# REGULATION OF ESTROGEN RECEPTOR-ALPHA UBIQUITINATION AND PROTEASOME-MEDIATED RECEPTOR DEGRADATION

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supplied the shouts of glee for me if my mode of celebration was simply an exhaled sigh of relief. I thank you for your constant voice of encouragement; “stay the course” and “you’re doing good things” have been words that have elevated my spirits. I thank Josh for providing a soundboard for ideas and a healthy scale in which to weigh the balance between school, science, and life. I thank Bizzle for providing laughter and perspective on progress, success, and celebration. I thank Gma and Gpa Wagner for the great conversations about school and life while enjoying the sun in Phoenix. To my great-aunt Jeanie, Uncle Tommy, and those other people in my life who have been touched by cancer, as fighters, survivors, or those who have lost their battle with cancer, you have continued to inspire me and encourage me to re-double my efforts towards understanding cancer biology.

**ABSTRACT**

**Nicholas Brandon Berry**

**Regulation of Estrogen Receptor-alpha (ERα) Ubiquitination and Proteasome-mediated Receptor Degradation**

Breast cancer is the most common cancer among women, and the majority (~70%) express estrogen receptor-alpha (ERα), thereby exhibiting estrogen-dependent growth. Antiestrogen therapies block ERα-mediated cell growth, either by blocking ERα function or by triggering ERα degradation. ERα is recognized for degradation by the 26S proteasome through the addition of ubiquitin protein tags onto ERα lysine residues. However, the specific receptor lysines that are ubiquitinated have not been identified. Two receptor lysines, K302 and K303, located in the hinge-region of ERα, serve multiple regulatory functions, and we examined whether these residues might also regulate receptor ubiquitination or are targets themselves for ubiquitination. An ERα protein was generated that contained lysine-to-alanine substitutions at these two residues. Comparisons were made between the unmodified ERα (wtERα) and the mutant receptor ERα-K302A, K303A (ERα-AA). The effect of the proteasome inhibitor MG132, Hsp90 inhibitor geldanamycin (GA), and ER ligands 17β-estradiol (E2), tamoxifen (OHT), and the pure anti-estrogen ICI 182,780 (ICI), were examined for their effect on receptor ubiquitination, degradation, and receptor activity. In the absence of ligand, ERα-AA displayed rapid ubiquitination and degradation due to elevated association with the ubiquitinylation enzyme CHIP and the proteasome-associated cochaperone Bag1. E2 or ICI induced rapid degradation of wtERα; however, ERα-AA was less efficiently degraded by these ligands. Furthermore, ERα-AA was also resistant to ICI-induced

ubiquitination, suggesting that these lysines are ubiquitinated in response to the antiestrogen. ERα-AA activity was decreased in the unliganded state and elevated in response to E2, concordant with receptor stability in these two states. These data provide the first evidence that K302/303 protect ERα from basal degradation and are necessary for efficient E2 and ICI-induced turnover in breast cancer cells, revealing a previously unexplored mechanism for regulating ERα stability and activity.

_____ KPN

_____ RMB

_____ JGF

_____ BJM

_____ HN

## TABLE OF CONTENTS

**LIST OF FIGURES**

## ABBREVIATIONS

| | |
|---|---|
| **αERKO** | estrogen receptor-alpha knock-out mouse |
| **AI** | aromatase inhibitor |
| **AIB1** | Amplified In Breast cancer 1 |
| **βERKO** | estrogen receptor-beta knockout mouse |
| **C4-12 cells** | ER-negative derivative of MCF7 cells |
| **CAT** | chloramphenicol acetyltransferase |
| **ChIP** | chromatin immunoprecipitation |
| **CHIP** | Carboxyl terminus of Hsc70-interacting protein |
| **CHIPi** | CHIP-siRNA expression construct; |
| **CHX** | cycloheximide |
| **CMV** | cytomegalovirus |
| **CS-FBS** | dextran-coated charcoal-stripped fetal bovine serum |
| **DMSO** | dimethyl sulfoxide |
| **E2** | 17β-estradiol |
| **EGFR** | epidermal growth factor receptor |
| **ERα** | estrogen receptor-alpha |
| **ERα-AA** | ERα-K302A,K303A |
| **ERα-RR** | ERα-K302R,K303R |
| **ERβ** | Estrogen receptor-beta |
| **ERE** | estrogen response element |
| **FRAP** | fluorescence recovery after photobleaching |
| **GAPDH** | glyceraldehyde-3-phosphate dehydrogenase |
| **HA** | hemagglutinin |
| **Hsp** | heat shock protein |
| **ICI** | ICI 182,780 |
| **LBD** | ligand binding domain |
| **Luc** | firefly luciferase |
| **LC-MS** | liquid chromatography coupled to mass spectrometry |
| **NLS** | nuclear localization sequence |
| **NR** | nuclear receptor |
| **OHT** | 4-hydroxytamoxifen |
| **PEST** | proline (P); glutamic acid (E); serine (S); threonine (T) |
| **PROTAC** | Protein Targeting Chimeric Molecules |
| **RT-qPCR** | Reverse transcriptase quantitative polymerase chain reaction |
| **SCF** | Skp1-cullin-F-box protein |
| **SRC** | steroid receptor coactivator |
| **SERD** | Selective Estrogen Receptor Downregulator |
| **SERM** | Selective Estrogen Receptor Modulator |
| **siRNA** | small interference RNA |
| **wtERα** | wild type estrogen receptor alpha |

## REVIEW OF ESTROGEN RECEPTOR LITERATURE

*Discovery of estrogen action*

Dr. Elwood Jensen pioneered the basic understanding of the molecular mechanisms of estrogen action, overturning conventional understanding of estrogen action and paving the way for the discovery of the nuclear receptor family. Traditionally, biochemists thought a hormone entered a cell, where a series of oxidation and reductions reactions with estrogen provided needed energy for the growth stimulation and other specific actions shown by estrogens. Using high specific-activity radiolabeled $H^3$-17β-estradiol, Jensen revealed the specific localization of estrogen to reproductive tissues as well as the binding of the steroid hormone to an immune complex termed "estrophilin" (1, 2). Jack Gorski and others revealed that this binding substance, with which estradiol associated without a chemical change, is a true receptor, and was the first steroid hormone receptor to be recognized (6, 7). These receptors were shown to be macromolecules that could be extracted from reproductive tissues which, when bound by estradiol, migrated to the nucleus where they activated specific genes by stimulating new RNA synthesis (7, 8).

Using Jensen's pioneering radiolabeling technique, the presence of estrogen receptors in clinical samples and breast cancer cells was able to be determined. It had been known for decades that about one-third of premenopausal women who had advanced breast cancer would respond to estrogen blockade brought about by removing their ovaries, the source of estrogen, but there was no way to predict which women would respond. ER-rich breast cancers were shown to be more likely to respond to endocrine ablation than ER-poor breast cancers (9). To address this challenge, Dr. Jensen and

Geoffrey Greene, at the University of Chicago's Ben May Institute, developed polyclonal (10) and monoclonal antibodies (9, 10) directed against ER, which enabled them to quickly and accurately detect estrogen receptors in breast and other tumors. This test transformed the treatment of breast cancer patients, allowing for rapid immunohistochemical determination of breast cancer patient tumor ER status and thus the use of hormonal therapy. This principle remains the basis of determining ER status today.

