protein (CREB) [6] (Fig. 3). Both in direct and in indirect transcriptional mechanism, ERα physically associates with co-regulators, like chromatin-remodeling complexes (*e.g.,* the SNF complex), histone acetyl transferases (HATs) [*e.g.,* CREB binding protein (CBP)/p300, pCAF, and steroid receptor co-activators (SRCs)], methyltransferases (*e.g.,* CARM1) and ubiquitin (Ub) ligases (*e.g.,* E6-AP and Rsp-5) [6]. These proteins allow the RNA polimerase II recruitment and facilitate ERα transcriptional activity. Many co-activators contain the ER-interaction domain consisting in the

conserved LXXLL (L= leucine, X= any amino acid) sequence, also called NR-box, which is primarily required for AF-2 association. Moreover, also AF-1 works as a docking site for co-activators (*e.g.,* SRC, CBP/p300).

ERα binding to estrogen responsive promoters occur cyclically both in the presence and in absence of E2. However, the E2-treatment increases the time of ERα:promoters association to guarantee the recruitment and the assembly of the transcription machinery. In each cycle, 26S proteasome-dependent degradation of ERα occurrs to allow some steps of the transcription process as well as to permit the association of newly synthesized receptors for further cycles. These events, in addition to allowing a rapid and limited response to E2, strongly link the ERα transcription and degradation mechanisms. Indeed, transcription inhibition blocks the receptor degradation and 26S proteasome inhibitors abolish ERα transcriptional activity [9, 10]. Remarkably, many signalling pathways control ERα-mediated gene expression by post-translationally modifying the receptor itself or its co-regulators, thus influencing the ERα folding or co-regulators recruitment.

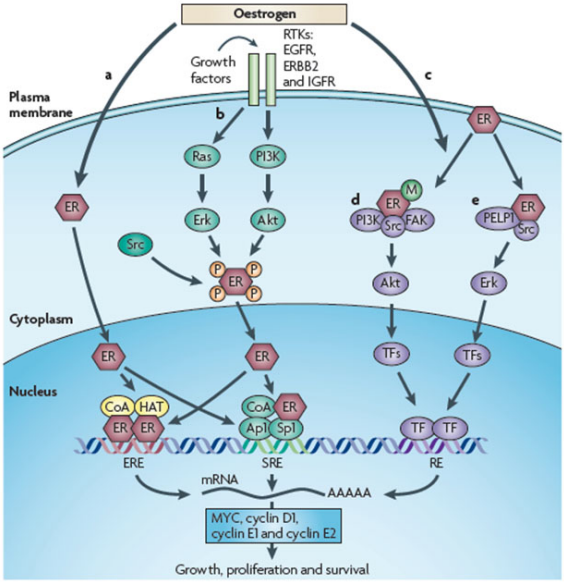

Figure 3. Nuclear and extra-nuclear signalling of the Estrogen receptor α (ERα). 17β-Estradiol (E2) binding to ERα activates different pathways that integrate to control several cellular processes (*e.g.,* cell proliferation an survival). **(a)** E2:ERα complex activate gene expression by directly binding the estrogen response elements (EREs) sequences on DNA or indirectly contacting other transcription factors [*e.g.,* the activator protein 1 (AP-1), the stimulating protein 1 (Sp-1)] that bind gene target promoters. Co-activators (CoA) and histone acetyl transferases (HATs) also associate to these protein complexes to activate gene transcription. **(b)** ERα can also be transcriptionally activated by growth factors that trigger the receptor phosphorylation. In addition, in the presence of E2, ERα increase its association with growth factors receptors (*e.g.,* IGF1-R) and activate rapid signalling pathways (*e.g.,* Shc/Src/Ras/ERK or PI3K/AKT) that converge to the nucleus to modulate gene expression. **(c)** E2 also triggers the ERα association with membrane signalling proteins that activate kinase cascades [*e.g.,* **(d)** methylated ERα (^{M}ERα)/PI3K/Src/focal adhesion kinase (FAK) for AKT activation; **(e)** ERα/PELP1/Src for ERK activation] resulting in the activation of transcription factors (TFs). [15]

1.2.3 ERα extra-nuclear signalling.

The E2:ERα nuclear effects occur at least 2 hours after hormone treatment [9, 10] and can explain only some E2 functions; indeed, rapid (*i.e.,* in seconds to minutes) E2-triggered effects have been reported that cannot be ascribed to the ERα nuclear activities [11]. These E2 rapid effects are independent on ERα transcriptional activity and are activated by membrane-impermeable E2-conjugates (*e.g.,* E2:BSA) [6]. The E2-triggered extra-nuclear signalling involve the activation of several signalling kinase cascades [*e.g.,* phospholipase C (PLC)/protein kinase C (PKC), avian Sarcoma virus (Src)/extracellular activated kinase, phosphatidyl-inositol 3 kinase (PI3K)/protein kinase B (PKB or AKT), p38/mitogen-activated protein kinase (MAPK)] that are selective for cell type as well as for ER sub-type. Remarkably, the PI3K/AKT and the ERK/MAPK pathways were found to be conserved in E2:ERα rapid signalling both in normal and transformed cell lines (*e.g.,* epithelial cells, breast cancer cells) [4].

These signalling pathways are activated by E2 after binding with a small portion of the total ERα, originating from the same gene as nuclear ERα, that localizes to the plasma membrane caveolae and lipid rafts. Since ERα is not a trans-membrane receptor, its ability to localize to the plasma membrane must be ascribed to membrane proteins association and/or lipidation. In fact, our group demonstrated that ERα undergoes to palmitoylation on the E domain. This post-translational modification is required for receptor plasma membrane association. Indeed, ERα palmitoylation site cysteine (Cys- C) 447 mutation [*i.e.,* C447 to alanine (A)] abrogates the receptor plasma membrane localization and the E2 ability to activate signal transduction pathways [12]. Moreover, S522 of ERα is necessary for receptor association with the caveolar protein caveolin-1, which facilitates ERα transport to the plasma membrane [13]. However, an unpalmitoylable ERα mutant is not able to interact with caveolin-1 [12].

Interestingly, in breast cancer cells E2 treatment induces a reduction of palmitate incorporation for ERα and

a parallel increase of its interaction with transmembrane growth factor receptors (*e.g.,* IGF1-R) as well as signalling proteins. In particular, the PI3K/AKT and the ERK/MAPK pathways require different membrane complexes for their activation. Indeed, ERK/MAPK pathway has been shown to be activated after ERα/PELP1/Src as well as ERα/Shc/Src/Ras complexes formation while the PI3K/AKT pathway requires for example methylated ERα/p85 subunit of PI3K p85/Src/focal adhesion kinase (FAK) or ERα/PELP1/Src/p85 complexes [4, 14, 15, 16] (Fig. 3).

As anticipated, the ERα association with IGF1-R plays a critical role for the assembly of some of these complexes and then for signalling activation. For example, the adaptor protein Shc has been reported to translocate ERα to Shc-binding sites of IGF1-R on plasma membrane where the ERα/Shc/Src/Ras complex is assembled and rapid signalling is activated [17]. Moreover, both ERα and IGF1-R recruit the p85 subunit and also the PI3K pathway can be activated after ERα:IGF1-R interaction without Shc requirement [17].

Although the occurrence of this membrane ERα dependent extra-nuclear signalling *in vivo* has long been questioned, the recent characterization of many phenotypes of knock-in mouse models, in which the ability of the ERα to localize to the plasma membrane was impaired by mutation of the palmitoylation site, strongly demonstrates the physiological relevance of such E2-triggered effects *in vivo* (*e.g.,* female and male fertility) [18, 19].

1.2.4 Integration of E2:ERα signalling to physiological processes.

In cells, the E2:ERα complex-dependent rapid and delayed signals integrate to control several physiological processes. For example, the activation of Src and PI3K leads to the Cyclin D1 expression, that promotes the G1-S phase transition [20] and to an increase of the pro-survival factor B-cell leukemia-2 (Bcl-2) [21]. In parallel, the E2-activated PI3K/AKT pathway induces the inhibitory

phosphorylation of the pro-apoptotic protein BAD, thus leading to an abrogation of the apoptotic processes [22]. The coordination of these events, induced by E2 and dependent on nuclear as well as extra-nuclear signalling activation results in cell survival and proliferation. The proliferative response of E2 target cells also depend on an adequate cholesterol sourcing (*i.e.,* synthesis and uptake of cholesterol), which represents an absolute requirement for the cell membrane fluidity and stability of the newly divided cells [23, 24, 25]. In particular, cholesterol homeostasis maintenance depends on the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), which is a key regulator for cholesterol biosynthesis, and the low-density lipoprotein receptor (LDLr), which controls cholesterol uptake [26]. E2 stimulation induces the direct transcription of the HMGR, which is an ERE-like containing gene [23] and the indirect, Sp1-mediated, transcription of the LDLr, which is a non-ERE containing gene [24]. Thus, the E2 cellular effects that lead cells to proliferate depend on the activation of several different intracellular pathways that require both nuclear and extra-nuclear ERα activities.

Recently, the generation of mutant mouse lacking the ERα membrane localization (*i.e.,* ERα-C451A mouse) or the activation function AF-2 (*i.e.,* ERα-AF2°) provided selective loss-of-function of the ERα extra-nuclear and nuclear activities, respectively [18]. The obtained data showed that the ERα membrane localization is critical for ovarian functions and thereby for fertility. On the contrary, while in ERα-C451A mouse E2-dependent uterine gene expression was preserved, it was abrogated in ERα-AF2° mouse, indicating that the nuclear and extra-nuclear cross-talk *in vivo* is modest in the uterus [18]. On the contrary, another group reported that nuclear and extra-nuclear signalling cross-talks *in vivo* in all the tested organs, including the uterus [19]. Thus, notwithstanding the discrepancies in the mice models, it is now very much accepted that nuclear and extra-nuclear activities of ERα play critical tissue-specific roles *in vivo* and are required

for organ development and function [19].

1.2.5 Modulation of ERα activities by post-translational modifications.

Membrane-originated E2 signals cross-talk to the nucleus by controlling ERα post-translational modifications that affect the receptor activities at different levels (*e.g.,* signalling cascades activation, subcellular localization, transcriptional activity, turnover). The best documented ERα post-translational modification is phosphorylation, which occurs in response to E2 predominantly at serine (Ser- S) 118 and to a lesser extent on Ser104 and Ser106, all located in the ERα A/B domain, by different pathway [6]. These modifications principally affect the nuclear signalling of ERα. For example, Ser118 phosphorylation causes ERα dissociation from co-repressors and allows ERα interaction with specific co-activators (*e.g.,* CBP/p300 and SRC-1); then, ligand-dependent as well as -independent activation of the receptor occurs [6]. Ser118 phosphorylation has been reported to be mediated by E2-induced PI3K/AKT signalling in MCF-7 cells [27]. Other phosphorylation residues include Ser167, regulating ERα transcriptional activity, Ser236, required for ERα dimerization, and Ser305, important for gene transcription (*e.g.,* Cyclin D1) [16]. Acetylation of ERα also occur at different lysine (Lys- K) residues by the ERα HAT co-activator p300 but while in some residues (*e.g.,* K299, K302, K303) it has an inhibitory effect on the receptor transcriptional activity in other (*e.g.,* K266, K268) is stimulatory [16].

In addition, ERα undergoes to other post-translational modifications that affect extra-nuclear signalling; among these, the previously described palmitoylation, which is required for ERα membrane localization and rapid signalling activation [12]. In addition, ERα arginine (Arg- R) 260 methylation by the protein arginine methyltransferase 1 (PRMT1) is necessary for the formation of the ERα complex with p85/Src/FAK required for the PI3K/AKT pathway activation [16, 28].

Finally, ERα is also ubiquitinated both for proteolytic (*i.e.,* polyubiquitination) and non-proteolytic (*i.e.,* monoubiquitination) functions. Of note, ERα modification with Ub affects both nuclear and extra-nuclear signalling. In particular, even if the exact site of ERα polyubiquitination is uncertain, it is well accepted that this modification regulates the receptor turnover and transcriptional activity [9, 10, 29]. On the contrary, ERα monoubiquitination on K302, K303 residues is mediated by the Ub ligase BRCA1/BARD1 [30] that could also be responsible for an impairment of p300-mediated ERα acetylation on the same residues [16, 31]. Our group found that ERα monoubiquitination is negatively modulated by E2 and control the E2-triggered PI3K/AKT pathway activation, transcriptional activity and cell proliferation [32-34].

Since this modification is the object of the present PhD project, it will be extensively described in the following paragraphs. Thus, in the absence and in the presence of E2, several post-translational modifications of ERα occur and control the receptor activities through the coordination of nuclear and extra-nuclear mechanisms [16, 27].

1.3 Ubiquitin (Ub)-based signalling network.

Ubiquitination is a highly controlled process that covalently labels proteins with the attachment of Ub to the target lysine (Lys- K) residues. Conjugation of Ub occurs through a cascade of enzymatic reactions mediated by the Ub-activating (E_1), the Ub-conjugating (E_2), and the Ub-ligases (E_3) enzymes. These reactions create an isopeptide bond between the Ub *C*-terminal glycine (G) residue and the K residue on the target protein [35] (Fig. 4). Three different kind of E_3 ligase exist: namely HECT (homologous with E6-associated protein *C*-terminus), which works as a single enzyme, U-box, and RING (really interesting new gene) ligases, which bind both the substrate and the Ub-loaded E_2 thus facilitating the formation of the

covalent binding between Ub and substrate. Accordingly, the action of the E_3 alone or in association with its E_2 guarantees the specificity of the ubiquitination cascade. Because Ub itself contains seven K residues (K6, K11, K27, K29, K33, K48 and K63) that can serve as acceptor sites for chain elongation (Fig. 4), chains that contain multiple Ub moieties can be formed by either homotypic or heterotypic linkages. In addition, Ub chains assembled head-to-tail exist. Linear polyUb chains have been found *in vivo* as the resulting action on monoUb of the E_3 ligase called LUBAC (linear Ub chain assembly complex). The difference between linear and K-linked chains has the physiological consequence to address selective recognition for specific interacting partners [36].

The ubiquitination pathway is further complicated by the possibility of the substrate to be differentially modified with Ub: 1) monoubiquitination, the attachment of a single Ub moiety, 2) multimonoubiquitination, the attachment of multiple Ub moiety to several K residues within the target protein and 3) polyubiquitination, a modification with a Ub chain [37] (Fig. 4). *In vivo*, all K residues are used for chain formation, but K48- and K63-based polyUb chains appear to be the most represented ones. Functionally, the modification of the target protein with a specific K-based modification has a specific signalling meaning and thus results in a particular Ub-dependent modulation of the physiological process, which is regulated by the ubiquitinated protein. Remarkably, all the Ub modifications are reversible and de-ubiquitination is achieved through the activity of specific proteases known as de-ubiquitinating enzymes (DUBs) [32]. This process reversibility renders ubiquitination a versatile post-translational modification.

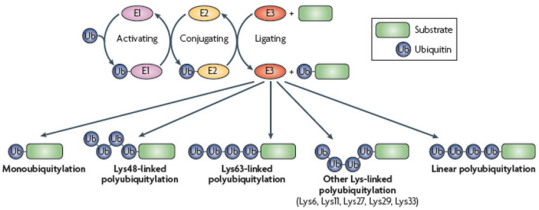

Figure 4. The ubiquitin (Ub)-system complexity. Protein modification with Ub occurs through a cascade of enzymatic reactions mediated by Ub-activating (E_1), Ub-conjugating (E_2) and Ub-ligating (E_3) enzymes. These reactions covalently attach Ub to target lysine (Lys) residues of the substrate. Diverse modifications with Ub can occur: monoubiquitination is the attachment of a single Ub to a Lys of the target protein while polyubiquitination is the modification of Lys residues on target proteins with a chain of Ub. Since Ub possesses seven Lys that can be target of Ub modification for chain formation, several chains with different functions can be built on proteins (Lys6-, Lys11-, Lys27-, Lys29-, Lys33-, Lys48-, Lys63-linked). Alternatively, ubiquitin molecules can be linked head to tail to form linear chains. [37]

1.3.1 Proteolytic and non-proteolytic ubiquitination.

It is known that each K-based polyUb chain assumes a particular three-dimensional topology, which in turn serves physiological processes. However, atypical Ub chains (*i.e.,* based on linkages other than K48 or K63) exist but possess roles which are still elusive and difficult to study [36].