*Estrogen*

The hypothalamic-pituitary-gonadal (HPG) axis directs integrated function among the endocrine organs to coordinate growth, development, and preparation for reproduction (11). GnRH from the hypothalamus stimulates the anterior pituitary to secrete FSH and LH, which act on the ovary to promote folliculogenesis and the concomitant synthesis of 17β-estradiol (E2) (12). The naturally occurring estrogens 17β-estradiol (E2), estrone (E1), and estriol (E3) are all C18 steroids derived from cholesterol. After binding to lipoprotein receptors, cholesterol is taken up by steroidogenic cells, stored, and used for steroid synthesis. Synthesis of endogenous estrogens is necessary for the development and regulation of reproductive systems, the maintenance of bone density, the modulation of cardiovascular system and lipid metabolism. Estrogens, as endocrine hormones, circulate systemically and diffuse into target tissues to exert their effects by interacting with steroid receptors, specifically estrogen receptors alpha and beta (ERα and ERβ). In the serum, E2 reversibly binds to sex-hormone-binding globulin, a β-globulin, while about 2 to 3 percent of estradiol is free (13). The free hormone is capable of diffusion across cell membranes.

## ESTROGEN RECEPTORS

ERα and ERβ are members of the steroid/nuclear receptor (NR) superfamily that includes over 150 members. This family shares a highly conserved structure and common mechanisms affecting transcription of a multitude of target genes in response to specific physiological and pathological signals (14). The NR superfamily includes class I NRs, the steroid receptors: glucocorticoid, mineralocorticoid, progesterone, estrogen, and androgen (GR, MR, PR, ER, and AR), and the class II NRs: retinoic acid receptor, retinoid X receptor, vitamin D receptor, thyroid receptor, and peroxisome proliferator activated receptor (RAR, RXR, VDR, TR, and PPAR). NRs function as dimeric transcription factors; class I NRs dimerize with themselves, while class II receptors typically dimerize with RXR. The NR superfamily also includes numerous "orphan receptors", known as such because their endogenous ligands are unknown. NRs act as intracellular transcription factors that, on association with ligand, bind to hormone-responsive target genes to modulate their expression.

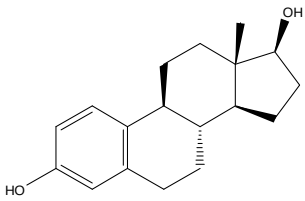
ERα (http://www.genenames.org/data/hgnc_data.php?match=ESR1) was first cloned from human breast cancer cells in 1986 (15), while ERβ (http://www.genenames.org/data/hgnc_data.php?match=ESR2) was discovered 10 years later (16). These two receptor subtypes vary in structure, and their encoding genes are on different chromosomes. ERα been mapped to chromosome 6 at the boundary between 6q24 and 6q27. ERβ maps to chromosome 14 at locus q23.2. The NR family shares multiple conserved functional domains and they exert their effects using relatively similar mechanisms.

17β-estradiol (E2) is the cognate ligand for ERs, and has the highest endogenous ligand binding coefficient ($K_d$) ~0.28 nM (17) (*Figure R1*). Both estrone and estriol bind ERs but with slightly lower affinity compared to E2 (18). 4-hydroxytamoxifen, the active metabolite of tamoxifen, has 100-fold greater affinity for ERα than E2, and directly competes with E2 for ERα binding (19). As ERα and ERβ share little homology in the ligand-binding domain (20), some ligands bind to the two receptors with different affinities. For example, raloxifene binds with higher affinity to ERα whereas several environmental pollutants, such as the alkylphenols, have a higher affinity ERβ (21). The short-acting 17α-estradiol and the biologically weak estrone have a higher affinity for ERα, while the majority of phytoestrogens bind with higher affinity to ERβ (22). The biological responses to phytoestrogens are therefore believed to be exerted through ERβ signaling (21).

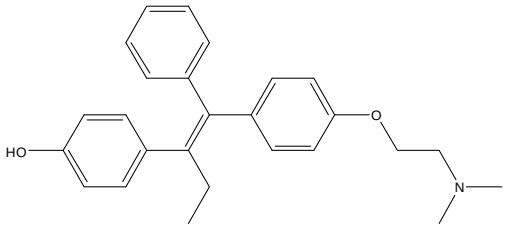
*ERα Structure*

The ERα protein contains six domains, A–F, which are conserved to varying degrees among members of the NR superfamily (23) (*Figure R2*). The N-terminal A/B domain is the least conserved among all members and demonstrates only 17% identity between human ERα and ERβ (24). This region contains a constitutive (ligand-independent) transcriptional activation function (AF1), the activity of which is regulated by growth factors via signal transduction cascades. The C domain is the most highly conserved among the different members of the family. This region encodes two zinc fingers that possess 97% homology between the ERα and ERβ genes (24, 25). These zinc fingers form a helix-loop-helix motif and function to bind ERs tightly to specific DNA sequences called estrogen response elements (ERE). The sequence of the consensus ERE element is

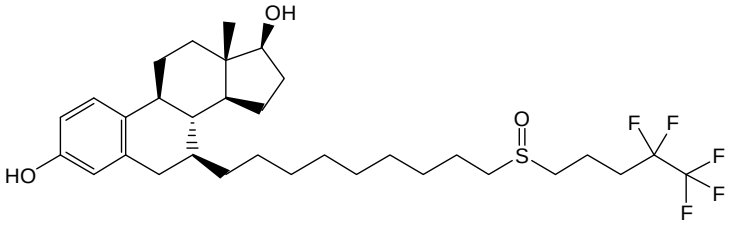
**1. 17β-estradiol (E2)**




**2. 4-hydroxy tamoxifen (OHT)**




**3. ICI 182,780 (ICI; Fulvestrant; Faslodex ®)**




**4. Raloxifene**

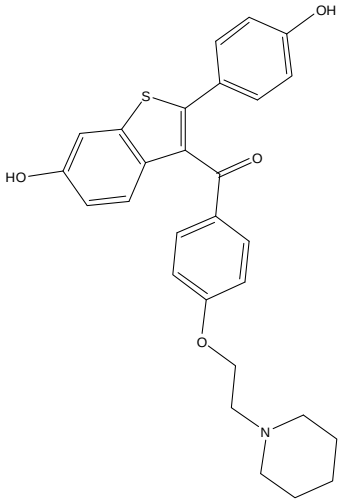



**Figure R1. Estrogen Receptor ligands**

1. The endogenous ER ligand 17β-Estradiol
2. SERM 4-hydroxytamoxifen
3. Pure antiestrogen (SERD) ICI 182,780
4. SERM raloxifene

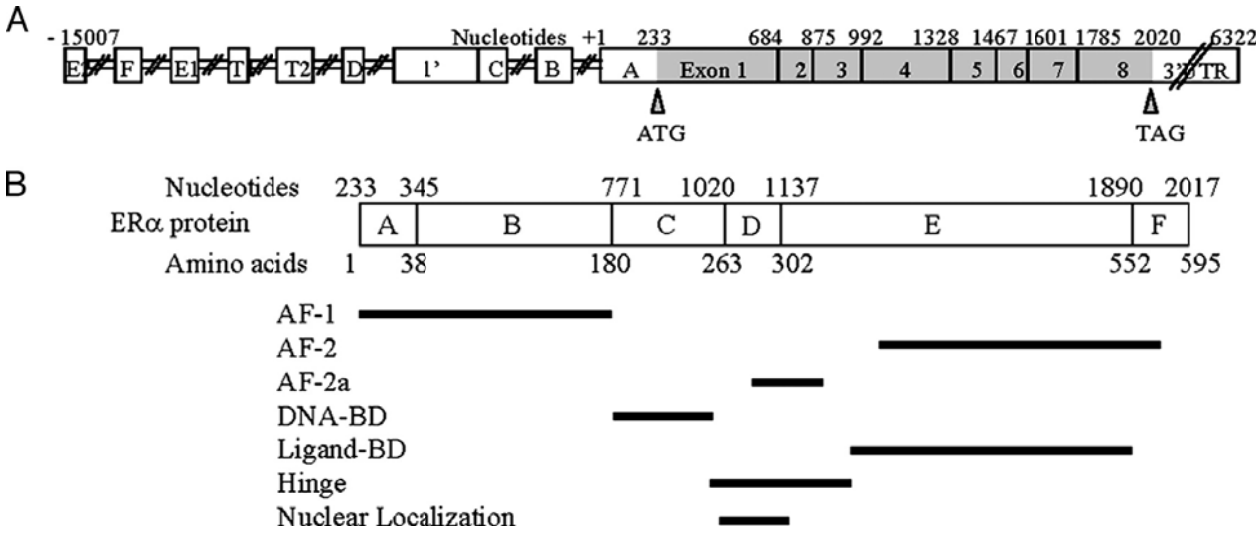



**Figure R2. ERα Domains.** A, The mRNA sequence of ERα. Alternative promoters are shown to the left of +1. The shaded box shows the ERα coding region. Exons are numbered in the corresponding blocked region with the nucleotide number above. ATG start codon and the TAG stop codon are shown below. B, The protein domains are labeled A–F, nucleotide numbers corresponding to the start of each domain are above, with amino acid numbers below. DNA sequence is shortened as indicated by // marks. Relative positions of some of the known functional domains are represented by solid bars below. BD, Binding domain Adapted from Herynk *et al.* Endocr Rev 2004 (4).

GGTCAnnnTGACC (26). As both ERα and ERβ bind to the same ERE, it is not suprising that they share such high homology in the DNA binding domain.

The D domain, or hinge-region, separates the DNA binding domain from the ligand binding domain. This region contains sequences for receptor dimerization and three prototypical nuclear localization sequences (pNLS) (27, 28). Although the D domain possesses high homology throughout the nuclear localization sequences for most nuclear receptors, the overall homology of this region is only 30% (24). The hinge region interacts with nuclear coregulatory proteins including L7/SPA, NCoR, and SMRT (29). Recent reports have placed several post-translational modifications at the hinge-region, including acetylation of ERα hinge-region lysines K266 and K268 (30), and K299, K302, K303 (31).

Sumoylation is also reported to occur on these same residues (32). Phosphorylation of S305 has been shown to regulate receptor acetylation at lysine K303 (33), revealing multiple mechanisms for receptor regulation in this region. A recent report has revealed monoubiquitination of K302 by BRCA1/BARD1 (34), adding further complexity to this regulatory region. The regulatory role of hinge-region lysines 302/303 on receptor ubiquitination and degradation serves as the basis of my thesis work.

The E domain contains the ligand-dependent activation function domain (AF-2) domain, which includes the ligand binding domain (LBD). The LBD is a compact structure consisting of 12 α-helices with a pocket into which the ligand fits (35, 36). Amino acids within the LBD interact with ligands, securing them within the pocket. Ligand binding alters the conformation of the LBD, bending helix 12 (H12), forming a lid over the pocket and trapping the ligand in a hydrophobic environment. The precise

positioning of H12 over the ligand binding pocket is dictated by the nature of the bound ligand (35). The position of H12 also determines whether ERα will be activated or repressed by the bound ligand. In this manner, H12 functions as a molecular switch, sensing the nature of the receptor ligand and translating this information into a receptor conformation that triggers a cellular response to the altered receptor structure (35, 36). For example, when E2 is the ligand, H12 is rotated into a position that exposes a surface on the LBD with which coactivator proteins interact, resulting in receptor-induced transactivation. On the contrary, antiestrogens like OHT or ICI, when bound to ERα, position H12 in conformations that occlude the coactivator recognition domain, resulting in transcriptional repression. These aspects of ligand binding and interaction with H12 make this α-helix indespensible for AF2 function (37). The ligand-binding domain best serves to distinguish ERα and ERβ, as only 55 percent of the amino acid sequence is shared within this region (24). However, each binds estradiol with nearly equal affinity, although xenoestrogens and synthetic ligands have differential binding affinities (22).

The C-terminal F domain is unique to ER among the nuclear receptors (38), but is not well conserved among the ERs of different species nor between the ERα and ERβ, sharing only ~18% homology (24). Studies using truncated ERα mutants (missing the C-terminus) have indicated a role for the F domain in modulating transcriptional activity of ERα when complexed with mixed agonist/antagonist ligands, suggesting this region may function alongside helix 12 to influence coregulator protein recruitment. This region may also facilitate receptor dimerization (39-41) or cross talk with signal transduction pathways (42). The F domain possesses a putative PEST (proline (P); glutamic acid (E);

serine (S); threonine (T)) sequence, which is thought to promote protein degradation. However, the ERα PEST sequence does not appear to influence receptor turnover (43).

*ERβ*

Studies with mice lacking ERα (ERα knock-out mice; αERKO mice) have shown that ERα but not ERβ is essential for proliferation in mammary gland, uterus, and prostate (44, 45). ERβ has been shown to antagonize the stimulation by ERα, (46) revealing a ying-yang relationship for these two receptors. ERα typically drives cell proliferation while ERβ influences differentiation of dividing cells (46). Reproductive-related cancers typically overexpress ERα, revealing the importance of maintaining an appropriate balance of ERα to ERβ (47). ERβ-selective agonists are considered anti-cancer compounds (phytoestrogens, etc), further suggesting ERβ signaling is necessary to offset ERα-mediated cell proliferation.

Perhaps the most significant disparity between ERα and ERβ is the tissue distribution. Although there is some overlap, the endometrium, mammary gland, testis, pituitary, liver, kidney, heart, and skeletal muscle contain mostly ERα, whereas ERβ transcripts are significantly expressed in in many nonclassic estrogen target tissues, including the kidney, intestinal mucosa, lung parenchyma, endothelial cells, and prostate gland (22, 24, 48, 49). Relatively equal and overlapping expression of ERα and ERβ is seen in the epididymis, thyroid, adrenals, bone, and various regions of the brain (22, 48, 50).

*Membrane-bound ERα*

In addition to conventional nuclear receptors, ERα can be anchored to the cell membrane. Membrane-bound ERα originates from the same gene as nuclear ER (51), and through palmitoylation of ERα C447, ERα is inserted into plasma membrane caveoli (52). These receptors cannot translocate to the nucleus but can elicit rapid, non-genomic actions through the cytosol. In breast cancer and endothelial cells, nuclear and membrane ERs were found to be the same proteins, providing evidence that classical ERα mediates rapid signals induced by E2 in these cells (53).

Alternativly, reports suggest that G protein-coupled estrogen receptor 1 (GPR30), an orphan receptor, is the membrane-bound ERα, and though it has high affinity for E2 and antiestrogens, it is otherwise unrelated to ERα (54). GPR30 is structurally unrelated to ERα as it is a 7-transmembrane protein that functions as a G-protein coupled receptor and therefore localized to the plasma membrane (55, 56), or the endoplasmic reticulum (57). This membrane-bound ERα has been suggested to be responsible for the numerous rapid effects of E2 on a number of target tissues, including vasodilation of blood vessels, calcium signaling in bone (58), and induction of numerous secondary messenger molecules such as cyclic AMP, $IP_3$, and phospholipase C (58).

## ESTROGEN ACTION ON TARGET TISSUES

The influence of estrogen is not restricted to reproduction and reproductive tissues. Estrogen is synthesized and secreted as endocrine steroid hormones, thereby exerting systemic effects, both positive and negative, on on hormone-sensitive tissues (*Figure R3*).