It is well known that the attachment of a K48-based polyUb chain labels proteins for 26S proteasome-mediated degradation. In addition, it has recently been reported that also K11-based polyUb chains serve as proteasomal degradation signal in yeast [36]. Thus, ubiquitination regulates protein half-life and turnover. In recent years it has become increasingly evident that the modification of proteins with a Ub chain based on K63 linkage or with the addition of a single Ub moiety (*i.e.,* monoubiquitination) fulfills non-degradative functions (*e.g.,* endocytosis, intracellular trafficking, DNA-damage response). For example, polyUb chains based on K6 linkages can also play a role in DNA repair. Indeed, the E_3 ligase breast

cancer-susceptibility protein (BRCA1) and BRCA1-associated ring domain 1 (BARD1) localizes at DNA lesions with a mechanism involving the binding to K6- (and K63-) linked Ub chains. At the present K27-linked Ub polymers remains without a defined function while linear Ub chains, which represent the source of cellular monoUb, play a physiological role in the nuclear factor-κB (NF-κB) pathway. Finally, some protein kinase [*e.g.,* members of the AMP-activated protein kinase-related family (AMPK)] activity has been found to be blocked after modification with K29- and K33-linkages [36].

1.3.2 The Ub-Binding Domains.

The recognition of the ubiquitination diversity depends on an array of Ub-binding domains (UBDs) that bind non-covalently to Ub and which are found in a plethora of effector proteins, called Ub-receptors, with different functions (*e.g.,* endocytosis, DNA repair). Ub-receptors generally have small (20-150 amino acids) UBDs, which do not possess any specific sequence conservation. However, the most common structural motifs in which an UBD folds are based on single or multiple α-helices [*e.g.,* UBA (Ub-associated), UIM (Ub-interacting motif), MIU (motif interacting with Ub), UMI (UIM- and MIU-related UBD) domains], zinc-fingers [*e.g.,* NZF (nuclear protein localization 4 zinc finger) domain], pleckstrin-homology (PH) fold [*e.g.,* pleckstrin-like receptor for Ub (PRU)] and Ub-conjugating-like (*e.g.,* UBC) structures [45]. The majority of the identified UBDs contact Ub on an hydrophobic patch around the isoleucine (I) 44 residue but also other Ub-surfaces contribute to binding specificity [38].

Interestingly, UBDs weakly bind to Ub (K_d=10-500 μM) thus avidity-based associations to ubiquitinated substrates are the mechanism by which high affinity interactions are obtained for efficient regulation of physiological processes. Indeed, protein oligomerization together with multiple UBDs on a single protein are often found in transient Ub-binding complexes [38] (Fig. 5). It has been reported that

many Ub-receptors possess UBDs with high binding selectivity toward polyUb chains rather than to monoUb *in vitro*. However, Ub-chains preference of an isolated UBD could differ from that of the full length Ub-receptor due to its subcellular location [38]. Moreover, selective binding of UBDs to specifically linked polyUb-chains has been reported and can depend on the topology of different polyUb. For example, K48-linked chains alternate in solution between a closed and an opened conformation, while K63 ones adopt an extended conformation [37]. Moreover, also UBDs organization can define Ub-binding specificity because linker regions of in tandem UBD repetitions arrange them in a way in which interactions are favored by one Ub chain type but not by others [37].

Interestingly, many Ub-receptors are ubiquitinated and this modification requires their UBDs. In this process, called coupled monoubiquitination, UBDs function as signals for ubiquitination because they recruit the ubiquitination machinery (*i.e.,* E_2-E_3 complex) [38, 40]. Most of the Ub-receptors are monoubiquitinated rather than polyubiquitinated maybe because the UBD:Ub-binding mask the I44 residue required for Ub chain formation or because of steric hindrance of the E_2-E_3 complex with polyUb chains. The ubiquitination of Ub-receptors might have a regulatory function: for example, to mantain the Ub-receptor in an autoinhibitory state by inducing intra-molecular interaction among the monoUb and UBDs (Fig. 6). In this respect, it has been reported that the monoubiquitination of the UIM-containing protein EPSIN, important for the regulation of endocytosis, negatively influences the *in vitro* binding to some binding partners, but not others. Thus, the regulation of Ub-receptor monoubiquitination deeply impacts on signal transduction. Moreover, coupled ubiquitination plays a crucial role also for the formation of a signal relay network in which the monoUb leads to interactions with other downstream Ub-receptors, thus amplifying the Ub-based signalling [38] (Fig. 6). Additional complexity is given by the fact that several post-translational modifications (*e.g.,*

phosphorylation) regulate the Ub:UBDs interactions thus controlling the Ub-receptor functions [38].

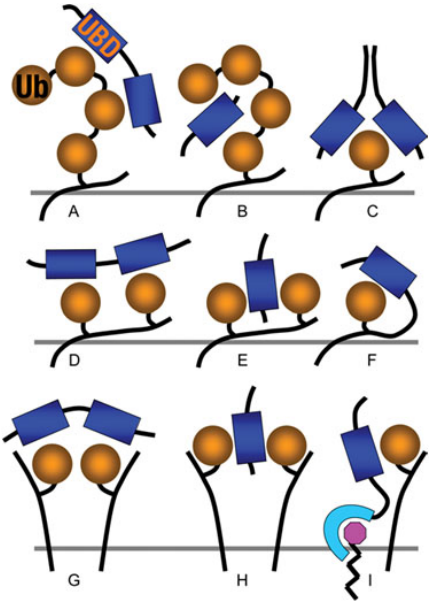

Figure 5. Mechanisms for ubiquitin (Ub):Ub-binding domains (UBDs) interaction. (A) A polyUb chain can be bound by tandem UBDs each contacting monoUb; alternatively, **(B)** a poly Ub chain can bind a single UBD by contacting it on two different surfaces. **(C)** MonoUb can be bound with high affinity from dimeric proteins that contact Ub on different surfaces through different faces of the UBDs. Protein modified with Ub in different Lys residues (*i.e.,* multi-monoubiquitination) can contact **(E)** one or **(D)** more UBDs. **(F)** Monoubiquitination can direct intra-molecular monoUb:UBD interactions that mantain the protein in an auto-inhibitory status. **(G)** MonoUb on clustered membrane proteins can be bound by tandem UBDs or **(H)** can contact a single UBD on different surfaces. **(I)** MonoUb on membrane protein can be bound by a lipid-modified membrane protein containing an UBD. [39]

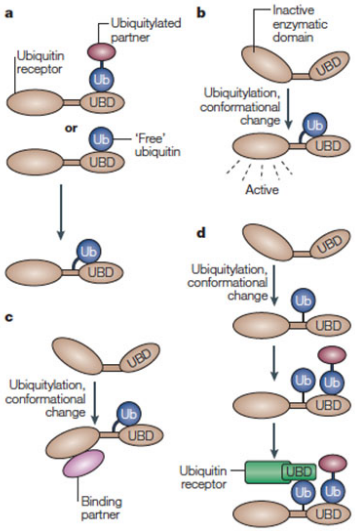

Figure 6. Models for coupled-monoubiquitination functions. (a) Ubiquitin (Ub)-receptors monoubiquitination can mantain the receptor in an auto-inhibitory state that prevent its association to free Ub or ubiquitinated partners. Monoubiquitination can also induce a conformational change on the Ub-receptor that **(b)** activate adjacent enzymatic reactions or that **(c)** expose previously masked binding sites. **(d)** Monoubiquitination of Ub-receptors can initiate a signal cascade in which the modification with monoUb could induce a conformational change that expose the UBD to the interaction with ubiquitinated partners or with other Ub-receptors. [38]

1.4 Interactions between the ERα and Ub signalling.

In recent years, several papers reported a strict correlation between the ERα and the proteolytic- as well as non-proteolytic Ub-based pathways. Moreover, mounting evidence indicates how targeting the Ub-system could be a pharmacological option for cancer therapy [41]. Several point of intervention can be considered; for example, at the levels of the enzymes of the ubiquitination cascade. In this respect, our group found a strong effect of 4[4-(5-nitro-furan-2-ylmethylene)-3,5-dioxo-pyrazolidin-1-yl]-benzoic

acid ethyl ester (Pyr-41), the first cell permeable inhibitor of the key initiating enzyme E_1 [42] on E2:ERα signalling and cellular processes (*i.e.*, cell proliferation, cell migration) in MCF-7 cells [43], thus supporting the notion of the intertwined nature between the Ub-based system and ERα signalling. Other known drugs target the E_3 ligase activity (*i.e.,* HLI98 to inhibit Hdm2), substrate recognition (*i.e.,* small compounds named RITA to block p53) or proteasomal activity (*i.e.,* bortezomib), while new putative drugs might be designed to specifically occupy the UBDs of specific effector proteins [37, 41].

1.4.1 Ub-dependent proteolytic control of ERα expression.

Ligand-induced ubiquitination and subsequent degradation appears to be a conserved pattern for limiting the cellular response to a given hormone. Indeed also in the case of E2, all the hormone effects occur in parallel while the ERα intracellular levels are regulated through a dynamic balance between ERα synthesis and ERα degradation. Proteolysis of ERα is principally under the control of the 26S proteasome since E2 binding induces the ERα polyubiquitination and subsequent degradation [29]. Furthermore, the un-liganded receptor is polyubiquitinated by the E_3 Ub ligase MDM2 (mouse double minute-2) and degraded by the 26S proteasome. Interestingly, while polyubiquitination of the apo-ERα has the function to address any mis-folded ERα molecule for 26S proteasome-based removal, the E2-induced ERα polyubiquitination and degradation are necessary for receptor transcriptional functions [9, 10]. In particular, in the nucleus, both in the absence and in the presence of E2, ERα undergoes to a cyclic recruitment to the ERE containing promoters where, through sequential and ordered 26S proteasome-dependent events, it produces the transcriptional response to E2. In this way, the receptor proteolytic breakdown guarantees the time- and space-dependent synchronization of the cellular responses with the variable E2 extra-cellular concentrations [9, 10]. Accordingly, as transcription progresses ERα is degraded as consequence of the recruitment of Ub ligases

(*e.g.,* E6-AP, MDM2, EFP) as well as coactivators (*e.g.,* SRC-1 and SRC-3) [9, 10, 16]. Thus, the 26S proteasome plays a pivotal function of in the molecular steps leading to the expression of estrogen-responsive genes [44].

Our group also reported a critical role for ERα plasma membrane localization and signalling in the regulation of ERα degradation in breast cancer cells [27]. Indeed, mutation of ERα in the palmitoylation site accelerates ERα degradation in response to E2 because the PI3K pathway is not activated. In parallel, irrespective of E2 treatment, inhibition of ERα palmitoylation constitutively addresses ERα to the nuclear matrix and induces the basal degradation of the neo-synthesized ERα, suggesting that the native ERα pool requires palmitoylation for stabilization [27].

Signalling modulation of the ERα proteasome-dependent pathway has been reported to occur possibly through ERα phosphorylation in the Ser118 residue [29]. Indeed, our group found that the lack of palmitoylation as well as the inhibition of the PI3K/AKT pathway prevents the E2-dependent ERα Ser118 phosphorylation, ERα association with ERE-containing promoter and ERα transcriptional activity [27]. Moreover, our group found that the receptor pool that is addressed to the nuclear matrix for degradation is not phosphorylated on Ser118 [27]. However, if Ser118 phosphorylation is an important regulator of E2-induced proteolytic ERα ubiquitination remains to be established.

Thus, the regulation of ERα intracellular levels depend on the plasma membrane-starting signals as well as on transcriptional activity.

1.4.2 Ub-dependent non-proteolytic control of ERα signalling.

Ub proteolytic functions in the E2-induced 26S proteasome-dependent control of ERα cellular levels exist but at the present the identity of the E_3 Ub ligase that directly polyubiquitinates ERα is unknown. Recent data have however defined that ERα is an actual substrate for the

E_3 Ub ligase BRCA1 [30]. Interestingly, BRCA1, which is often mutated and amplified in several kinds of breast cancers, catalyzes the monoubiquitination of the ERα [30]. In particular, BRCA1 monoubiquitination has been shown to occur in *in vitro* assays on the K302 and K303 of the ERα E domain. Moreover, BRCA1 mutations frequently associated with cancer development prevent ERα monoubiquitination [30]. According to this initial evidence, our group found that ERα monoubiquitination also occurs in cell lines [32-34] and found that it is required for the activation of the E2-induced ERα rapid signalling (*i.e.,* AKT activation) that controls the key steps for E2-induced cell proliferation (*i.e.,* Cyclin D1 transcription, G1-to-S phase transition, cell cycle progression). Furthermore, we observed that the lack of ERα monoubiquitination impairs the E2-dependent increase of ERα:IGF1-R association, blocks ERα Ser118 phosphorylation and the ERα ability to regulate gene expression. Thus, endogenous ERα monoubiquitination regulates E2-dependent nuclear and extra-nuclear ERα activities and monoubiquitination seems to work as a negative feedback signal for the ERα-mediated E2 effects (*e.g.,* cell proliferation) activation [32-34]. This evidence strongly suggests non-proteolytic functions (*i.e.,* monoubiquitination-dependent) of the Ub-system in the regulation of E2:ERα signalling. However, the exact mechanism by which monoUb modulates ERα has not been defined.

For example, it cannot be excluded that the ERα monoubiquitination could depend on an UBD on the receptor itself. In that case the UBD:monoUb interaction could maintain ERα in an autoinhibitory state until the E2 binding allows deubiquitination and activation of several pathways. However, no information are available on the presence of UBDs on ERα. Another possibility is that monoUb creates new binding surfaces on ERα that guarantee the receptor association with specific interactors. The Ub-mediated interactions should then occur between ERα and the UBD of an Ub-receptor; however, the putative ERα Ub-receptor partner(s) has not been identified yet.

2. AIM.

Non-covalent binding between ubiquitinated proteins and Ub-receptors generates a complex network of interactions that is critical for signal transduction and controls many cellular processes. Indeed, the deregulation of this intricate system has been associated with several pathological conditions including several types of cancer [41].

Recent papers reported that the Ub system deeply impacts the E2:ERα signalling; indeed, while ERα polyubiquitination controls the receptor turnover and transcriptional activity [9, 10, 29], ERα monoubiquitination is required for the E2-dependent activation of rapid signalling to cell proliferation [34]. Even if it has not been clarified how the Ub modification on ERα modulates the receptor activities, it is possible that it creates new surfaces for the interaction with other molecules that possess an UBD and transfer the signal to the final outcome (*e.g.,* gene expression, cell proliferation). On the other hand, it is also possible that ERα could recognize and transduce the Ub modification on itself or on interacting proteins through an UBD; however, no information are available on this issue.

Interestingly, monoubiquitinated proteins often possess and require an UBD to correctly function (*e.g.,* to be monoubiquitinated, to interact with other proteins) [32]; thus, ERα monoubiquitination could depend on the presence of a UBD within the receptor structure. Moreover, among the six domains of ERα structure only the C and the E ones display a folded structure: two zinc-finger motifs for C domain and 12 α-helices for E domain [6]. Remarkably, the structure of most of the UBDs identified so far is based either on zinc-finger or α-helices [38]. These evidence suggests that ERα could behave as an Ub-receptor that decodes the Ub signal on other proteins and is itself modulated by it.