*Fetal development*

The female reproductive tract is the default phenotypical sex and will differentiate and develop normally in the absence of ovaries and adrenal glands (67). Therefore, estrogen is not required for differentiation and initial development of the female reproductive tract, whereas testosterone is critical to differentiation of the male genitalia (68, 69). Estrogen receptors are expressed during development of fetal brains of both males and females, however estrogens are only produced in fetal males due to aromatization of testosterone secreted by the neonatal testis at specific stages during development (70). Activation of ERα in males leads to neural development and differentiation resulting in morphological and biochemical sex differences (71). Exposure of females to exogenous estrogen during development also masculinizes the brain (72). Other actions of testosterone on sexual differentiation of the brain are mediated by androgen receptors that, like estrogen receptors, are also expressed equally in male and female brains but are differentially occupied in males because of the testicular secretion of testosterone during perinatal development (71).

Because the developing brains of both sexes are highly sensitive to gonadal hormones, the fetus must be protected from maternal and endogenous estrogens until developmentally appropriate. The developing fetus therefore expresses α-fetoprotein

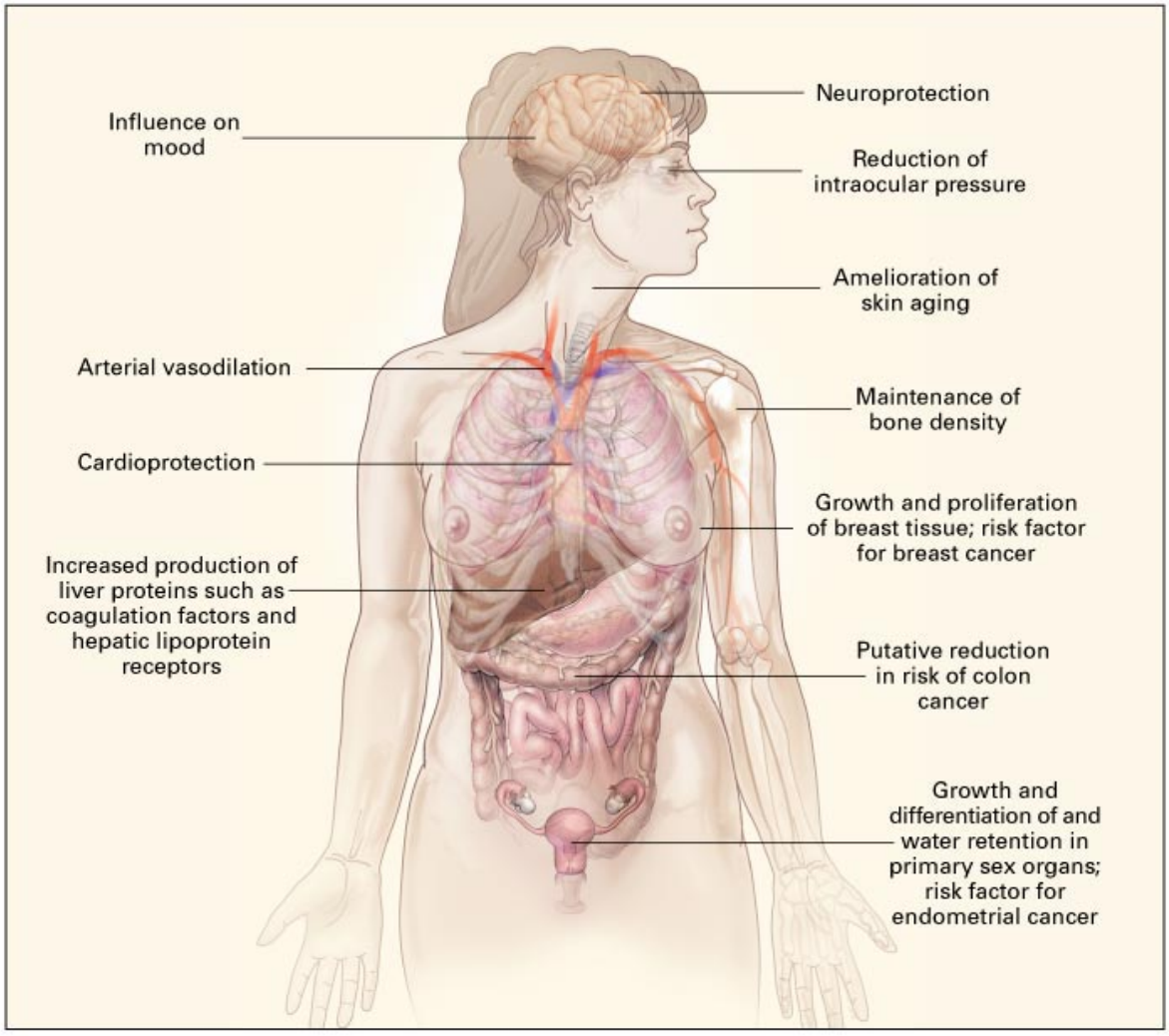



**Figure R3. Effects of Estrogens in Different Organ Systems**

Estrogens have neuroprotective effects and reduce perimenopausal mood fluctuations in women. Estrogens are arterial vasodilators and may have cardioprotective actions. In the liver, estrogens stimulate the uptake of serum lipoproteins as well as the production of coagulation factors. Estrogens also prevent and reverse osteoporosis and increase cell viability in various tissues. When applied topically, estrogens increase skin turgor and collagen production and reduce the depth of wrinkles. These benefits are offset by the risk that estrogens stimulate growth of endometrial and breast tumors. Adapted from Gruber *et al.* NEJM 2002 (5).

(AFP), which binds estradiol and prevents hormonal stimulation (73). The capacity to produce and utilize estrogens clearly exists before birth, as the fetal ovary expresses aromatase and ERα (74). A key element in understanding how xenoestrogens exert teratogenic effects was realized when it was discovered that AFP does not bind synthetic estrogens such as diethylstilbesterol (DES) (73); DES is therefore capable of bypassing AFP-mediated estrogen blockage and stimulate brain sexual differentiation (73). Many insights into actions of estrogens on brain development have come from studies of offspring of mothers exposed to DES during pregnancy, while other studies have revealed that prenatal DES exposure can alter general personality and altered patterns of sexual behavior in adolescence and adulthood (75, 76). DES is not an androgen, although DES has been reported to have a masculinizing effect (76), and some DES-exposed women show elevated testosterone levels as adults (75). DES induces genetic imprinting and epigenetic silencing of tumor suppressor genes and is implicated in the development of breast and uterine cancer (77). Frighteningly, DES-induced epigenetic events and hormonal response reprogramming have been shown to exert physiological effects to generations well beyond the initial fetal and maternal exposure. The US Center for Disease control maintains information and support for DES-treated women, children exposed *in utero*, and their offspring (http://www.cdc.gov/DES/).

*Ovary*

Puberty in girls is initiated by low-amplitude nocturnal pulses of gonadotropin, stimulating steroidogenic enzymes that raise serum E2 concentrations (78). E2 production varies cyclically during menstrual cycles, with the highest rates and serum

concentrations in the preovulatory phase, resulting in follicle maturation, rupture, and oocyte release (79). The precise roles of estrogen in ovarian function has been extensively studied using ER and aromatase knockout animal models (50, 80-82). There are defects in ovulation in both αERKO and βERKO mice. αERKO mice are anovulatory and βERKO mice have a severe reduction in ovulation (50, 80), and both αERKO male and female mice are infertile (50). Both ERα and ERβ are therefore required for the correct functioning of the ovaries. The ovarian defect in αERKO mice can be corrected by normalization of luteinizing hormone levels (50, 82), which suggests that ovarian defects are indirectly related to the loss of estrogen feedback on the pituitary. In double knockout mice (αERKO/βERKO), the females have ovaries that contain seminiferous tubule-like structures that are filled with Sertoli-like cells, and the males are infertile because of a reduction in the number and motility of epididymal sperm, a phenotype similar to that in male αERKO mice (81).