Thus, the main goal of the present PhD project was to understand the non-covalent Ub-binding abilities of ERα

and their regulatory role in E2-dependent cellular processes.

To this purpose, *in vitro* experiments were performed by using purified ERα domains and recombinant or cell lysates-derived ubiquitinated molecules. Next, the Human Embrionic Kidney (HEK293) and the cervix adenocarcinoma HeLa cell lines, which we endowed with the wt or mutant ERα, were used to analyze the impact of the ERα Ub-binding on E2:ERα signalling. We also used human breast cancer MCF-7 cells and treated them with specific inhibitors to block pathways that we found to be required for the ERα Ub-binding functions and to verify that they were conserved also in breast cancer cells.

3. IDENTIFICATION OF NON-COVALENT Ub-BINDING SURFACE (UBS) ON ERα.

3.1 Introduction.

Monoubiquitinated proteins are often Ub-receptors that possess at least one UBD [40]. Although many different UBDs exist, no specific conservation in terms of UBDs 3D-structure has been recognized. In fact, structural folds that have a regular secondary structure (*e.g.,* α-helix and/or zinc-finger) are thought to be the only UBDs common features [37, 40, 45]. Interestingly, ERα biochemical anatomy consists in six modular domains (Fig. 2): while the A/B, D and F domains do not display a folded structure, the C domain consists in two zinc-finger motifs and the E domain is composed of 12 α-helices [6].

On this basis, we speculated that an UBD could be present in ERα. To this purpose, pull-down assays have been performed by using all ERα domains against recombinant or cell lysate-derived ubiquitinated species.

3.2 Results.

3.2.1 Indentification of two UBSs on ERα.

Initial *in vitro* experiments were performed to understand if ERα could possess an UBD. The ERα A/BC, C and E domains were cloned, expressed and purified as GST-fusion proteins as described in [46] except that all ERα-E domain encoding constructs were prepared in the presence of 20 μM E2. Next, GST-fusion proteins were used in pull-down assays by incubating them with HeLa cell lysates as source of ubiquitinated species. Subsequent anti-Ub immunoblot revealed that the A/B and the E domains but not the C domain were able to pull-down ubiquitinated species from total cellular lysates (Fig. 7A). Interestingly, the A/B and E domains showed different abilities to pull-down ubiquitinated species. The comassie (Com.) normalization, corresponding to 1/10 of the pull-down, indicate no differences between A/B and E protein

levels used for the experiment; thus, the observed variation in Ub-binding was not an artifact. Next, to exclude the possibility that the ubiquitinated species were pulled down through surface different than Ub, we performed pull-down experiments by using only the A/B or E domains against recombinant polyUb chains linked through K63. We found that either these domains were able to pull-down recombinant K63-linked polyUb chains (Supplemental Fig. 1 and Fig. 7B), thus demonstrating that the ERα A/B and E domains non-covalently contact Ub on the Ub-modified proteins. Next, to understand the reason for the observed differences between the A/B and E domain Ub-binding, we analyzed their ability to pull-down all the other known recombinant Ub chains (*i.e.,* K6-, K11-, K27-, K29-, K33-, K48-linked). Interestingly, we found that while the E domain bound all (Fig. 2) but recombinant K33-linked Ub chains (data not shown), the A/B domain was able to pull-down only K6-linked Ub chains (Supplemental Fig. 1). Thus, the ERα Ub-binding A/B and E domains display preferential Ub-binding ability.

Because the A/B domain is non-structured and displays weak association to ubiquitinated species when compared to the 12 α helices-containing E domain, we focused on the E domain Ub-binding ability. Thus, we investigated which portion(s) of the ERα E domain is necessary for Ub-binding. We found that the *N*-terminal part of the E domain (*i.e.,* amino acids 301-439) but not the E domain *C*-terminus (*i.e.,* amino acids 439-547) was able to pull-down ubiquitinated species from total cellular lysates (Fig. 7C). This evidence indicates that the region of the ERα E domain that non-covalently associates *in vitro* with Ub is located within the protein region encompassing the amino acids 301-439. Unfortunately, we could not narrow down a smaller section of the E domain that associated to ubiquitinated species by using rational deletions of the *N*-terminal protein portion (*i.e.,* 301-439) (data not shown). It is most likely that the ERα E domain Ub-binding portion requires an intact E domain 3D-folding. Thus, we considered the 301-439 region of the E domain as

the minimal binding region to Ub and called it ERα-Ub-binding surface (ERα-UBS).

In turn, *in silico* molecular modelling experiments would help rationalize the biochemical structural requirements for ERα E domain:Ub interaction. For these reasons, we decided to introduce specific point mutations within the minimal Ub-binding region (*i.e.,* 301-439) in the context of the full length E domain in order to find the critical residues required for Ub-binding.

Interestingly, bioinformatic analysis identified L428, L429 and A430 in the E domain to have a spatial distribution reminiscent of the UBD called UMI [47], with L428 buried in the E domain core protein matrix and L429 and A430 at least partially exposed to solvent (Fig. 7D). Consistently, while single substitution of either L429 or A430 barely affected the Ub-binding abilities of the E domain in pull-down assays, double mutation of L429 and A430 (L429A, A430 to glycine (G)- LAAG) strongly prevented the E domain Ub-binding (Fig. 7E). To understand if the observed reduction in LAAG E domain Ub-binding was due to an impaired association to specific Ub chains, we performed pull-down experiments by using the wt and mutated E domain against all but K33-linked recombinant Ub chains. Remarkably, we found that the introduction of the LAAG mutations reduced the E domain ability to bind all but K48-linked Ub chains (Fig. 8). Thus, the observed reduction in Ub-binding ability of the mutant UBS depends on an overall lower ability to bind Ub chains. Next, we wondered if the reduced Ub-binding of the LAAG E domain could be due to an impairment of the E domain structure caused by the introduction of the mutations. To solve this problem, we performed circular dichroism (CD) experiments on the E-domain GST-fusion proteins, which showed that the introduction of the LAAG mutations in ERα did not significantly affect proper receptor folding (Supplemental figure 2).

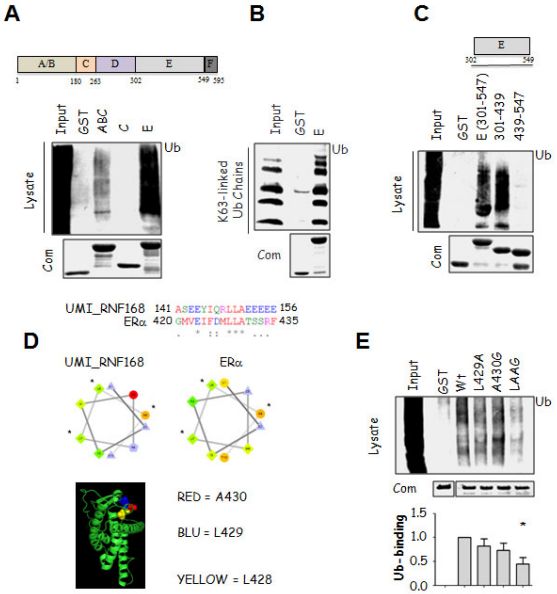

Figure 7. Ub-binding ability of ERα. (A, B, C, E) Schematic of ERα and E domain structure, in which the aminoacid positions are depicted. *In vitro* pull-down assays were performed using the indicated GST-tagged ERα constructs. GST-fusion proteins were incubated with **(A, C, E)** total cellular lysates extracted by growing HeLa cells or **(B)** with synthetic polyUb2-7 linked by Lys (K)63 and analyzed by immunoblot as indicated. *indicates significant differences with respect to the relative wild-type sample. **(D)** Top: ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) alignment of ERα E domain with RNF168 UMI domain [47]. Middle: Projected helices using the Helical Wheel Projections software tool (http://rzlab.ucr.edu/scripts/wheel/wheel.cgi). Bottom: Three-dimensional structure of the human ERα LBD-E2 complex (1ERE) [48]; E2 has been removed. The structure is drawn in green; conserved amino acids between the ERα E domain and RNF168 UMI domain are space-filled and stained as indicated.

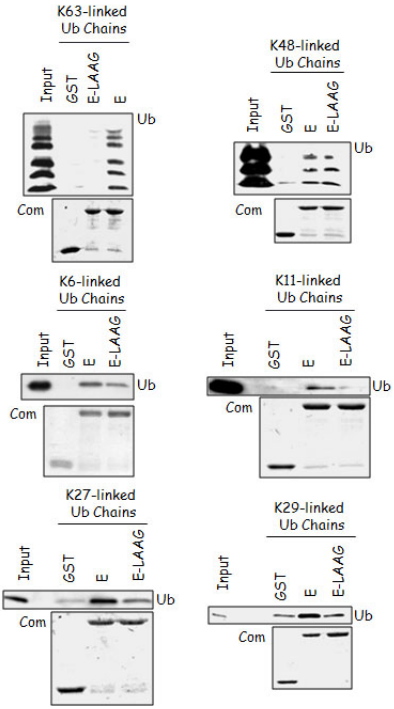

Figure 8. Ub-binding ability of the wt and L429A, A430G (LAAG) ERα E domain. *In vitro* pull-down assays using the GST-tagged ERα E domain constructs. GST-fusion proteins were incubated with synthetic polyUb2-7 K48- or K63-linked chains, or K6-, K11-, K27-, K29-linked di-Ub and analyzed in immunoblot as indicated.

3.2.2 Characterization of the full lenght ERα-UBS mutant in cells.

The pull-down experiments helped us identifying the *in vitro* key residues of the ERα E domain UBS. However, to understand if these residues are required also for the full length ERα binding to ubiquitinated species we introduced the LAAG mutations in wt ERα and transiently transfected it in HeLa or HEK293 cells.

Next, the wt and LAAG mutant receptor expressing cell lysates were treated with SDS in order to reduce the amount of receptor interactors that could associate to the receptor through a protein surface different from UBS [40]. Then, immunoprecipitation analysis was performed and the obtained results revealed that the introduction of the double LAAG mutation in ERα strongly prevents the receptor ability to associate with ubiquitinated proteins (Fig. 9A). These data demonstrate that the ERα-UBS directs Ub-binding also in cells.

Next, we sought to determine the role of ERα non-covalent Ub-binding on receptor activities. Since the ERα-based signalling is a function of receptor intracellular localization (*i.e.,* nuclear and extra-nuclear) [6, 27], we tested the effect of the LAAG mutation on ERα sub-cellular distribution. Immunofluorescence analysis showed that both GFP-tagged wt and LAAG mutant ERα have the same nuclear, cytoplasmic and membrane tethered localization (Fig. 9B). Thus we conclude that mutation of L429 and A430 residues does not influence basal ERα sub-cellular localization.

Because the LAAG mutation is within the LBD, we also evaluated E2 binding affinity to the ERα-UBS mutant. Importantly, we found that the wt ERα E2 binding affinity was not impaired by the introduction of the LAAG mutations (Table 1).

In cells, ERα dimerizes and binds DNA to ERE sequences and induces gene transcription after E2-treatment [6]. To evaluate if the LAAG mutation could impair the receptor ability to dimerize under basal conditions, we transiently co-transfected flag-ERα wt and

GFP-ERα wt, or flag-ERα LAAG and GFP-ERα LAAG in HEK293 cells. Then, anti-flag immunoprecipitation and anti-ERα immunoblot were performed and showed that the wt and LAAG ERα were equally able to dimerize (data not shown).

Next, we also determined whether the reduction in ERα Ub-binding could affect basal ERα transcriptional activity. As shown in figure 9C, equal expression of either wt or LAAG mutant ERα triggered the activation of the artificial ERE-containing reporter constructs (*i.e.* 3xERE-TATA- ERE promoter) to comparable levels.

Finally, to gain insights in the LAAG ability to respond to E2 with an increased expression of target proteins, we investigated the physiological expression of the endogenous E2-responsive Bcl-2 gene [49]. Remarkably, dose-response analysis revealed that the LAAG mutation reduced the ability of the E2:ERα complex to increase the Bcl-2 cellular levels as compared with the wt ERα (Fig. 9D).

These data indicate that the UBS on ERα E domain identified *in vitro* plays a critical role for Ub-binding also in cells in the context of the full length receptor. The mutation of key residues for Ub-binding does not impair the receptor basal activities, as cellular localization and ability to dimerize as well as DNA binding to ERE sequences. Moreover, even if the LAAG mutant ERα binds E2 as the wt, it showed a reduced ability to mediate the E2-induced increase of Bcl-2 gene expression. Thus, since E2-induced ERα transcriptional activity deeply impacts on cellular function, it is possible that the ERα-UBS could be required for E2-dependent physiological processes.

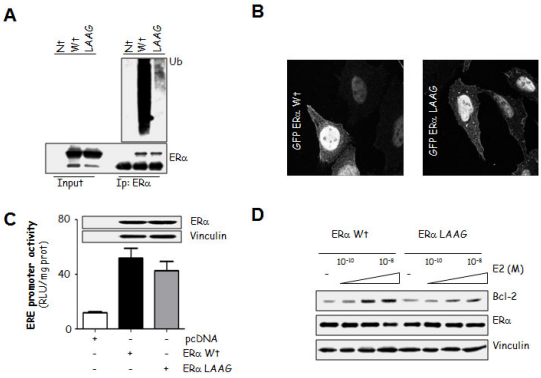

Table1

	IC_{50} (M)			
	Kit	ERα Rec	ERα wt	ERα LAAG
E2	1.0×10^{-8}	$3.0\pm0.24\times10^{-8}$	$7.0\pm0.91\times10^{-10}$	$2.6\pm0.40\times10^{-10}$

Figure 9. Characterization of the full length ERα-UBS mutant. (A) Ub and ERα western blot analysis of ERα immunoprecipitation in non-transfected (Nt) and pcDNA flag wild-type ERα (wt) HEK293 cells and in pcDNA flag-ERα L429A, A430G (LAAG) mutant transfected HEK293 cells. SDS (0.05%) was added to the lysates before immunoprecipitation and the immunoprecipitated complexes were washed in RIPA buffer containing 0.1% SDS. **(B)** Confocal miscrocopy analysis of the HeLa cells transiently transfected with pEGFP-ERα (wt) or the pEGFP-ERα (LAAG) mutant kept in growing conditions. **(C)** Luciferase assay on the HeLa cells transiently transfected with pcDNA flag, pcDNA flag-ERα (wt) or the pcDNA flag-ERα LAAG mutant together with the 3xERE TATA reporter plasmid. Insets show wt and mutant flag-ERα expression normalized on vinculin. **(D)** Western blot analysis of the Bcl-2 protein levels in the HeLa cells transiently transfected with pcDNA flag-ERα (wt) or the pcDNA flag-ERα (LAAG) mutant and then treated for 24 hours with different doses of E2 (10^{-10}-10^{-8} M). Loading control was done by evaluating vinculin expression in the same filter.

Table1. E2 binding to wt and mutant ERα. *In vitro* ERα affinity (as indicated by the IC_{50} value) was determined using the HitHunter EFC assay (Discoverx) according to manufacturer instructions for E2 towards either recombinant ERα protein (ERα Rec), wt or LAAG mutant receptors (ERα wt or ERα LAAG, respectively) extracted from transfected HEK293 cells. Kit indicates the value of E2 affinity to ERα as reported in the manufacturer's specification, and has been introduced as a positive control reference; the value is similar to that measured with ERα Rec (Panvera). Results show that the E2 affinity to ERα wt is the same as that for the mutant ERα LAAG. Overall affinity of ERα wt and ERα LAAG with respect to ERα Rec can be ascribed to the fact that ERα wt and ERα LAAG E2 binding abilities were measured in equivalent amounts of total cellular lysates.

4. ROLE OF THE ERα-UBS IN E2-INDUCED CELL PROLIFERATION.

4.1 Introduction.

The E2-controlled ERα-mediated physiological processes depends on the integration of nuclear (*i.e.,* gene transcription) and extra-nuclear (*i.e.,* activation of rapid signalling) signalling pathways [6, 27] (Fig. 3). In particular, cell proliferation is increased by E2 treatment thanks to its ability to induce rapid signalling that controls the transcription of the Cyclin D1 protein, which is required for the G1-S progression [50, 12].

In addition, E2 regulates cholesterol homeostasis protein machinery, which in turn is important for cell proliferation [23]. The modulation of cholesterol sourcing by E2 depends on the co-ordinate transcriptional regulation of the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), an ERE-like containing gene [23], and of the low-density lipoprotein receptor (LDLr), a non-ERE containing gene [24].

In this section, we evaluated the putative role of the ERα-UBS in E2-dependent cell proliferation by analyzing both pathways responsible for cell cycle progression and cholesterol sourcing. To this purpose, the wt and LAAG mutant ERα was stably introduced in HEK293 cells and the E2-triggered signalling to cell proliferation was evaluated.

4.2 Results.

4.2.1 The ERα-UBS is required for E2-induced cell proliferation.

The residues L429 and A430 are critical for ERα to associate to ubiquitinated species *in vitro* and in cell lines. Therefore, to begin unravel the role of the ERα-UBS in ERα signalling, the ERα-UBS mutant LAAG was transfected in HEK293 cells and stable cell lines expressing the LAAG mutant receptor were generated and selected on the basis of the wt ERα cellular levels (Fig. 10A, inset) and

of a growth rate similar to that of wt ERα (Fig. 10A) and next compared with the already characterized wt ERα expressing cells [34]. Initial experiments were performed in these cell lines to evaluate the role of ERα-UBS in E2-induced cell proliferation. In wt ERα expressing cells, E2 induced a significant increase in cell number compared to the un-stimulated cells (Fig. 10B). On the contrary, E2 did not raise the number of the LAAG mutant receptor expressing cells (Fig. 10B). Thus, the ERα-UBS plays a critical role for E2-induced cell proliferation.

E2 mitogenic effects depend on ERα extra-nuclear signalling [4]. Thus, E2-dependent ERK and AKT activation was evaluated. As shown in figure 10C (upper panels and 10C'), E2 induced the rapid and transient increase in ERK1/2 phosphorylation in both cell lines. On the other hand, while E2 evoked a rapid and persistent increase in AKT phosphorylation in wt ERα expressing cells, the hormone failed to trigger it in LAAG mutant receptor expressing cells (Figs. 10C- lower panels and 10C'). Notably, no significant changes in the total ERK2 and AKT levels were detected in cells treated with E2 (Figs. 10C and 10C'). The LAAG ERα inability to mediate PI3K/AKT activation after E2 treatment could be due to a general impairment of the kinase cascades activation in mutant expressing cells. To exclude this possibility, we analyzed the epidermal growth factor (EGF)-dependent ERK1/2 and AKT activation in both wt and LAAG receptor expressing cells and found that they were intact in either cell lines (Supplemental figure 3). Thus, the impairment of AKT activation after E2-treatment in LAAG ERα cells was specifically dependent on the introduction of double mutation on ERα, which does not affect the PI3K/AKT activation induced by other stimuli (*e.g.,* growth factors). Moreover, these data demonstrate that ERα-UBS is necessary for E2-induced AKT activation while it is dispensable for ERK1/2 phosphorylation.

The mechanism by which the mutation in ERα-UBS prevents E2-induced AKT phosphorylation remains obscure. However, activation of the PI3K/AKT pathway by

E2 occurs through the physical recruitment of ERα to IGF-1R [17]. Thus, the lack of the E2-dependent AKT activation in LAAG mutant receptor expressing cells (Figs. 10C- lower panels and 10C') could depend on deregulated ERα:IGF-1R association. As expected, in wt ERα expressing cells, E2 treatment triggers the rapid ERα:IGF-1R association while E2 administration reduced the LAAG mutant ERα association with the IGF-1R (Fig. 10D), thus indicating that mutation of the ERα-UBS alters E2-induced ERα:IGF-1R interactions. Thus these data suggest that the reason for the defect of the ERα-UBS mutant in the activation of the PI3K/AKT pathway is ascribable to an E2-dependent dissociation of the LAAG mutant receptor from IGF-1R (Fig. 10D), a mechanistic event that impedes the E2-dependent AKT activation (Figs. 10C- lower panels and 10C') [17] and cell proliferation (Fig. 10B).

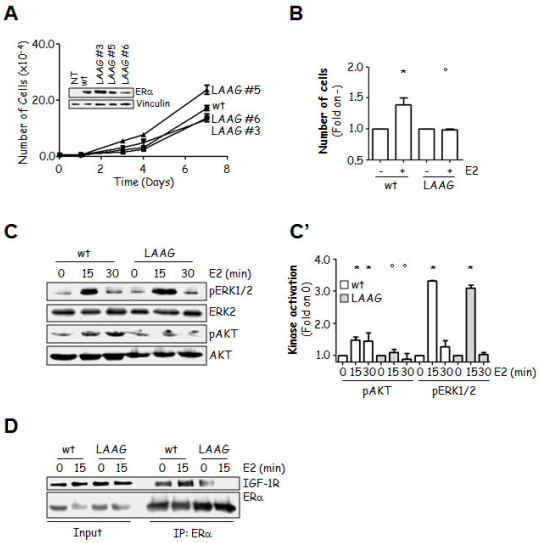

Figure 10. The role of ERα-UBS on E2-induced cell proliferation. (A) Western blot analysis of ERα protein levels in HEK293 stable cells expressing the pcDNA-flag- ERα (wt) and the pcDNA-flag-ERα L429A, A430G (LAAG) (inset) and growth curves of HEK293 cells stably expressing the pcDNA-flag-ERα (wt) and the pcDNA-flag-ERα LAAG. **(B)** Number of HEK293 cells stably expressing the pcDNA-flag-ERα (wt) and the pcDNA-flag-ERα LAAG mutant both in the presence or in the absence of E2 (10^{-8} M- 48 hours). **(C)** Western blot and **(C')** relative densitometric analysis of AKT and ERK1/2 phosphorylation in HEK293 stable cells expressing the pcDNA-flag-ERα (wt) and the pcDNA-flag-ERα LAAG treated with E2 (10^{-8} M) at the indicated time points. **(D)** ERα:IGF-1R coimmunoprecipitation analysis in HEK293 cells stably expressing pcDNA-flag-ERα (wt) and pcDNA-flag- ERα LAAG treated with E2 (10^{-8} M) at the indicated time points * indicates significant differences with respect to (– or 0) wt sample; ° indicates significant differences with respect to (+ E2) wt sample ($p<0.01$).

4.2.2 The ERα-UBS functions are necessary for the E2-induced cholesterol sourcing.

E2 activates several pathways to guarantee to the cells the all requirement necessary to proliferate (*e.g.,* cyclins expression, cholesterol sourcing). For example, proliferating cells have an increased requirement of cholesterol that is obtained through an enhancement of the lipid biosynthesis as well as of the uptake from the bloodstream. E2 transcriptionally regulates the expression of both the HMGR enzyme [23], which controls the cholesterol synthesis [26], and the LDLr [24], which is responsible for the cholesterol uptake [26]. Because the E2-induced cell proliferation was blocked in LAAG ERα expressing HEK293 cells, we evaluated if also the cholesterol sourcing was affected by the ERα-UBS mutation.

Thus, we analyzed the effect of E2 stimulation on the cellular levels of the LDLr and HMGR proteins in wt and LAAG ERα HEK293 cells. We found that E2 increased both LDLr and HMGR levels in wt but not LAAG ERα expressing cells (Figs. 11A and 11B). These results indicate that the ERα-UBS is required to mediate the E2-induced transcription of the master regulators of the cholesterol sourcing.

Since E2-induced cell proliferation requires the activation of the PI3K/AKT signalling activation, which we found to be impaired in LAAG ERα HEK293 expressing cells, we evaluated if this pathway could play a critical role for cell proliferation and cholesterol sourcing also in MCF-7 cells. To this purpose, we analyzed the effects of E2 on LDLr and HMGR cellular levels in the presence or absence of the PI3K inhibitor LY 294002 (LY). Remarkably, we found that the LY blocked the E2-induced increase of both LDLr and HMGR also in this breast cancer cell line (Figs. 11C and 11D). Next, to understand if this effect could reflect a role of the PI3K/AKT for cell proliferation, we evaluated the ability of E2 to induce an increase of the cell number when the pathway was inhibited by LY. Interestingly, we found that the LY prevented the E2-

induced MCF-7 cell proliferation (Fig. 11E). Thus, in MCF-7 cells the E2-induced cholesterol sourcing as well as cell proliferation requires the activation of the PI3K/AKT signalling.

Thus, data obtained in LAAG ERα HEK293 as well as in LY treated MCF-7 cells helped us defining a conserved pathway in which the ERα-UBS, *via* E2-activated extra-nuclear PI3K/AKT pathway, controls the nuclear ERα-mediated transcription of HMGR and LDLr [23, 24], which modulate the cholesterol sourcing required for cells to proliferate.

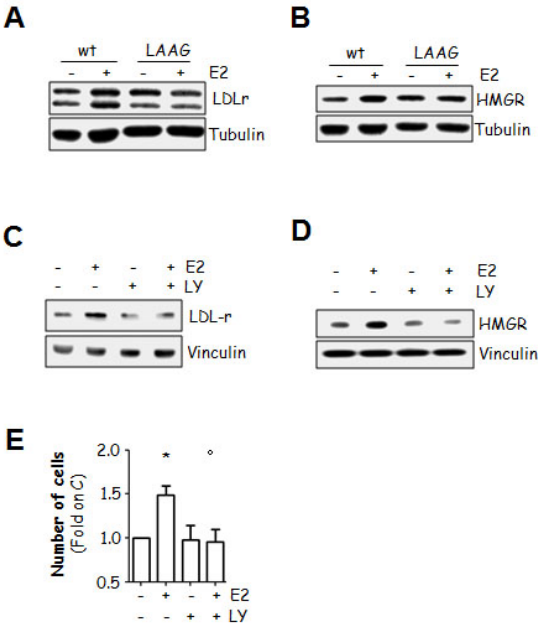

Figure 11. The role of ERα-UBS on cholesterol metabolism. (A-D) Western blot of the **(A)** LDL receptor (LDLr) and **(B)** HMG-CoA reductase (HMGR) protein levels in HEK293 stable cells expressing pcDNA-flag-ERα (wt) and pcDNA-flag-ERα L429A, A430G (LAAG) or **(C, D)** in MCF-7 cells treated with E2 (10^{-8} M) for 24 hours. Loading control was done by evaluating tubulin or vinculin expression in the same filter. **(E)** Number of MCF-7 cells treated with E2 (10^{-8} M- 48 hours) both in presence or in absence of LY 294002 (LY) (10^{-6} M). * indicates significant differences with respect to (–) wt sample; ° indicates significant differences to the relative (+ E2) wt sample ($p<0.05$; $p<0.01$ for cell number).

4.2.3 The ERα-UBS controls the receptor full transcriptional activation.

The E2-induced AKT signalling also controls ERα Ser118 phosphorylation [27, 34], which is required for the full transcriptional activation of the receptor. Indeed, this modification increases the receptor association with ERE sequences as well as the assembly of transcriptional co-regulators (*e.g.,* CBP/p300 and SRC-1) [51] that mediate ligand-dependent and independent transcription [16].

Thus, we evaluated the ERα Ser118 phosphorylation status in both wt and LAAG receptor expressing cells. As expected, E2 treatment increased the phosphorylation of wt ERα on the Ser118 residue but not in the LAAG mutant receptor and no changes in the total ERα levels were observed (Fig. 12A), thus corroborating the concept that E2-dependent ERα Ser118 phosphorylation is under the control of the PI3K pathway [27, 34]. Next, to evaluate if the lack of Ser118 phosphorylation in LAAG ERα expressing cells after E2 treatment resulted in a reduced transcriptional activity of the receptor, we analyzed the E2-induced expression of the Cyclin D1 gene [50, 51]. We found that in wt ERα expressing cells E2 increased the mRNA levels of Cyclin D1 while in the presence of the LAAG mutant ERα E2 did not modify them (Fig. 12B). Because the increase of the Cyclin D1 protein levels promote the G1-S progression of the cell cycle, these results further sustain that the LAAG ERα is not able to mediate the E2 mitogenic effects. Moreover, these data together with that on Bcl-2 protein levels obtained in HeLa cells transiently transfected with wt and LAAG ERα (Fig. 9D), suggest that the ERα-UBS also controls the expression of some E2 target genes.

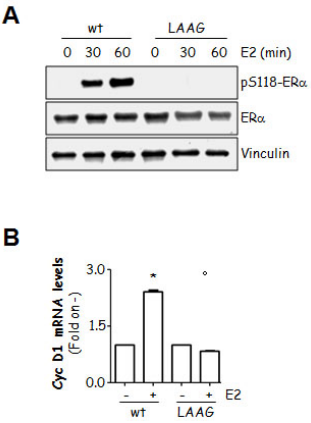

Figure 12. The ERα-UBS mutant phosphorylation and transcriptional activation. (A) Western blot analysis of ERα and ERα Ser118 phosphorylation in HEK293 stable cells expressing the pcDNA-flag-ERα (wt) and the pcDNA-flag-ERα L429A, A430G (LAAG) treated with E2 (10^{-8} M) at the indicated time points. Loading control was done by evaluating vinculin expression in the same filter. **(B)** RT qPCR analysis of Cyclin D1 (Cycl D1) mRNA expression normalized on the GAPDH mRNA expression in HEK293 cells stably expressing pcDNA-flag-ERα (wt) and pcDNA-flag-ERα LAAG treated with E2 (10^{-8} M) for 24 hours. * indicates significant differences with respect to (–) wt or LAAG sample; ° indicates significant differences with respect to the corresponding E2 ERα wt treated sample ($p<0.05$).

5. ROLE OF THE ERα-UBS IN E2-INDUCED GENE EXPRESSION.

5.1 Introduction.

Data reported in the previous sections showed that the ERα mutated on UBS is not phosphorylated on Ser118 after E2 treatment and seems to be less transcriptionally active than the wt counterpart. The ERα transcriptional activity is dependent on the Ser118 phosphorylation but the E2-induced gene expression also relies on other transcription factors [6] whose activity could be independent on the ERα-UBS functions. Moreover, it is possible that E2 *via* the ERα-UBS selectively modulates specific pathways at transcriptional level. To takcle these problems, we decided to perform high through-output experiments. In particular, DNA Micro Arrays were chosen because this technique allows to evaluate both the global LAAG ERα mutant transcriptional activity and the impact of this mutation on the activation of pathways to cellular effects. Thus, DNA Micro Arrays were performed on HEK293 cells stably expressing the wt and LAAG ERα in the presence or in the absence of E2.

Different kinds of analysis were conducted on the obtained data; among these, the Ingenuity Pathway Analysis, which on the basis of the upregulated or down-regulated genes predicts the activation or the inhibition of upstream transcription factors.

In this section, we analyzed the Micro Arrays data and evaluated the impact of the ERα-UBS mutation on the E2-induced gene expression.

5.2 Results.

5.2.1 DNA Micro Arrays: the ERα-UBS is necessary for E2-induced gene expression.

In order to identify pathways whose E2-induced transcriptional activation requires the ERα-UBS, a global gene expression profiling was carried out using the

Affymetrix GeneChip Human Gene 1.0 ST Array. The HEK293 cells stably expressing wt or UBS mutant ERα were treated with E2 for 24 hours and DNA Micro Array experiments were performed.