*Uterus*

Several reports describe the initial appearance of ER immunoreactivity in the developing uterus as early as fetal day 15 (83). ER was first detectable in mesenchymal cells, whereas induction in the epithelial cells occurs during the late fetal stages and increases significantly during the neonatal period (83). The fully developed uterus is composed of many heterogeneous cell types comprising three major anatomical compartments, the outer myometrium, endometrial stroma, and luminal/glandular epithelium. Under the influence of FSH from the anterior pituitary, the ovarian follicular cells produce estrogen, stimulating early estrogen-responsive genes c-*fos* and c-*jun*,

which results in proliferation, increased blood flow, and water retention in the uterus (84, 85). The importance of ERα in the uterus is evident from the extremely small uteri in αERKO mice and the almost complete inability of this tissue to respond to E2. ERβ may be responsible for some estrogenic stimulation in αERKO mice, as ERβ is present in the uterus (86), particularly in the stroma. The transformation of the uterine stroma in response to implantation of the embryo (decidualization) is normal in αERKO mice, indicating that ERα is not involved in this process, and may be mediated in part by ERβ (87). The uteri of immature βERKO mice are hypersensitive to the proliferative actions of E2 (86), recapitulating the hypothesis that ERβ modulates growth effects mediated by ERα.

Using αERKO tissue recombination techniques, estrogen-induced proliferation in the uterine epithelium was shown to occur through an indirect mechanism involving activation of ERα in the underlying stromal cells (88). Clarification using similar tissue recombination techniques, the stromal response was shown to occur only if the overlying epithelium expressed ERα (89). ERα is temporally down-regulated in the epithelia while simultaneously up-regulated in the uterine stromal compartment, suggesting ERα plays a role in early events associated with E2-induced cell proliferation of the uterine epithelia (85). Based on these results, and with what is currently known about ERα biology, these results suggest that early epithelial stimulation (and subsequent receptor loss) precedes ERα-mediated signals transduction to the underlying stroma, which by upregulation of ERα, may signal through ER-dependent pathways. Indeed, an extension of earlier αERKO tissue recombination studies revealed that the uterine epithelia are directly

stimulated by E2 and that the epithelia stimulate stromal cell proliferation by transmiting an epithelial ERα-dependent, systemic factor to the underlying stroma (90).

*Breast*

In mammals, the mammary gland is essentially undeveloped at birth and does not undergo full growth until the completion of puberty and remains undifferentiated until pregnancy and lactation. Development of the mammary gland may be divided into five distinct stages: embryonic and fetal, prepubertal, pubertal, sexually mature adult, and pregnancy/lactation (59). The later four stages of mammary gland development occur after birth and terminate in a gland capable of milk production. These stages are strongly regulated by the endogenous ovarian steroid hormones and are characterized by massive growth of the glandular ducts that emanate from the nipple until they have progressed through the fat pad composing the bulk of the breast. Upon pregnancy and the onset of lactation, the gland undergoes dramatic differentiation to produce milk-secreting structures, termed alveoli, throughout the ductal network (59).

The lobular units of the terminal ducts of the breast tissue of young women are highly responsive to estrogen. The density of breast tissue and ERα expression is highest in the follicular phase of the menstrual cycle and falls after ovulation (60). In the normal breast, there appears to be a discordance between cells that express ERα and those that are actively proliferating (61, 62). Cells that contain proliferation markers (cyclin A, Ki67, or proliferating cell nuclear antigen (PCNA) do not appear to express ERα (63, 64). As E2 stimulates cellular proliferation of the mammary ductal epithelium (65), the basic question was raised: How do breast epithelial cells respond to E2? The first proposed

mechanism, using αERKO mice, revealed that E2 stimulated ERα in stromal cells (66). According to that study, stromal-derived growth factors would then interact with growth factor receptors on epithelial cells and stimulate their proliferation. However, these findings do not agree with the clinical responsiveness of epithelial-derived ERα-positive breast cancers to anti-estrogen therapy, raising the need for re-evaluation and clarification of this important issue. Furthermore, the original αERKO mouse was found to express a shorter isoform of ERα and was therefore not entirely ERα null (67). Recent findings by Feng *et al.* provide a more direct explanation of E2 action in the breast. Using a novel mammary gland-specific ERα knockout mouse, expression of ERα in the mammary epithelia was found to be required for ductal and alveolar morphogenesis, as well as mammary fat pad infiltration and terminal end bud formation (45), providing strong evidence that E2 acts directly through ERα in breast epithelia. Together, results from αERKO tissue recombination experiments (68) and mammary gland-specific αERKO mice (45), a common mechanism has been established in which estrogen stimulates epithelial growth and underlying stroma through an epithelial ERα-dependent pathway.

*Liver*

Estrogen and estrogen-like compounds are extensively metabolized in the liver into catechol and methoxylated estrogens (100). Estrogens undergo secondary metabolism in the liver resulting in conjugation via sulfation or glucuronidation, allowing excretion into the bile or urine (101). Hydrolysis of these conjugates by the intestinal flora and subsequent reabsorption of the estrogen result in an enterohepatic circulation. Estrogen can acti directly on the liver, increasing lipoprotein receptors (28, 54), resulting

in a decrease in serum concentrations of low density lipoprotein cholesterol (102). Although ERα-negative, ERα-transfected HepG2 liver cancer cells are commonly used to screen for estrogenic compounds as well as determine the hepatic enzymes that are responsible for generating functionally estrogenic or antiestrogenic metabolites of novel compounds (103).

The active metabolite of tamoxifen; 4-hydroxy-tamoxifen (OHT) is catalyzed by the liver P450 enzyme CYP2D6 (104). Interestingly, OHT is a minor metabolite, and the 4-hydroxy-*N*-desmethyl-tamoxifen metabolite (endoxifen), also catalyzed by CYP2D6, is the major metabolite of tamoxifen and is essentially equivalent in antiestrogen activity as OHT, suggesting this major metabolite contributes to the overall activity of tamoxifen (105, 106). Patients with CYP2D6 polymorphisms who were coadministered antidepressants and other drugs that are CYP2D6 inhibitors (paroxitene, others) had significantly lower plasma concentrations of tamoxifen and its metabolites (107). Polymorphic CYP2D6 patients also have fewer hot flashes associated with tamoxifen treatment, but have a higher recurrence rate, presumably due to lower activation of the parent tamoxifen drug (108, 109).

*Bone*

Both osteoclasts and osteoblasts express ERα and are direct targets for estrogens (110, 111). Bone is a dynamic tissue that is constantly being resorbed to serve as a mineral source for the body and remodeled to replace this reservoir as well as to maintain skeletal strength. Osteoporosis is a defined pathology characterized by a loss in bone mass and strength and is believed to be due to a disruption in the equilibrium between bone resorption and formation (112). Estrogens directly inhibit the function of osteoclasts (*i.e.*

"hardens the target"), and are therefore classified as antiresorptive agents (113). E2 may exert this effect by inhibiting PTH-induced cAMP formation thereby blocking the ability of parathyroid hormone (PTH) to stimulate osteoclast formation (114).

Numerous other studies have provided considerable evidence that both estrogen and testosterone both prolong the lifespan of the osteoblast by inhibiting osteoblast apoptosis (115). Sex steroid effects on osteoblast apoptosis appear to be mediated by activation of the Src/Shc/ERK signaling pathway (115). Steroids also suppress osteoclast development and survival, mediated through suppression of RANKL (116, 117) and induction of the Fas/FasL pathway (113). Estrogen also regulates the production of additional cytokines in osteoblasts which signals to modulate osteoclastic activity in a paracrine fashion (118).