Several types of analysis were conducted to identify specific pathways selectively modulated by E2 *via* the ERα-UBS. Figure 13A shows the gene cluster analysis in which each column represents a wt or LAAG ERα expressing sample treated or not with E2 and contains all the regulated genes. In particular, the up-regulated genes are in red while the down-regulated ones are in green; the pattern for each cluster of genes can be observed horizontally. This analysis shows that there are many genes stimulated by E2 in wt but not in LAAG ERα expressing cells (Fig. 13A).

In the second kind of analysis, scatter plots were produced to understand the amount of genes stimulated or inhibited by E2 in wt and LAAG ERα cells. In Figure 13B, the localization of each dot depends on the value obtained by normalizing the signal of each E2-regulated gene (y-axis) on the control signal (x-axis). This type of analysis indicated that the LAAG mutant ERα was less transcriptionally active than the wt receptor after 24 hours of E2 treatment (Fig. 13B).

In particular, it was found that the wt ERα modulated 623 unique genes while the LAAG ERα modulated only 285 unique genes. However, a set of 8 genes (*e.g.*, CCAAT/enhancer binding protein beta-CEBPB) were found to be activated by E2 in wt as well as in LAAG ERα expressing cells (Table 2). Actually, the validation of the CEBPB mRNA levels in both cell lines showed that they were significantly increased by E2, even if at different levels (lower in LAAG ERα expressing cells) (Fig. 13C).

Moreover, Ingenuity Pathways Analysis was applied to the obtained data to predict the activation and inhibition of upstream transcription factors. Among these, as expected, the ERα was identified as an E2-activated transcription factor transcriptionally active in wt but not in

LAAG ERα cells (Fig. 14A). To validate these data, we performed real time qPCR experiments and confirmed that wt but not LAAG ERα mediated the E2-induced transcription of pS2, Cathepsin D and Progesterone Receptor (PR) genes (Fig. 14B-D), which are well known target genes predicted to be activated in wt HEK293 cells but not in LAAG mutant expressing cells. These data confirm the Micro Arrays results and indicate that the ERα-UBS integrity is required for the E2-induced ERα-mediated target genes expression.

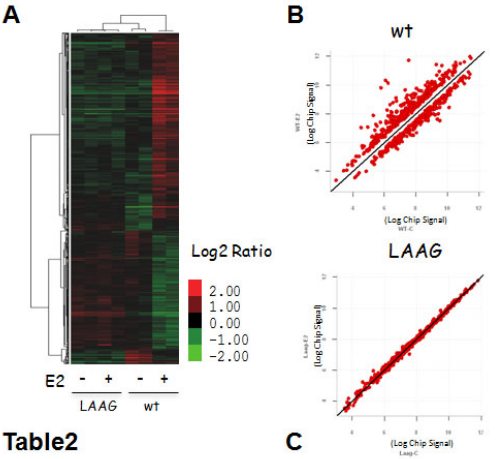

Table2

Overlap wt-LAAG (p<0,05)
CEACAM8
CEBPB
G3BP1
GFER
OCLN
PTMA
SKP1
TUBB2A

Figure 13. Gene expression profile of the wt and LAAG ERα expressing HEK293 cells. HEK293 cells stably expressing pcDNA-flag-ERα (wt) and pcDNA-flag-ERα LAAG were treated with 10^{-8} M E2 for 24 hours before cells were harvested, RNA extracted and Affymetrix GeneChip Micro Array analysis performed. **(A)** Gene cluster analysis was performed for genes found to be significantly regulated by E2. Stimulated genes are shown in red and inhibited genes in green. **(B)** Scatterplots were produced using the normalized signal for each of the E2-regulated genes, plotted against the control signal for that gene on the x-axis. **(C)** RT qPCR analysis of the CCAAT/enhancer binding protein beta (CEBPB) mRNA expression normalized on the GAPDH mRNA expression in HEK293 cells stably expressing pcDNA-flag-ERα (wt) and pcDNA-flag-ERα LAAG treated with E2 (10^{-8} M) for 24 hours. * indicates significant differences with respect to (–) wt sample; ° indicates significant differences with respect to (+ E2) wt sample ($p<0.01$).

Table2. Overlap between E2-activated genes in wt and LAAG ERα expressing cells. List of the only 8 genes found to be significatively activated by E2 in wt and LAAG ERα expressing cells ($p<0.05$).

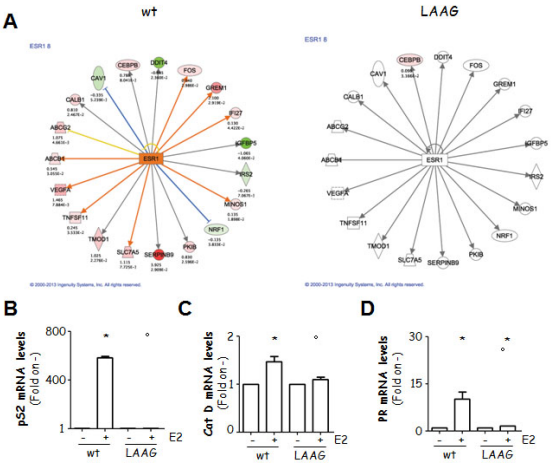

Figure 14. The role of ERα-UBS on E2-induced ERα-mediated gene expression. (A) The Ingenuity Pathway Analysis applied to the data obtained from GeneChip Micro Arrays predicted the activation or inhibition of upstream transcription factors (located at the center of the circle) in HEK293 cells stably expressing pcDNA-flag-ERα (wt) and pcDNA-flag-ERα L429A, A430G (LAAG) treated with E2 (10^{-8} M) for 24 hours. Colored lines indicate the level of consistency of the relationships with the state of the downstream molecule (orange: the prediction leads to activation, blue: the prediction leads to inhibition, yellow: the prediction is inconsistent and gray: the effect is not predicted). ERα is predicted to be activated only in pcDNA-flag-ERα (wt) expressing cells. RT qPCR analysis of **(B)** presenilin 2 (pS2), **(C)** cathepsin D (Cat D), and **(D)** progesterone receptor (PR) mRNA expression normalized on the GAPDH mRNA expression in HEK293 cells stably expressing pcDNA-flag-ERα (wt) and pcDNA-flag-ERα LAAG treated with E2 (10^{-8} M) for 24 hours. * indicates significant differences with respect to (–) wt sample; ° indicates significant differences with respect to (+ E2) wt sample ($p<0.01$).

5.2.2 Ingenuity Pathways Analysis: CREB1 activation by E2 relies on a functional UBS on ERα.

In addition to ERα, the Ingenuity Pathways Analysis also predicted the activation of CREB1 by E2 for gene transcription. Data obtained from Micro Arrays analysis showed that also E2-induced CREB1-mediated target genes were transcribed in response to E2 in wt but not in LAAG ERα expressing cells (Figs. 15A). Accordingly, real time qPCR experiments performed on the Vascular Endothelial Growth Factor A (VEGFA), a CREB1-regulated gene, mRNA levels in these cells confirmed the Micro Array data (Fig. 15B).

Next, to evaluate the impact of CREB1 activation on E2:ERα activities we tested on wt ERα HEK293 cells the 2-naphthol-AS-E phosphate (*i.e.*, KG-501- KG), an inhibitor of the CBP/CREB1 interaction, which blocks CREB1-mediated gene transcription [52]. Among others, CREB1 controls the E2-induced transcription of the pro-survival factor Bcl-2 [53]; thus, to select the best dose of KG to block CREB1-mediated transcription we performed a dose-response curve of the inhibitor on the Bcl-2 cellular levels in HEK293 expressing wt ERα (data not shown). We found that 10 μM of KG is able to block the E2-induced increase of the Bcl-2 cellular levels (Fig. 15D) in wt ERα expressing HEK293. Interestingly, both in transiently (Fig. 9D) and in stably (data not shown) transfected HEK293 cells E2 increased the levels of the anti-apoptotic protein Bcl-2 in the presence of the wt receptor [49] but not in the presence of the LAAG mutant ERα.

Then, to further test the KG ability to block CREB1-mediated gene transcription, we analyzed the VEGFA mRNA levels in the presence or in the absence of the selected dose of KG in wt ERα HEK293 cells. Figure 10D shows that the 10 μM of the KG inhibitor effectively reduced the E2-induced CREB1 target gene VEGFA transcription (Fig. 15C).

Thus, the E2-dependent control of VEGFA and Bcl-2 genes *via* CREB1 is blocked by the LAAG mutations as

well as by KG. These data indicate that the KG could be an useful tool to mimic the ERα-UBS mutant phenotype on CREB1 pathway in order to evaluate if the pathway is conserved in other cell lines and to understand its impact on cell functions.

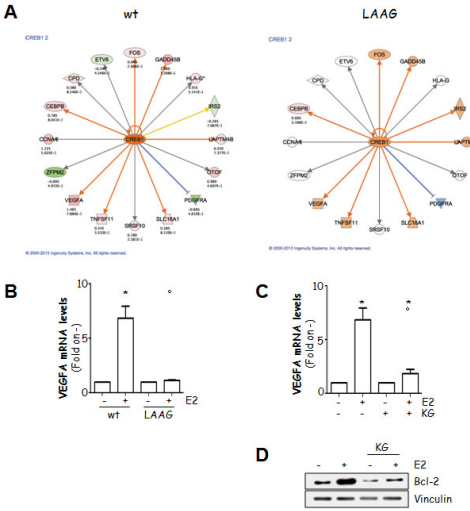

Figure 15. The role of ERα-UBS on E2-induced CREB1-mediated gene expression. (A) The Ingenuity Pathway Analysis applied to the data obtained from GeneChip Micro Arrays predicted the activation or inhibition of upstream transcription factors (located at the center of the circle) in HEK293 cells stably expressing pcDNA-flag-ERα (wt) and pcDNA-flag-ERα L429A, A430G (LAAG) treated with E2 (10^{-8} M) for 24 hours. Colored lines indicate the level of consistency of the relationships with the state of the downstream molecule (orange: the prediction leads to activation, blue: the prediction leads to inhibition, yellow: the prediction is inconsistent and gray: the effect is not predicted). CREB1 is predicted to be activated only in pcDNA-flag-ERα (wt) expressing cells.**(B)** RT qPCR analysis of the vascular endothelial growth factor A (VEGFA) mRNA expression normalized on the GAPDH mRNA expression in HEK293 cells stably expressing pcDNA-flag-ERα (wt) and pcDNA-flag-ERα L429A, A430G (LAAG) treated with E2 (10^{-8} M) for 24 hours. **(C)** RT qPCR analysis of the vascular endothelial growth factor A (VEGFA) mRNA expression normalized on the GAPDH mRNA expression in HEK293 cells stably expressing pcDNA-flag-ERα (wt) treated with E2 (10^{-8} M) for 24 hours in the presence or absence of KG-501 (KG- 10^{-5} M). * indicates significant differences with respect to (–) wt sample; ° indicates significant differences with respect to (+ E2) wt sample ($p<0.01$). **(D)** Western blot analysis of Bcl-2 in HEK293 stable cells expressing the pcDNA-flag-ERα (wt) treated with E2 (10^{-8} M) for 24 hours in the presence or absence of KG-501 (KG- 10^{-5} M). Loading control was done by evaluating vinculin expression in the same filter.

5.2.3 Dissection of the E2-triggered ERα-UBS dependent CREB1 pathway.

The activation of CREB1 depends on its phosphorylation on Ser133 residue by different kinases (*e.g.*, MAPK, PKA) [54, 55]. After phosphorylation, CREB1 migrates to the nucleus where it binds the DNA CRE sequence and associates with the CBP and other transcription components to activate target genes transcription [55].

To evaluate if the lack of E2-induced CREB1-mediated gene transcription in LAAG ERα cells could depend on an altered activation of CREB1, we analyzed the effect of E2 treatment on CREB1 Ser133 phosphorylation in wt and mutant ERα-UBS cells (Fig. 16A). We found that CREB1 is activated by E2 in 30 minutes in wt but not in LAAG ERα cells. These data not only indicates that E2 controls CREB1 phosphorylation in ERα expressing HEK293 cells but also that the ERα-UBS is required for this activation.

To understand if the E2-triggered PI3K/AKT pathway could be responsible for CREB1 activation, we analyzed the effects of the PI3K inhibitor LY on the E2-induced CREB1 Ser133 phosphorylation in wt ERα expressing cells. We found that the E2-dependent increase of CREB1 Ser133 phosphorylation was blocked when wt ERα cells were pre-treated with the PI3K inhibitor (Fig. 16B and 16B'). However, we found that the CREB1 Ser133 phosphorylation were higher after LY treatment alone respect to untreated cells. This result suggests that other kinases could be basally activated as compensatory mechanism to guarantee CREB1 activation.

In addition, we next found that the PI3K inhibitor was able to block the E2-triggered increase of the CREB1-target gene Bcl-2 as also the KG inhibitor did (Fig. 16C). Importantly, also the E2-triggered cell proliferation was blocked by both PI3K and CREB1 inhibitors (Fig. 16D). These data strongly support that the E2-induced PI3K/AKT signalling, whose activation is impaired by the LAAG

mutations on ERα, is required for CREB1 phosphorylation and transcriptional activity in wt ERα cells.

However, we were interested in understanding if this pathway (*i.e.,* E2:ERα-UBS/activation of the PI3K/AKT signalling/CREB1 phosphorylation/gene transcription) could be equally important for breast cancer MCF-7 cells E2-induced proliferation. Thus, we performed in MCF-7 cells experiments similar to that previously described for HEK293 wt ERα cells. In particular, we analyzed if E2 induced CREB1 activation and if the PI3K signalling was responsible for its phosphorylation on Ser133. We found that CREB1 phosphorylation on Ser133 was induced by E2 and the LY inhibitor blocked this increase (Fig. 17A). As observed in HEK293 wt ERα cells, we found that LY inhibitor induced a basal increase of CREB1 Ser133 phosphorylation, further supporting the concept of a compensatory contribute of other kinases. We also evaluated the impact of the PI3K inhibition on the E2-induced and CREB1-mediated expression of the Bcl-2 and Cyclin D1 proteins. We found that the E2-dependent Bcl-2 and Cyclin D1 expression increase was blocked by LY (Fig. 17B-left panels).

Next, we performed a dose-response analysis of the KG effect also in these cells (data not shown) and found that 1 μM of KG was able to block the E2-induced CREB1-mediated Bcl-2 expression (Fig. 17B-right panels). Moreover 1 μM of the KG inhibitor blocked the E2-dependent increase of Cyclin D1 protein levels also in this cell line (Fig. 17B-right panels). Finally, we evaluated if the E2-induced CREB1-mediated transcription could be required for the E2 mitogenic effects. To this purpose MCF-7 cells were pre-treated with KG and after 48 hours of E2 treatment were counted. Figure 17C indicates that the KG inhibitor impedes the E2-induced increase of the cell number (Fig. 17B-right panels- and 17C).

Thus, the LAAG ERα inability to mediate the PI3K/AKT pathway activation in response to E2 could be responsible for the lack of CREB1 phosphorylation, which is required for E2-induced CREB1-dependent gene

transcription. This pathway in turn is critical for E2-dependent cell proliferation and is conserved also in MCF-7 breast cancer cells.