In mice, though ERα appears to be the major receptor in most estrogen target tissues including bone (119), neither clear bone loss nor high bone turnover is detectable in αERKO or αERKO/βERKO double-knockout females (120, 121). This unexpected maintenance of bone mass in female mutants is presumed to be due to unphysiologically elevated levels of other osteoprotective hormones, namely androgens. Systemic defects in the hypothalamus caused by ER inactivation were shown to impair the negative feedback system of hormone production leading to an excess of androgens (120). The anabolic effects of androgens mediated by the androgen receptor (AR) are evident in female mice (119, 122).

Upwards of 200-fold differences in ERα-expression level have been noted between breast and bone cells (123), due in part to preferential utilization of the distal ERα F promoter, which lies approximately 117 kb upstream of the ERα transcription start

site (124). Interestingly, use of this promoter commonly results in splicing of the 5' UTR of ERα to the splice acceptor site in exon 2, effectively skipping exon 1 and resulting in the formation of a shorter ERα. The translation of this variant mRNA results in the expression of the 46-kDa ERα isoform (123, 124). Whereas the ERα-46 corresponds to approximately one-third of the transcripts expressed in osteoblasts,it represents around one-tenth of the total ERα mRNA transcripts in MCF7 cells (124). In apparent compensation for the lower ERα expression level seen in bone, ERα-46 can form heterodimers with full-length ERα (ERα-66) as well as homodimerize. Homodimers show a higher affinity for an ERE than ERα-66 dimers. Furthermore, the ERα-46/66 heterodimer forms preferentially as compared to the ERα-66 homodimer (123, 124), possibly allowing minimal ERα expression to accomplish high levels of estrogen response. Regardless of the relative expression level of ERα in bone, the FDA approval of raloxifene, a selective ERα modulator (SERM), for ther treatment of osteoporosis clearly highlights the contribution of estrogen and ERα signaling in regulating bone density. Membrane-bound ERα has also been implicated as a mediator of rapid E2 effects in bone such as rapid calcium signaling (60). E2 rapidly causes the influx of $Ca^{2+}$, and elevated $IP_3$, cAMP, cGMP, and diacylglycerol formation (125, 126). A cell-impermeant E2 conjugate demonstrated the cell-surface ER-mediated stimulation of alkaline phosphatase in chondrocytes (127).

Aromatase is also expressed in osteoblasts and chondrocytes (128), and aromatase activity in cultured osteoblasts is comparable to that present in adipose tissue (129). Thus, it appears that in bone, local aromatase expression is a major source of E2 and participates in the maintenance of mineralization. Several males with aromatase

deficiency have been described (130, 131), with identical skeletal phenotypes as the ER knockout male (132), revealing the importance of aromatase and localized estrogen action in bone. Osteopenia is evident in a male patient genetically deficient in ERα (132), and in patients with aromatase mutations (133).

Because estrogen has pleiotropic effects on virtually all aspects of osteoclast development, activity, and lifespan, it is not surprising that the consequence of estrogen deficiency in humans is a marked stimulation of bone resorption (134). In bone extracts from postmenopausal women with osteoporosis, the concentrations of interleukin-6, interleukin-1, and TNF mRNA were high (135), which have been shown to stimulate osteoclast differentiation (136). A single case exists in which a human man lacks functional estrogen receptors (132). He was found to have severe osteoporosis and reduced fertility, with no target-tissue responses to estrogen therapy. Genetic analysis showed that the patient was homozygous for a mutation in the second exon (R157X) of the ER gene, generating a premature stop codon. This man was exceptionally tall due to incomplete epiphyseal closure and continued linear bone growth, revealing the importance of ER is epiphyseal closure (132). Furthermore, despite elevated testosterone levels, high rates of bone resorption and skeletal osteopenia were evident (132). Similar to the observations in the αERKO mice, these findings reveal E2 action in both bone maturation and mineralization.

Osteoporotic bone loss is the result of high bone turnover in which bone resorption outpaces bone deposition (137, 138). This imbalance in bone turnover that is induced by estrogen deficiency in women and female rodents can be ameliorated with bio-available estrogens including selective estrogen receptor modulators (SERMs) (139).

Current evidence supports the hypothesis that excess bone resorption occurs in the postmenopausal years, acting to strip the bone of mass and further remove the foundation upon which new bone may be formed (112). Several therapies are known to reduce the postmenopausal increases in bone resorption, including the intake of calcium and vitamin D, calcitonin, bisphosphates, and estrogens (112). Breast cancer bone lesions span a spectrum in which the majority are osteolytic, but up to 15% are osteoblastic or mixed (140). Both osteoblastic and osteolytic bone metastases lead to numerous skeletal complications, including bone pain, hypercalcemia, pathologic fractures, and spinal cord and nerve compression syndromes (141). Such complications increase morbidity and diminish quality of life in these patients. Tumor cells and bone cells may rely on the same signaling pathways and transcription factors to facilitate their cooperative interactions at sites of metastases. This phenomenon has been suggested to represent "osteomimicry" on the part of the tumor cells (142) by expressing bone proteins such as bone cell surface proteins and secreted factors. Osteolytic MDA-MB231 breast cancer cells express PTHrP which may allow breast cancer cells to grow into the bone microenvironment by stimulating the bone resorption axis (143), suggesting breast cancer cells may possess or aquire similar signaling pathways to facilitate metastasis to bone.

*Cardiovascular*

There exists a remarkable gender-related contrast in the risk of cardiovascular disease. Women generally possess a greater incidence of the multiple risk factors associated with cardiovascular disease when compared with men, *e.g.*, obesity, diabetes, elevated blood pressure, and plasma cholesterol, however, epidemiological studies

indicate their relative risk of developing this disease is significantly lower (69, 70). It is now believed that the protective factor against cardiovascular disease in females is their inherently increased exposure to estrogens (69, 70)

Estrogens are thought to be natural vasoprotective agents and participate in maintenance of the cardiovascular system (71), mediated through expression of ERα in smooth-muscle cells of coronary arteries and endothelial cells (72). Estrogens may exert vasoprotective effects by causing short-term vasodilation through formation and release of nitric oxide and prostacyclin in endothelial cells (73). E2 has been shown to decrease LDL and inhibit plasminogen activator and vasorestrictive peptides while increasing HDL and VEGF synthesis (74, 75). A protective role of estrogens against atherosclerosis is suggested by the finding that estrogen treatment reduced the progression of coronary-artery atherosclerosis in oophorectomized monkeys, though it does not appear that E2 has an effect on preexisting plaques (76). An unclarified role remains whether estrogen treatment during the postmenopausal period prevents atherosclerosis (77). Favorable findings from epidemiologic studies are counter-balanced by the lack of benefit of estrogen seen for protection against cardiovascular disease (Heart and Estrogen/Progestin Replacement Study) (78).

*Central Nervous System*

The most important role of estrogens in the brain is feedback control of the menstrual cycle. In the adult, estrogens exert a neuroprotective and supportive effects on the central nervous system, which is most evident later in life (79, 80). Some epidemiologic data suggest that in postmenopausal women, estrogen deficiency is

associated with a decline in cognitive function and an increased risk of Alzheimer's disease (81). Unfortunately, estrogen administration does not appear to have a beneficial effect in women with established Alzheimer's disease (82).

Recently, nuclear receptor gene expression has been mapped according to their temporal and spatial, and functional aspects in the mouse brain (83). This project has generated a unique, comprehensive dataset, that covers both the quantitative and spatial aspects of NR gene expression in the adult mouse brain, and is available online in the form of an interactive database (http://www-mci.u-strasbg.fr/mousepat/), allowing for data mining to potentially reveal the role of specific nuclear receptors in brain development, function, and behavior. ERα and ERβ appear to have distinct roles in the brain. Behavioral studies with αERKO and βERKO mice show that ERα mediates aggressive and sexual behavior whereas ERβ regulates emotional and cognitive behavior (84-86).

## ESTROGEN AND BREAST CANCER RISK

In 1896, George Beatson reported that removal of the ovaries from premenopausal women with advanced breast cancer produced a dramatic decrease in tumor size and improved the patient's prognosis (87). Since then, a substantial amount of evidence has accumulated to demonstrate that estrogens play a major role in the etiology and progression of breast cancer (88-91). Beginning in the early 1970s, studies began reporting significant increases in incidence of endometrial cancer in Western caucasian women who had undergone estrogen therapy (92), triggering investigation that continues to this day into what levels of estrogen exposure are considered safe. The concern that postmenopausal hormone replacement therapy (HRT) may cause breast cancer has lead to an enormous volume of research in epidemiology, endocrinology and tumor cell biology. Whether there exists a positive correlation with the risk of breast cancer and prolonged use of synthetic estrogen and progestin contraceptive pills remains controversial (93-95), and is further complicated by the influence that environmental exposures, geography, diet, body weight, and genetics also play in individual risk of developing breast cancer (96). Polymorphisms in the genes coding for steroidogenic enzymes influence estrogen production and should also be taken into consideration for individual response to contraceptives and HRT and their respective increase in cancer risk (97, 98). Based on these findings, a prudent and carefully individualized therapeutic approach to contraceptives and HRT is warranted.

## CLINICAL MANAGEMENT OF BREAST CANCER

Breast cancer is the most common cancer and the second leading cause of cancer mortality among women. More than 214,000 women were diagnosed with breast cancer and with an estimated 41,430 related deaths in America in 2006 (http://www.cancer.org/). Thankfully, these statistics represent an overall decline in new cases and related deaths, primarily attributed to the results of the 2002 Women's Health Initative (WHI) study, which recommended that women stop taking the hormones Premarin and Provera (99). The decrease in numbers was greatest in ER+ breast cancers.

Numerous studies have been carried out concerning the levels of ER and PR in neoplastic breast tissue and the prognostic value that these parameters may provide (100, 101). The consensus is that ER status is a clear predictor of response to hormone therapy (102). Reports indicate that more than 70% of primary breast tumors are ERα-positive and exhibit estrogen-dependent growth (101) while the remainder of mammary tumors are often ERα-negative and exhibit aggressive, estrogen-independent growth (100).

### *SERMs*

Early studies showed reduction in breast tumor growth following adrenalectomy (9) or ovariectomy (87). With the discovery of ER and estrogen action, hormone therapy has replaced aggressive surgery as the preferred treatment for ER-positive breast cancers.

Dr. Jensen, known for concluding his lectures in verse, neatly summed up decades of his discoveries:

"A lady with growth neoplastic
Thought surgical ablation too drastic.
She preferred that her ill
Could be cured with a pill,
Which today is no longer fantastic."

The term "selective estrogen-receptor modulator" (SERM) was introduced to define nonsteroidal ligands such as tamoxifen that antagonize the action of estrogen in some tissues, such as the breast, and mimic its action in others, such as the uterus. Among postmenopausal women, the agonist action of estrogen is desired in bone for the maintenance of density and in the cardiovascular system and brain for the maintenance of function, but not in the breast or endometrium.

Tamoxifen has been the first-line standard therapy for ERα-positive breast cancer treatment for the past 40 years (103). Tamoxifen exerts its antiestrogenic effect by competing with estrogen for ligand binding with ERα, causing attenuated transcription of estrogen-responsive genes that are involved in the development and growth of breast malignancies. Unfortunately, a side effect of tamoxifen therapy is the increased risk in the development of endometrial carcinoma. Alternative SERMs like raloxifene retain the beneficial estrogenic effects on bone and lipids but are not estrogenic in the uterus (104).

*SERDs*

Inevitably, tumors develop resistance to tamoxifen. This occurs via multiple mechanisms but it appears it is not simply due to loss of ER expression (105). This, in

turn, provides a rationale for continued hormone treatment after tamoxifen resistance has developed. However, new endocrine therapies must lack cross-resistance with prior treatments. Of the SERMs that have been developed, none have shown clinically relevant activity following development of resistance to tamoxifen (106-110). ICI 182, 780 (fulvestrant, or Faslodex) belongs to a new class of ERα antagonists that rapidly triggers ERα protein degradation and is thereby classified as a Selective Estrogen Receptor Downregulator (SERD).

This mechanism of action of fulvestrant is different from that of tamoxifen, reducing the risk of cross-resistance, which allows this drug to have an important role in the hormonal treatment of breast cancer. Fulvestrant competitively binds to ERα thus preventing endogenous estrogen from exerting its effect in target cells with no known agonist effects (111, 112). Fulvestrant causes ERα downregulation and reduced shuttling of ERα from the cytoplasm (113), and increased receptor immobilization in the nuclear matrix (114, 115). For patients who have progressed on tamoxifen, fulvestrant produces good response rates (116). Fulvestrant is therefore considered second-line therapy for treatment of ER+ breast cancers that have failed tamoxifen therapy.

*Aromatase Inhibitors*

At menopause, the synthesis of ovarian hormones ceases. However, estrogen continues to be converted from androgen (produced by the adrenal glands) by aromatase in mammary adipose (117), which can cause high estrogen levels locally (118). This biological pathway served as the basis for the development of an aromatase inhibitor class of compounds. Current AI therapy includes usage of third-generation non-steroidal

AIs such as anastrozole, letrozole, and exemestane. These AIs have been widely used in the treatment of advanced breast cancer in postmenopausal women with disease progression following tamoxifen therapy. AIs have also been proposed as as first-line treatment for advanced disease, and eventually adjuvant therapy. More data on the efficacy of fulvestrant after AIs is essential to determine the most appropriate sequence.

*Hormone-resistant Breast Cancer Therapy*

Overexpression of many growth factor receptors, as well as growth factors, has been shown to confer varying degrees of estrogen-independent growth on ER-positive breast cancer cells. The majority of hormone-resistant breast cancers maintain ERα expression, although ERα expression may be lost due to progressive methylation and silencing of the ERα promoter region (119-122). Epidermal growth factor receptor (EGFR) and erbB2 are increased in tamoxifen-resistant breast cancer cell lines (123). Herceptin, a monoclonal antibody specific for erbB2, a member of the EGFR-tyrosine kinase (EGFR-TK) family, has shown promising results in the treatment of women with metastatic breast cancer (124). Breast cancer cells resistant to fulvestrant become ERα-negative and upregulate alternative growth signals, including erbB2, Wnt/β-catenin, and EGFR pathways (125). The EGFR-TK inhibitor ZD1839 (Iressa) is also an effective inhibitor of cell proliferation (126) and when used in combination with antiestrogens, has been shown to be more effective at inhibiting proliferation of breast carcinoma cell lines than either drug alone (127). Although the concept of combination biologic/hormonal therapy is still in its infancy, the future of biological therapies and their potential role in extending the window of effective endocrine therapies will require a more complete

understanding of the mechanisms by which breast cancer cells transition from estrogen-dependent growth to hormone-independent growth. Effective "management" of breast cancer will require drugs that can target each of these pathways and that are used in the order that follows the transition of breast cancer growth from one pathway to another.