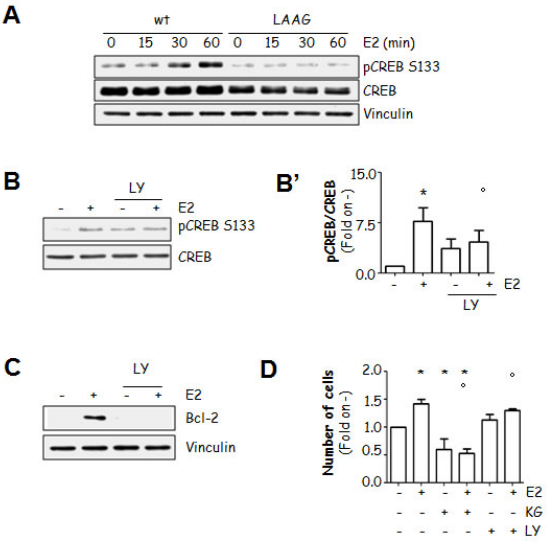

Figure 16. The E2-induced CREB1-mediated pathway in wt and L429A, A430G (LAAG) ERα cells. (A) Western blot analysis of CREB1 and CREB1 Ser133 phosphorylation in HEK293 stable cells expressing the pcDNA-flag-ERα (wt) and the pcDNA-flag-ERα LAAG treated with E2 (10^{-8} M) at the indicated time points. Loading control was done by evaluating vinculin expression in the same filter. **(B)** Western blot and relative densitometric analysis of CREB1 and CREB1 Ser133 phosphorylation in HEK293 stable cells expressing the pcDNA-flag-ERα (wt) treated with E2 (10^{-8} M) in the presence or absence of LY 294002 (LY- 10^{-6} M) after 1 hour of E2 treatment. Loading control was done by evaluating vinculin expression in the same filter. **(C)** Western blot analysis of Bcl-2 protein in HEK293 stable cells expressing the pcDNA-flag-ERα (wt) treated with E2 (10^{-8} M- 24 hours) in the presence or absence of LY 294002 (LY- 10^{-6} M). Loading control was done by evaluating vinculin expression in the same filter. **(B)** Number of HEK293 cells stably expressing the pcDNA-flag-ERα (wt) treated with E2 (10^{-8} M- 48 hours) both in the presence or in the absence of KG-501 (KG- 10^{-5} M) or LY (10^{-6} M). * indicates significant differences with respect to (–) wt or LAAG sample; ° indicates significant differences with respect to the corresponding E2 ERα wt treated sample ($p<0.05$).

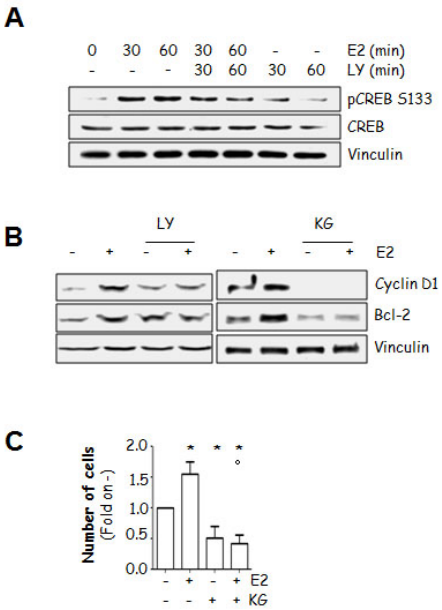

Figure 17. The E2-induced CREB1-mediated pathway in MCF-7 cells. **(A)** Western blot analysis of CREB1 and CREB1 Ser133 phosphorylation in MCF-7 cells treated with E2 (10^{-8} M) in the presence of absence of LY 294002 (LY) (10^{-6} M) at the indicated time points. Loading control was done by evaluating vinculin expression in the same filter. **(B)** Western blot Cyclin D1 and Bcl-2 in MCF-7 cells treated with E2 (10^{-8} M) in the presence or absence of LY 294002 (LY- 10^{-6} M) or KG-501 (KG- 10^{-6} M) after 24 hours of E2 treatment. Loading control was done by evaluating vinculin expression in the same filter. **(C)** Western blot analysis of Bcl-2 protein in HEK293 stable cells expressing the pcDNA-flag-ERα (wt) treated with E2 (10^{-8} M - 24 hours) in the presence or absence of LY 294002 (LY- 10^{-6} M). Loading control was done by evaluating vinculin expression in the same filter. **(B)** Number of MCF-7 cells treated with E2 (10^{-8} M - 48 hours) both in the presence or in the absence of KG-501 (KG). * indicates significant differences with respect to (–) wt or LAAG sample; ° indicates significant differences with respect to the corresponding E2 ERα wt treated sample ($p<0.05$).

6. DISCUSSION AND CONCLUSIONS.

The E2:ERα signalling controls a plethora of physiological processes but plays also a critical role in breast cancer progression, thus the comprehension of the mechanisms that control E2-induced cell proliferation would help to identify putative druggable targets for the treatment of breast cancer. The Ub-system is gaining much attention for cancer therapies because its deregulation has been associated to different kind of cancers. Several blocking point of the system have been proposed, some drugs have just been synthesized and are being tested [41]. One of the proposed future druggable target is the UBD:Ub interaction, which is used by cells to transduce the Ub signal and/or to create a network of molecular interactions required for the regulation of cellular processes. Even if several evidence reported a deep correlation between the E2:ERα and Ub signalling [32], no information are available on the possibility that ERα could behave as an Ub-receptor that bind through an UBD the Ub molecule on itself or on interacting proteins thus transducing the Ub-based signal.

The main goal of this PhD project was to understand the non-covalent Ub-binding abilities of ERα and their regulatory role in E2-dependent cellular processes. To this purpose, initial experiments were performed *in vitro* by using purified ERα domains and recombinant or cell lysates-derived Ub molecules.

We evaluated the putative presence of an UBD on ERα and we found that ERα has three domains (*i.e.,* A/B and E domains) that non-covalently bind Ub. These domains showed different Ub-binding abilities; in particular, the A/B domain possesses a weaker Ub-binding ability than E domain (Fig. 7A). This evidence can be explained by the fact that A/B and E domains show selective binding properties towards specific Ub-based chains. Indeed, while the E domain is able to non-covalently bind all but K33-linked Ub chains (Fig. 8), the A/B domain contact only the K6- and K63-linked Ub chains (Supplemental Fig. 1). These data, in addition to explain the

Ub-binding ability differences between the domains also demonstrate that they directly contact Ub on ubiquitinated proteins. Interestingly, as the A/B and the E domains are located at the *N*-terminus and at the *C*-terminus of the ERα structure respectively, they could cooperate to contact monoUb on K302,K303 residues [30] or an interactor modified with K6- and K63-linked Ub chains, to increase the ERα Ub-binding regions avidity towards Ub. In this respect, structural crosstalk between distant ERα A/B and E domains has been reported [56] and our findings open the possibility that this intra-molecular crosstalk could depend on an Ub:UBD interaction. Next, we focused on the E domain Ub-binding ability since it displays a well-known folded structure and we mapped the Ub-binding on its *N*-terminal portion (*i.e.,* 301-439) (Fig. 7C). Since most of the identified UBDs are small (20-150 amino acids), we tried to narrow down a smaller section of the E domain that associated to ubiquitinated species by using rational deletions of the *N*-terminal protein portion; however, we could not map the minimal UBD possibly because an intact E domain 3D folding is required. Thus, we called it the ERα-Ub-binding surface (UBS).

On this UBS, through a bioinformatic approach, we searched for the amino acids that could create an Ub-binding surface similar to that of the known UBDs. Remarkably, we found that L428, L429 and A430 residues on the ERα-UBS showed a spatial distribution reminiscent the UBD called UMI [47] (Fig. 7D). Since only the L429 and A430 residues were at least partially exposed to the solvent, we mutated them. Surprisingly, while single substitution of L429 (*i.e.,* L429A) or A430 (*i.e.,* A430G) barely affected the Ub-binding ability of E domain, double mutation (*i.e.,* LAAG) strongly prevented the E domain Ub-binding (Fig. 7E). Thus, the L429, A430 residues are the structural determinants required for ERα-UBS to non-covalently associate to Ub *in vitro*. Moreover, circular dichroism experiments demonstrated that the introduction of LAAG mutations on ERα E domain did not affect the proper domain folding (Supplemental Fig. 2).

Next, experiments were performed to evaluate if the L429,A430 double mutations (*i.e.,* LAAG) in the context of the full length ERα reduces Ub-binding also in cells. To this purpose, HEK293 cells were transiently transfected with the wt or LAAG receptor and a reduction of Ub-binding abilities of the ERα-UBS mutant was found (Fig. 9A). Thus, we report here that the residues L429 and A430 within the E domain ERα-UBS are necessary for Ub-binding both *in vitro* and in cells. Interestingly, a characterization the ERα-UBS mutant under basal conditions indicated that its intracellular localization as well as transcriptional activity (*i.e.,* ERE binding) was the same as that of the wt ERα (Figs. 9B and 9C). This means that the ERα-UBS does not play a critical role for the receptor cellular distribution or DNA binding ability. Moreover, the E2 binding affinity of ERα was not affected by the introduction of LAAG mutations (Table 1). Thus, the E2-induced LAAG ERα activities, which have been next analyzed, do not depend on an impaired binding to E2. To gain insights on the impact of the ERα-UBS on the E2-induced gene expression, we analyzed the cellular levels of the E2 target gene Bcl-2 whose transcription involve multiple enhancer elements, including that bound by ERα-Sp1 or ATF-1/CREB1 [53]. Remarkably, when we analyzed the E2-induced cellular levels of the Bcl-2 protein, we found that LAAG ERα showed a reduced ability to mediate the increase of this protein levels (Fig. 9D). These data suggest that the ERα-UBS mutant could have a transcriptional activity lower than the wt receptor in response to E2. E2-dependent productive gene transcription involves the sequential formation of macromolecular complexes centered on ERα [1, 57]. As many of the ERα binding partners are either ubiquitinated proteins or Ub-ligases [9, 10, 32], the observed reduction of E2-induced Bcl-2 expression opens the possibility that the assembly of E2-dependent ERα-based macromolecular complexes could be due to the presence of ERα-UBS, ERα monoubiquitination [30, 33] or both. Remarkably, our finding about the *in vitro* LAAG E domain reduced ability

to bind several differently linked recombinant Ub chains suggest that the mutant ERα-UBS activities in cells could be impaired at several levels. Thus, at transcriptional level, the lack of one of the expected Ub-mediated associations between ERα and its interactors could be responsible for the observed reduced expression of the Bcl-2 protein under E2 treatment.

Prompted by these results, we next evaluated the regulatory role of the ERα-UBS in E2-dependent cellular processes. To this purpose, we transfected HEK293 cells with wt and LAAG ERα and selected clones stably expressing that receptors (Fig. 10A). In these cell lines, we analyzed the E2-induced cell proliferation. As mentioned before, E2 activates several pathways to guarantee to the cells all requirements necessary to proliferate (*e.g.,* cyclins expression, cholesterol sourcing). Initially, we evaluated the ability of E2 to induce the increase of the cell number in wt and LAAG ERα expressing cells. Surprisingly, we found that the ERα-UBS mutation blocks the E2-induced cell proliferation (Fig. 10B). To strengthen our finding we analyze also the E2-triggered increase of the master regulators of the cholestrol sourcing, which is required from cells to proliferate [58]. These regulators are the HMGR enzyme, which controls the cholesterol synthesis, and the LDLr, which is responsible for the cholesterol uptake [26]. We found that the HMGR and the LDLr levels were increased by E2 in wt ERα HEK293 as well as in MCF-7 cells (Figs. 11A-D). However, when we analyzed the cellular levels of these proteins in LAAG ERα expressing HEK293 cells, we found no increase after E2 treatment (Figs. 11A-D). Thus, ERα-UBS is required for E2-induced increase of cholesterol biosynthesis and uptake, which in turn is necessary to these cells to proliferate. The E2-triggered cell proliferation has been reported to be dependent on the rapid activation of rapid signalling from the membrane ERα [4]. Thus, we evaluated the rapid signalling activation by E2 in wt and LAAG ERα cells to understand which pathway was responsible for LAAG ERα cells inability to proliferate after E2 treatment. We found

that the ERK/MAPK pathway was equally induced by E2 in both the cell lines (Figs. 10C and 10C'); thus it is not responsible for the observed blockade of cell proliferation in LAAG ERα cells. Remarkably, we also found that while the wt ERα cells rapidly activate PI3K/AKT after E2 treatment, the LAAG ERα was not able to do so (Figs. 10C and 10C'). Thus, the ERα-UBS is required to mediate the E2-triggered PI3K/AKT pathway activation, which in turn is crucial for E2-induced cell proliferation.

Interestingly, we also found that in breast cancer MCF-7 cells, the pharmacological inhibition of the PI3K/AKT pathway blocked the E2-dependent cell proliferation (Fig. 11E). These data were supported by the finding that also the E2-induced increase of the HMGR and LDLr proteins was blocked by the PI3K inhibitor (Figs. 11C and 11D). Thus, the E2-triggered PI3K/AKT control of the HMGR and LDLr expression is conserved in transfected as well as in endogenously expressing ERα cells. In addition, the LAAG ERα inability to mediate the E2-dependent increase of these proteins and proliferation can be ascribed to its impaired AKT signalling activation. All these data suggest that the ERα-UBS could be required for E2-triggered cell proliferation also in this breast cancer cell line. A possible explanation of the PI3K/AKT pathway role on E2-induced cholesterol sourcing is that it affects the ERα ability to directly {in the case of the HMGR-ERE containing promoter [23]} or indirectly {for the LDLr non-ERE containing promoter [24]} activate these protein transcription. However, it is also possible that the PI3K/AKT signalling could affect the activation of other transcription factors, like the sterol-regulatory element binding protein (*i.e.,* SREBP-2), which was found to dependent on this pathway to mediate the HMGR as well as LDLr expression in some cell lines [59].

Taken together, these data indicate that the UBS on ERα E domain plays a critical role in E2 mitogenic effects. In particular, the LAAG ERα inability to activate the PI3K/AKT pathway seems to be the principal responsible for this effect in MCF-7 cells as well as in HEK293

transfected cells. Notably, it is becoming increasingly accepted that in many normal, transformed or artificial ERα expressing cell lines the ERK/MAPK and PI3K/AKT pathways are consistently activated by E2 through a mechanism that requires the E2-dependent association of the ERα with transmembrane growth factor receptors (*e.g.,* IGF1-R) as well as with several downstream signalling proteins {*e.g.,* PELP1/Src and Shc/Src/Ras for ERK/MAPK; p85/Src/FAK and PELP1/Src/p85 for PI3K/AKT [4, 14-16]} and leads to the modulation of many physiological processes including cell proliferation [4, 17]. Thus, the impairment of the ERα-UBS mutant to associate to a putative Ub-modified interactor could be responsible for the lack of PI3K/AKT pathway activation. In addition, because we observed that in the presence of the ERα-UBS mutant E2 fails to induce AKT phosphorylation but still determines ERK1/2 activation, it is possible that the mechanism by which the E2:ERα complex transduces the intracellular signal to the activation of ERK and AKT could be potentially separable. A differential recruitment of ERα extra-nuclear Ub-dependent binding partner(s) could allow signalling specificity, as in the case of G-protein coupled receptors (GPCR) [60]. Of note, the fact that the ERK/MAPK pathway activation in response to E2 was not affected by the introduction of LAAG mutations (Fig. 10C and 10C'), beside supporting the notion that the ERα-UBS mutant binds E2 with the same affinity than the wt receptor (Table 1), further suggests that the ERα-UBS interactors may be dispensable for ERK/MAPK activation. Next, we reported that the mutation of ERα-UBS impaired the E2-induced increased association with the IGF1-R (Fig. 10D). Interestingly, the basal levels of ERα:IGF1-R interaction were similar between the wt and LAAG receptor. This evidence indicates that IGF1-R could be excluded from the ERα-UBS putative ubiquitinated interactors that under basal condition do not associate to LAAG-ERα (Fig. 9A). However, the rapid dissociation of ERα from IGF1-R suggest that another component of the membrane complex, required to stabilize ERα:IGF1-R interaction, could contact

ERα on its E domain UBS through Ub. Thus, the lack of the association between this ubiquitinated partner and ERα-UBS under E2 treatment could be responsible for the blockade of the AKT activation in LAAG cells.

One of the putative ERα-UBS ubiquitinated partner could be the AKT itself. It is ubiquitinated via K48 [61, 62] and K63 [63, 64] by different ligases in different cell types. As expected, while the K48 AKT modification addresses it to the proteasomal degradation, the K63 one codes for non-proteolytic functions. Indeed, K63-based AKT ubiquitination induced by various stimuli (*e.g.,* IGF-1) was found to be required for its membrane recruitment and activation [63, 65]. Notably, aberrant AKT activation, due to its mutation [*e.g.,* glutamate (E) 17 to K] is observed in various human cancers, and results from hyperubiquitination and hyper-PIP3 binding [65]. Even if it is still unknown how AKT K63-linked ubiquitination results in its membrane recruitment and activation, it has been proposed that this modification could enhance AKT binding to membrane bound Ub-receptors [65]. However, more studies are required to evaluate this possibility.

In addition, the UBS requirement for the E2:ERα PI3K/AKT activation could also depend on an altered association of the ERα-UBS with enzymes that promote the receptor post-translational modifications. However, protein modification with non-conventional Ub chains (*i.e.,* all but K48-linked Ub chains) is poorly known; thus, the hypothesis of putative interactors of the ERα-UBS can be made on the basis of their role in the activation of the PI3K/AKT signalling in response to E2 or their site of interaction with ERα. For example, ERα methylation by PRMT1 on Arg260 residue is necessary for the E2-induced plasma membrane interaction with the p85 and Src and for the PI3K/AKT pathway activation [28]. We can speculate that a putative PRMT1:ERα-UBS interaction could be required to the ERα methylation, which is necessary for the membrane complex assembly to PI3K/AKT activation. Thus, a crosstalk between methylation and ubiquitination pathway cannot be excluded.

In addition, the observed mutant ERα-UBS-dependent phenotype is super-imposable to the one described for the mutation in the ERα monoubiquitination sites (*i.e.,* K302R,K303R- KKRR) [34]. Indeed, in both LAAG ERα and KKRR ERα expressing cells the E2-induced activation of PI3K/AKT and cell proliferation were blocked. Moreover, these mutant receptors were similarly unable to increase IGF1-R association after E2-treatment [34]. In monoubiquitinated proteins that contain an UBD, monoubiquitination and Ub-binding are often mechanistically linked through the coupled monoubiquitination process [40]. Several functions have been associated to this process, like the recruitment of the ubiquitination machinery on the UBD, the intra-molecular UBD:Ub interaction that prevents the receptor association with other proteins or the creation of a network of an Ub-based interactions [38]. The fact that ERα is both a monoubiquitinated protein [30] and contains an UBS and our findings indicate that their mutations similarly affect the E2:ERα activities opens the possibility that the ERα-UBS could sustain the coupled monoubiquitination process. In this respect, for example ERα-UBS could direct ERα monoubiquitination.

The PI3K/AKT signalling also control the ERα activities by determining the phosphorylating the receptor in response to E2. In particular, the receptor phosphorylation on Ser118 residue is required for the recruitment of co-activators and for the assembly of the macromolecular complex necessary for E2-induced target gene expression [6]. This modification on ERα depends on the PI3K/AKT pathway activation [27]. As expected, the LAAG ERα inability to activate this pathway also prevents the receptor Ser118 phosphorylation (Fig. 12A). This data is in accordance with the previous finding on the E2-induced Bcl-2 protein expression that was reduced in LAAG respect to wt ERα cells (Fig. 9D). Moreover, also the transcription of the Cyclin D1 protein, important for the G1-S phase cell cycle progression, was blocked by the introduction of the LAAG mutations (Fig. 12B), thus

further sustaining the requirement of ERα-UBS for the E2-induced cell proliferation (Fig. 10B) as well as transcriptional activity.

In summary, the loss of ERα-UBS function impairs the E2-triggered pathways to mitogenic effects, which depend on the PI3K/AKT pathway extra-nuclear activation that controls ERα Ser118 phosphorylation, which in turn is required for the transcription of the Cyclin D1 gene into the nucleus. Although the molecular details by which the ERα-UBS associates with specific ubiquitinated proteins in the control of these ERα activities and cell proliferation have not been clarified here, these data reveals that non-covalent Ub-binding to the ERα is a critical determinant for E2:ERα cellular functions.

DNA Micro Arrays experiments definitively demonstrated that the E2-induced ERα transcriptional activity was strongly compromised by the introduction of the LAAG mutation (Figs. 13A and 13B). Indeed, wt ERα modulated 623 unique genes while the LAAG ERα only 285; only a set of 8 unrelated genes were activated by both the receptors (Table 2). However, when we validated the transcription of one of these genes (*i.e.,* CEBPB), we found that the wt ERα-mediated increase was significantly higher than that mediated by the LAAG receptor (Fig. 13C), indicating an overall reduced ability to activate gene transcription. Next, the Ingenuity Pathway Analysis was applied to these data to predict the E2-induced activation or inhibition of transcription factor in wt and LAAG ERα expressing cells.

As expected, the Ingenuity Pathway Analysis indicated ERα as a transcription factor activated by E2 in wt but not LAAG ERα expressing cells (Figs. 14A). We validated these data by analyzing the expression of some E2:ERα target genes. We found that the pS2, Cathepsin D and PR expression was induced by E2 only in wt ERα expressing cells (Fig. 14B-D). Thus, we confirmed the Micro Arrays data indicating that the ERα-UBS is required for E2-induced target genes transcription. The impaired transcriptional activity of the LAAG ERα after E2

treatment could depend on several factors; for example on the lack of ERα Ser118 phosphorylation, which impairs the co-activators recruitment. Moreover, it is also possible that the E2-dependent ERα association with the components of the transcriptional complex requires an intact UBS. In this respect, several ubiquitinated proteins and Ub-ligases have been found in these complexes [9, 10, 32] and may represent the putative ERα-UBS interactors required for both direct and indirect transcription.

The Ingenuity Pathway Analysis extrapolated also another transcription factor activated by E2 through wt but not LAAG ERα, CREB1 (Figs. 15A). CREB1 transcriptional activity depends on its phosphorylation on Ser133 residue, which is induced by different signals (*e.g.,* hormones, growth factors) after the activation of several kinases (*e.g.,* PKA, AKT, MAPK) on the basis of the cell type [54]. CREB1 phosphorylation on this residue is required for its binding to the co-activator CBP, which enables the recruitment of the transcriptional machinery on the CREB1 gene promoters and then the gene expression [66]. CREB1 directly controls the transcription of genes involved in cell proliferation (*i.e.,* Cyclin D1), cell survival (*i.e.,* Bcl-2) and metastasis (*i.e.,* VEGF) [66]; thus, it is not surprising that its iperactivation has been found in breast cancer [66, 67] as well as TAM-resistant MCF-7 cells [68]. Given the key role played by CREB1 in cancer, some compounds have just been synthesized to block the CREB1-mediated gene expression. Among the known CREB1 inhibitors there is the KG, which interferes with the CBP/CREB1 interaction [52].

Thus, we initially validated the Micro Array data by analyzing the mRNA levels of the E2-induced CREB1-mediated VEGFA gene and found that they were effectively blocked in mutant ERα-UBS cells (Fig. 15B). Next, since we also found that the KG effectively blocked the E2-induced VEGFA and Bcl-2 CREB1-mediated transcription (Fig. 15C and 15D), we decided to use it as tool to mimic the LAAG ERα transcriptional phenotype in wt HEK293 as well as MCF-7 cells. However, initial

experiments were performed to understand if the impaired activation of E2-induced CREB1-mediated gene transcription was due to a defect in its phosphorylation or to a defect in transcriptional complex components binding. We found that the CREB1 activating phosphorylation on Ser133 residue was induced by E2 in wt but not in LAAG ERα cells (Fig. 16A). Thus, the inability of CREB1 to activate the E2-induced gene expression is principally due to a lack of its activation. Next, to understand the regulatory role of the ERα-UBS in the CREB1 activation pathway we used the PI3K inhibitor LY. Remarkably, we found that the PI3K/AKT pathway contributed to the E2-induced CREB1 phosphorylation (Fig. 16B and 16B'), responsible for transcriptional activation and target genes expression (*e.g.,* Bcl-2, Cyclin D1) in wt ERα HEK293 as well as MCF-7 cells (Figs. 16C and 17B- left panels).

Thus, we identified a conserved pathway for E2-induced CREB1 activation and downstream gene transcription. Remarkably, the activation of this pathway requires an intact UBS on ERα. Finally, by blocking the E2-activated CREB1 pathway upstream or downstream through the LY and KG inhibitor, respectively, we found an inability of wt ERα HEK293 as well as of MCF-7 cells to proliferate (Figs. 16D and 17C). Thus, it is tempting to speculate that the CREB1 activation by E2 strongly depends on the ERα-UBS-based membrane complex responsible for the PI3K/AKT signalling activation.

Overall, the data reported in this PhD project indicate that the ERα possesses an UBS on its E domain that plays a critical role for E2-induced nuclear and extra-nuclear signalling pathways to cell proliferation.

Even if we found a strong reduction of the ERα non-covalent Ub-binding when the receptor UBS was mutated, our experiments were not focused on the basal receptor activities since we were more interested in the impact of the ERα-UBS on E2 mitogenic effects. In this respect, the observed LAAG phenotype may be dependent on a loss of interaction with differently Ub-modified proteins that affect ERα functions at various levels (*e.g.,* membrane signalling

complex assembly, nuclear transcriptional machinery recruitment). Given the difficulty to individually test the putative ERα-UBS interactors, a Mass Spectometry approach would be the best tool to better understand which protein(s) associates to ERα-UBS both in the presence and in the absence of E2.

Moreover, the study of a putative K6- and K63-Ub-dependent crosstalk between the A/B and E domain could help defining the role of these Ub-linked chains on E2:ERα signalling.

In conclusion, our findings open new avenues in the field of E2-activated molecular mechanisms to physiological effects that now have to include also the non-covalent Ub-binding abilities of ERα. Recent studies indicates that the initiation and progression of cancer include events that alter the Ub-system balance; thus, targeting the Ub-system can be a promising approach to fight breast cancer [41]. In this respect, one of the proposed future druggable point of the Ub-system will be the interference of the UBD:Ub interaction by using specific small molecules [37, 41]. Thus, the comprehension of the ERα Ub-binding ability and its regulatory roles on E2 mitogenic effects would be useful to design future drugs that aim to block breast cancer cell proliferation.

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APPENDIX A.
MATHERIALS AND METHODS.

Reagents.

E2, Simvastatin, G418 disulfate salt, Pen-Strep solution, L-glutamine, $CaCl_2$, Dulbecco's modified Eagle medium (DMEM) without phenol red, charcoal-stripped fetal calf serum, protease inhibitor cocktail, phosphatase inhibitor cocktail 3, bovine serum albumin fraction V (BSA), CREB1 inhibitor 2-naphthol-AS-E-phosphate (KG-501), anti-flag®M2 and anti-vinculin antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Optimem and Lipofectamine®2000 Transfection Reagent were purchased from Gibco-BRL Life technology (Gaithersburg, MD, USA). The AKT inhibitor Ly 294002 and the MAPK cascade inhibitor PD 98059 was obtained from Calbiochem (San Diego, CA, USA). Bradford protein assay was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Human recombinant ERα was obtained by Pan-Vera (Madison, WI, USA). Anti-phospho-ERK1/2, anti-ERK1/2, anti-AKT, anti-ERα (HC-20), anti-Ub (P4D1), anti-IGF1-R (C-20), anti-Cyclin D1 (H295), and anti-Bcl-2 (C-2) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-α-tubulin was purchased from MP Biomedical (Solon, Ohio, USA). The anti-phospho-ERα (16J4), anti-phospho-CREB1 (87G3), anti-CREB1 (48H2) and anti-phospho-AKT (193H12) antibodies were purchased by Cell Signalling Technology Inc. (Beverly, MA, USA). Anti-LDLr was purchased from Abcam (Cambridge, UK). Anti-HMGR was purchased from Upstate (Lake Placid, NY). The luciferase kit was obtained from Promega (Madison, WI). Chemiluminescence reagent for Western blot ECL was obtained from GE Healthcare (Little Chalfont, UK). All the other products were from Sigma-Aldrich. Analytical or reagent grade products were used without further purification.

Cells.

Human breast cancer MCF-7 cell line. MCF-7 cells were routinely grown in air containing 5% CO2 in modified, phenol red-free, DMEM medium, containing 10% (v/v) charcoal-stripped fetal calf serum, L-glutamine (2 mM), gentamicin (0.1 mg/ml), and penicillin (100 U/ml). Cells were passaged every 2 days and media changed every 2 days.

Human cervix epithelioid carcinoma (HeLa) cell line. HeLa cells were routinely grown in air containing 5% CO2 in modified, phenol red-free, DMEM medium, containing 10% (v/v) charcoal-stripped fetal calf serum, L-glutamine (2 mM), gentamicin (0.1 mg/ml), and penicillin (100 U/ml). Cells were passaged every 2 days and media changed every 2 days.

Human Embryonic Kidney (HEK) 293 cell line stably transfected. HEK293 cells were routinely grown in air containing 5% CO2 in modified, phenol red-free, DMEM medium, containing 10% (v/v) charcoal-stripped fetal calf serum, L-glutamine (2 mM), gentamicin (0.1 mg/ml), and penicillin (100 U/ml). The growth medium was additionated with G418 disulfate salt (400 µg/ml), cells were passaged every 2 days and media changed every 2 days.

Cell stimulation. Cells were simultaneously treated with vehicle and/or E2 (0.1-10 nM), the AKT inhibitor LY(1 µM), the CREB1 inhibitor KG (1-10 µM) were added 30 min before E2.

Plasmids and constructs.

The reporter plasmid 3xERE TATA and the pcDNA flag-ERα have been described [34]. The pEGFP-ERα was obtained by subcloning the ERα ORF from the pSG5-HE0 [12] into the pEGFP-C2. Site-directed mutagenesis of the ERα L429 and A430 residues were performed by using the QuikChange kit (Stratagene, La Jolla, CA) and the following oligonucleotides: 5'-GAGATCTTCGACATGCTG**GC**GGCTACATCATCTCG GTTC-3' (for single L429A mutation) on the wt ERα ORF

used as template; 5'-ATCTTCGACATGCTGCTGG**G**TACATCATCTCGGTTCCGC-3' (for single A430G mutation) on the wt ERα ORF used as template; 5' ATCTTCGACATGCTG**GC**GG**G**TACATCATCTCGGTTCCGC-3' (for double L429A, A430G mutation) on the ERα ORF L429A mutant used as template (bold underlined nucleotides differ from the wt ERα ORF). Plasmids were then sequenced to verify the introduction of the desired mutations. To engineer constructs harboring ERα deletions domains, PCR amplification was performed with the following primers: for **ABC** (3-256) 5'-GCGGATCCCGGACCCTCCACACCAAAGCATC-3' (forward) and 5'-CCGCTCGAGCGGGTCTTTTCGTATCCCACCTTT-3' (reverse), for **C** (181-256) 5'-GCGGATCCCGGGAGACTCGCTACTGTGCAGTG-3' (forward) and 5'-CCGCTCGAGCGGGTCTTTTCGTATCCCACCTTT-3' (reverse), for **E** (301-547) 5'-GCGGATCCCGGTCTAAGAAGAACAGCCTGGCC-3' (forward) and 5'-CCGCTCGAGCGGGTGGCTTTGGTCCGTCTCCTC-3' (reverse), for **AB** (3-180) 5'-GCGGATCCCGGACCCTCCACACCAAAGCATC-3' (forward) and 5'-CCGCTCGAGCGGCACTGCACAGTAGCGAGTCTC-3' (reverse), for **ERα 301-439** 5'-GCGGATCCCGGTCTAAGAAGAACAGCCTGGCC-3' (forward) and 5'-CCGCTCGAGCGGATTCATCATGCGGAACCGAGA-3' (reverse), for **ERα 439-547** 5'-GCGGATCCCGGAATCTGCAGGGAGAGGAGTTT-3' (forward) and 5'-CCGCTCGAGCGGGTGGCTTTGGTCCGTCTCCTC-3' (reverse).

PCR products were then cloned into pGEX-6P-3 using BamHI/XhoI sites. All constructs were sequence verified.

GST pull-down assays and circular dichroism.
GST fusion proteins were expressed, purified and as described in [46] except that all ERα-E domain encoding constructs were prepared in the presence of 20 μM E2. Pull-down experiments were performed as previously reported [34, 69] by incubating 30 μg of GST-fusion proteins with either 300 μg of growing HeLa cells total lysate for 3 hours at 4°C or with 0.5 μg of polyUb2-7 linked by K48 and K63 (Boston Biochem) or with 0.5 μg of di-Ub linked by K6, K11, K27, K29, K33 for 1 hour at 4°C in the presence of 20 μM E2. All experiments were normalized by running 1/10 of the pull-down on an SDS-PAGE gel. Proteins were detected by Comassie Brilliant Blue staining (Com.). For circular dichroism analysis GST-fusion proteins were eluted and recovered from the beads by treatment with 20 mM free glutathione in 50 mM Tris HCl (pH 9) buffer. The elution buffer, which is unsuitable for spectral measurement in the far UV region, was exchanged by dialyzing the proteins (two times) against 100 volumes of 25 mM sodium phosphate (pH 7.5), 150 mM NaF at 4°C, for 10 hours.

UV absorption spectroscopy.
The molar extinction coefficients at 280 nm (ε_{280}) of GST-wt and GST-LAAG fusion proteins (476 residues, 54.8 kDa), calculated by the method of Gill and von Hipple [70], resulted to be 65,110 $cm^{-1}M^{-1}$. The proteins were centrifuged (16°C, 10 minutes, 15,000 rpm) to remove traces of scattering particles, and their absorption spectra were recorded on an Agilent HP8453 diode array spectrophotometer, using 1 cm quartz cell. Samples were perfectly transparent at all wavelengths greater than 300 nm. The protein concentrations were measured by reading the absorption at 280 nm.

Circular dichroism measurements.
CD spectra in the far-UV region were recorded at 16°C using a Jasco J-600 spectropolarimeter and 0.05 cm quartz cells. GST-wt and GST-LAAG fusion proteins were

measured at the concentration of 0.075 mg/ml. For each sample, nine sequential spectra were recorded and averaged. Data are shown, without smoothing, in terms of mean molar ellipticity per residue. Measures were performed in collaboration with Dr. Pasquale Stano (University of Roma TRE- Rome).

E2 Binding Assays.
In vitro ERα affinity (as indicated by the IC_{50} value) was determined using the HitHunter EFC assay (Discoverx) according to manufacturer instructions for E2 towards either recombinant ERα protein (ERα Rec), wild-type or LAAG mutant receptors (ERα wt or ERα LAAG, respectively) extracted from transfected HEK293 cells. Kit indicates the value of E2 affinity to ERα as reported in the manufacturer's specification, and has been introduced as a positive control reference; the value is similar to that measured with ERα Rec (Panvera).

Transient transfection and luciferase assay.
HeLa cells were grown to ~70% confluence and then transfected with pcDNA-flag-ERα plasmid using Lipofectamine®2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Three hours after transfection, the medium was changed and 24 hours after the cells were 24 hours serum starved and then stimulated with either vehicle and E2 (10 nM). 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 as previously described [12].

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

$Na_2HPO_4x7H_2O$ 1.5 mM). Sixteen hours after trasfection medium was changed and the selection antibiotic was added. In particular, HEK293 cells stably expressing ERα were generated by using G418 (400 μg/ml), as previously reported [34]. For the ERα LAAG three individual clones were selected on the basis of the wt ERα expression levels and with similar wt ERα growth rate. Experiments are shown for one of HEK293 ERα LAAG expressing clone and for the already characterized wt ERα clone [34].

RNA isolation and quantitative Real-Time PCR (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 cyclin D1 5'-AACTACCTGGACCGCTTCCT-3' (forward) and 5'-CCACTTGAGCTTGTTCACCA-3' (reverse), for CEBPB 5'-TACTACGAGGCGGACTGCTT-3' (forward) and 5'-CGTAGTCGTCGGAGAAAGAGG-3' (reverse), for human presenilin 2 (pS2) 5'-CATCGACGTCCCTCCAGAAGAG-3' (forward) and 5'-CTCTGGGACTAATCACCGTGCTG-3' (reverse), for human cathepsin D 5'-GTACATGATCCCCTGTGAGAAGGT-3' (forward) and 5'-GGGACAGCTTGTAGCCTTTGC-3' (reverse), for human progesterone receptor (PR) 5'-AAATCATTGCCAGGTTTTCG-3' (forward) and 5'-TGCCACATGGTAAGGCATAA-3' (reverse), for VEGFA 5'-AGGCCAGCACATAGGAGAGA-3' (forward) and 5'-TTTCTTGCGCTTTCGTTTTT-3' (reverse), 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 gene expression levels, cDNA synthesis and qPCR were performed using the GoTaq two-step RT-qPCR system (Promega, Madison, MA, USA) in a ABI Prism 7900HT Sequence Detection

System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Each sample was tested in triplicate and the experiment repeated twice. All primers used were optimized for real-time amplification in a standard curve amplification (>98% for each pair of primers) and the production of a single amplicon was verified in a melting curve assay. Results were normalized for GAPDH mRNA levels. The relative level for each gene reported in arbitrary units, was calculated using the $2^{-\Delta\Delta Ct}$ method.

DNA Micro Arrays.
Total RNA was extracted from cells using the RNAese extraction kit (Quiagen) according to the manufacturer's instructions.
The Micro Array experiment, including quality control of the total RNA conducted by Agilent Bioanalyzer 2100, was performed by Congentech (IFOM, Milan)- cod YYAFFA1. Briefly, preparation of 1 biotinylated cDNA from at least 500 ng of total RNA. control of the synthesis reaction efficacy and fragmentation procedure. Hybridization, washing, labeling and image acquisition of 1 GeneChip arrays Gene ST Human 1.0. Analysis were performed in collaboration with Dr. Fabrizio Bianchi (IEO- Milan).

Western Blot Assays.
After treatments, cells were lysed in YY buffer (50 mM HEPES at pH 7.5, 10% glycerol, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA) plus protease and phosphatase inhibitors. Total proteins were quantified using the Bradford protein assay. Solubilized proteins (20 μg) were resolved by 7 or 10% SDS-PAGE at 100 V for 1 h at 25°C and then electrophoretically transferred to nitrocellulose for 7 min at 25 V by using Trans Blot Turbo Transfer System (Biorad Laboratories, Hercules, CA, USA). The nitrocellulose was blocked with 5% non-fat dry milk in TBS-T solution (150 mM NaCl, 50 mM Tris HCl (pH 8.0), 0.1% (w/v), Tween-20) for 1 hour at RT and then probed overnight at 4°C with either anti-ERα HC-20 (final

dilution 1:1000), anti-flag®M2 (final dilution 1:5000), anti-phospho Ser118-ERα (final dilution 1:1000), anti-cyclin D1 (final dilution 1:500), anti-phospho-ERK1/2 (final dilution 1:200), anti-ERK1/2 (final dilution 1:1000), anti-phospho-AKT (final dilution 1:1000), anti-AKT (final dilution 1:1000), anti-Bcl-2 (final dilution 1:500), anti-phospho-CREB1 (final dilution 1:1000), anti-CREB1 (final dilution 1:1000), anti-IGF1-R (final dilution 1:500), anti-SREBP-2 (final dilution 1:1000), anti-LDLr (final dilution 1:1000) and anti-HMGR (final dilution 1:1000) antibodies. The nitrocellulose was also probed with anti-α-tubulin (final dilution 1:10000) or anti-vinculin (final diluition 1:10000) to normalize total lysate. The PVDF was blocked with 5% bovine serum albumin (BSA) in TBS-T solution for 1 hour at RT or overnight at 4°C and then probed 3 hours at RT with anti-Ub (final diluition 1:1000) antibody. Moreover, the nitrocellulose incubated with either anti-phospho-ERK1/2 and anti-phospho-AKT was stripped by Restore Western Blot Stripping Buffer (Pierce Chemical, Rockford, IL, USA) for 10 min at room temperature and then probed with anti-ERK1/2 (final dilution 1:200) and anti-AKT (final dilution 1:100) respectively. Antibody reaction was visualized with ECL chemiluminescence. Next, filters were washed twice in TBS-T for 10 minutes and then incubated with the anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody diluted in TBS-T for 60 minutes at RT. After incubation with the secondary antibody, the filter was washed three times in TBS-T (5 minutes each) and the bound secondary antibody was visualized with ECL chemiluminescence. Densitometric analyses were performed by ImageJ software for Microsoft Windows.

Confocal Microscopy Analysis.

HeLa cells were transiently transfected with the pEGFP-ERα wt or the pEGFP-ERα LAAG mutant kept in growing conditions. Cells were processed in chamber slides and rinsed with PBS, pH 7.4, followed by fixation in formaldehyde 4% (v/v) for 1 hour. Following extensive

washes coverslips were mounted using Prolong® Gold anti-fade reagent and confocal analysis (63x magnification) was performed using LCS (Leica Microsystems, Heidelberg, Germany).

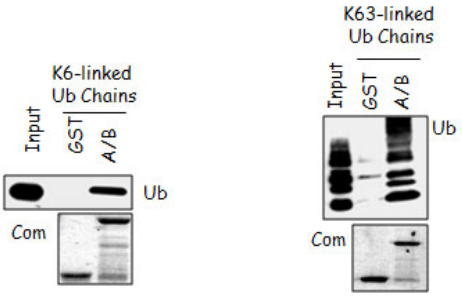
Cell proliferation analysis.
Growth curves and cell proliferation analysis were performed as previously reported [34].

Co-immunoprecipitation and ERα non-covalent Ub-binding.
After stimulation, HEK293 cells transfected with wt or LAAG ERα were lysed in YY buffer plus protease inhibitor. Total proteins were quantified using the Bradford protein assay. Next, co-immunoprecipitation were performed as previously reported [34].
For ERα non-covalent Ub-binding immunoprecipitation, 0.05% SDS as well as 20 μM E2 were added to cell lysates before immunoprecipitation and freeze (-80°C)/thawing (RT) cycles of 5 minutes were repeated twice to reduce the amount of receptor interactors. Next, equal amounts (250 μg) of soluble cell extracts were incubated with 15 μg of anti-ERα (HC-20) at 4°C for 1 h, then 50 μl of protein G sepharose beads were added and samples incubated for 1 hour on a rocking platform at 4°C. Samples were centrifuged at 10000 g for 1 min, the supernatant was removed completely and beads (pelleted) were washed 3 times with 1 ml of RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholat, 5 mM EDTA, 1 mM EGTA) containing 0.01% SDS.
SDS-Reducing 4x Laemli buffer (30 μl) was added and samples were boiled at 100°C for 5 min. 9/10 of total immunoprecipitated were resolved using 7% SDS-PAGE at 100 V for 1 hour and then electrophoretically transferred to PVDF for 7 min at 25 V by using Trans Blot Turbo Transfer System (Biorad Laboratories, Hercules, CA, USA). 1/10 of total immunoprecipitated were resolved using 7% SDS-PAGE at 100 V for 1 hour and then electrophoretically transferred to nitrocellulose for 7 min at

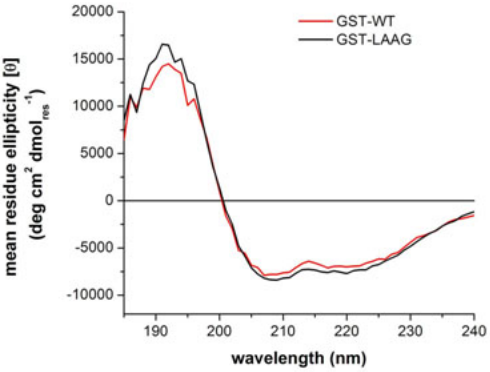
25 V by using Trans Blot Turbo Transfer System.
Both filters were treated with 5% (w/v) non-fat dry milk in TBS-T solution (150 mM NaCl, 50 mM Tris HCl (pH 8.0), 0.1% (w/v), Tween-20) and then the PVDF was probed at RT 3 hours with anti-Ub (1:1000) antibody while the nitrocellulose was probed at 4°C overnight with anti-ERα (HC-20) (1:1000) antibody to normalize the immunoprecipitate. The antibody reaction was visualized with the ECL chemiluminescence reagent for Western blot.

Statistical Analysis.
A statistical analysis was performed by using ANOVA followed by Tukey-Kramer post-test with the GraphPad InStat3 software system (GraphPad Software Inc., San Diego, CA). In all analyses, $p<0.01$ was considered significant, but for densitometric analysis $p<0.05$ was considered significant. Data are means±s.d. of three independent experiments.

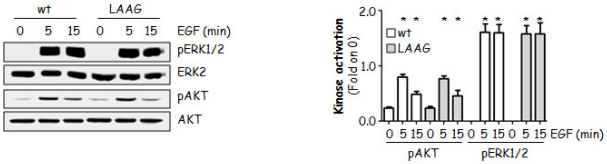
APPENDIX B.
SUPPLEMENTAL FIGURES.



Supplemental Figure 1. Ub-binding ability of the ERα A/B domains. *In vitro* pull-down assays using the GST-tagged ERα A/B domains. GST-fusion proteins were incubated with synthetic polyUb2-7 K63-linked chains, or K6-linked di-Ub and analyzed in immunoblot as indicated.



Supplemental Figure 2. Structure of wt and LAAG E domain. Circular dichroism spectra of GST-WT and GST-LAAG fusion proteins in 25 mM sodium phosphate (pH 7.5), 150 mM NaF, 16 °C. The spectra, which overlap almost perfectly at all wavelengths, have the typical shape of α-helix rich proteins, strongly supporting the fact that both fusion proteins are well-folded.



Supplemental Figure 3. The role of ERα-UBS on EGF-induced signalling. Western blot and relative densitometric analysis of AKT and ERK1/2 phosphorylation in HEK293 stable cells expressing the pcDNA-flag-ERα (wt) and the pcDNA-flag-ERα LAAG treated with EGF (1 μg/ml) at the indicated time points. * indicates significant differences with respect to (–) wt sample; ° indicates significant differences with respect to (+ E2) wt sample ($p<0.01$).

APPENDIX C.
LIST OF THE PEER REVIEWED PUBLICATIONS.

Pesiri V, Totta P, Marino M, Acconcia F. The role of endocytic pathways on estrogen receptor α intracellular trafficking and 17β-estradiol signalling. IEMAMC 2014;14(2). 10.2174/1871522214666141029234030.

Pesiri V, Totta P, Marino M, Acconcia F. Ubiquitin-activating enzyme is necessary for 17β-estradiol-induced breast cancer cell proliferation and migration. IUBMB Life 2014;doi: 10.1002/iub.1296.

Totta P°, **Pesiri V°**, Marino M, Acconcia F. Lysosomal function is involved in 17 β-estradiol-induced estrogen receptor α degradation and cell proliferation. PLOSone 2014;9(4): e94880. doi: 10.1371/journal.pone.0094880. (°**Co-first Authorship).**

Pesiri V, La Rosa P, Stano P, Acconcia F. Identification of an estrogen receptor alpha non-covalent ubiquitin binding surface: role in 17beta-estradiol-induced transcriptional activity. Journal of Cell Science 2013;126:2577-82.

La Rosa P, **Pesiri V**, Leclercq G, Marino M, Acconcia F Palmitoylation regulates 17β-Estradiol-induced Estrogen Receptor α Degradation and Transcriptional Activity. Molecular Endocrinology 2012;26(5): 762-74.