## ERα-MEDIATED TRANSCRIPTION

The specific nuclear actions of estrogens are determined by the structure of the hormone, the subtype or isoform of the ER involved, the characteristics of the target gene promoter, and the balance of coactivators and corepressors that modulate the final transcriptional response to the complexes of estrogen and ER. ER uses three distinct mechanisms to exert its effects on its target genes (***Figure R4***; (5)). E2 can stimulate receptor binding to DNA at estrogen response elements (128). Estrogen-occupied ERα can also be tethered to other transcription factors that bind their respective response elements, such as AP-1 sites for *fos/jun* heterodimers (129, 130), Sp1 (131, 132), NF-κB sites, and others (133). ERα can also be activated via phophorylation by other growth signaling pathways, or can activate other pathways through growth-signaling crosstalk (134), acting in non-nuclear or non-genomic fashion.

### *Ligand-Dependent Activation of ERα at Estrogen Response Elements*

ERα binds to estrogen-responsive elements (ERE) to initiate transcription or repression of estrogen target genes (135). ERα and ERβ can bind an ERE as a homodimer or heterodimer (136), and both bind the same ERE sequence and exert transcriptional regulation through the ERE (16). Direct nuclear interaction is achieved by

recognition of the DNA estrogen-response elements by receptor zinc fingers. Estrogen-response elements are present in the regulatory regions of estrogen target genes and are responsible for confering estrogen regulation of genes. Highly estrogen-responsive and perfectly palindromic sequences have been found in the African clawed frog *Xenopus laevis* genes encoding vitellogenin (137). From these natural EREs, a minimal consensus sequence for EREs has been derived. The perfect ERE sequence is a 13 bp perfect palindromic inverted repeat with a 3 bp spacing of variable bases contained within the regulatory regions of target genes: GGTCAnnnTGACC (26, 138-140). ERα recognizes this sequence with high affinity. The receptor-DNA complex then interacts with basal transcription factors, coregulator proteins, and other transcription factors to ultimately regulate transcription of the target gene (138, 140, 141).

Only a small number of most estrogen-inducible genes contain perfect consensus EREs. In most cases, variant ERE elements have been described. Variant EREs or even half-EREs, often separated by many base pairs, can still confer estrogen responsiveness (142). For instance, the sequence 5'GGTCAnnnTGGCC3', which differs from the consensus sequence by 1 bp, mediates the estrogen induction of the Bcl-xl gene (143). These variant sequences bind ERs with less affinity, depending on the flanking bases (144). Even as such, both consensus and imperfect EREs within the promoters of the human genes pS2 (145), c-*fos* (146), c-*myc* (147), PR (148), vitellogenin (149), and cathepsin D (150), are all used as experimental read-outs of ERα-mediated transcription, either as gene promoters driving E2-induced reporter genes or as induction of the gene mRNA.

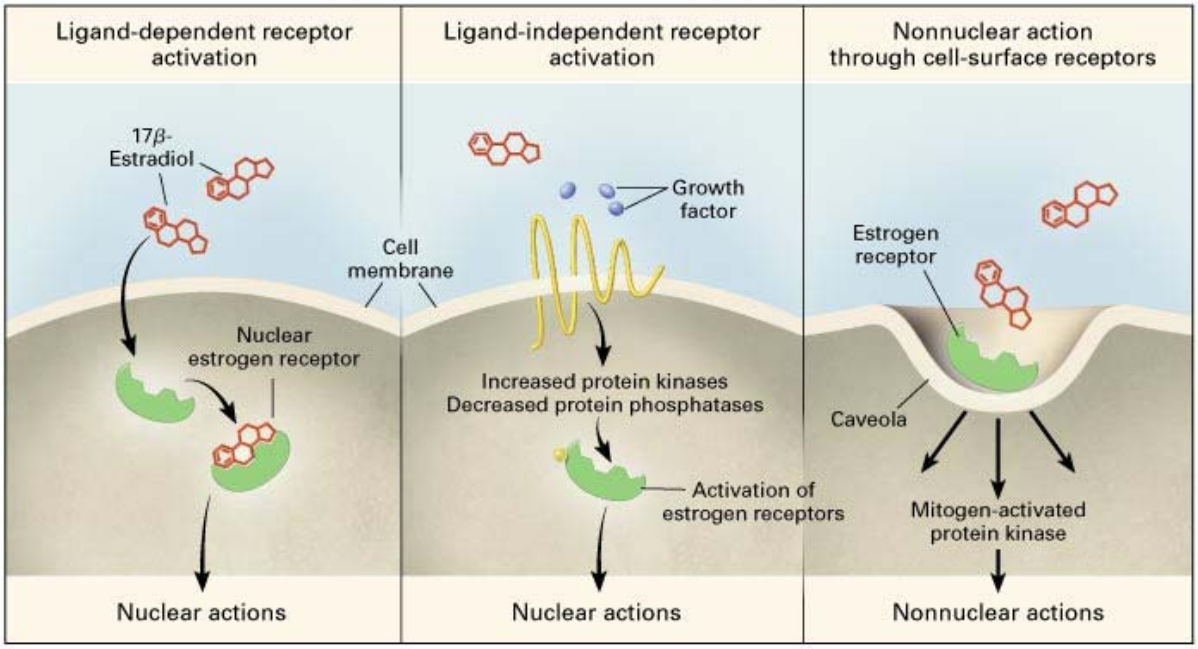



**Figure R4. Ligand-Dependent and Ligand-Independent Estrogen-Receptor Activation.** The estrogen receptor can be activated by estrogen (left-hand panel) or independently of estrogen — for example, by growth factors that increase the activity of protein kinases that phosphorylate different sites on the receptor molecule. In this model (center panel), the unbound but activated receptor will then exert transcriptional effects. In the case of the nonnuclear estrogen-signaling pathway (right-hand panel), cell-membrane estrogen receptors are located in cell-membrane invaginations called caveolae. Their activity is linked to the mitogen-activated protein kinase pathway, resulting in a rapid, nonnuclear effect. Adapted from Gruber *et al.* NEJM 2002 (5).

Using ChIP assays, ERα and several cofactors were shown to bind endogenous target genes in a cyclical manner (151, 152), establishing a kinetic model for transcriptional activation by ERα that integrates cofactor involvement and histone modification. Further studies built on the cyclical model of transcriptional activation with a comprehensive analysis of the coordinated recruitment of up to 46 regulatory factors to the estrogen-responsive pS2 gene promoter (153). By performing sequential immunoprecipitations that simultaneously measure occupancy of the promoter by several factors, termed re-ChIP, multiple transcriptional complexes were identified that assembled with ERα at an endogenous promoter. α-Amanitin-synchronized cells were used to measure multiple ERα cycles with a resolution of 1-min time intervals. Unliganded receptor also exhibits cyclical binding but with faster kinetics than shown by ligand-bound receptor (152), revealing the possibility that the frequency of receptor-cofactor cycles on a promoter may provide yet another element of regulation. The importance of time as a variable in receptor transactivation resulted in the introduction of the "transcriptional clock" concept (153). Both nonproductive and transcriptionally productive cycles exist for ERα and cofactor occupancy (153). The ERα-DNA transcriptional clock has been summarized in an animation (153).

A major discrepancy in the timing of receptor cycling has been noted between ChIP studies and studies performed using fluorescence recovery after photobleaching (FRAP). The mobile nature of nuclear receptors, and of other transcriptional components, revealed by FRAP demonstrated rapid (within seconds) exchange with chromatin targets. These studies argued against the idea of stable association of transcriptional complexes with promoter elements (154-156). Cycles measured by ChIP

are on the order of 35 to 40 min (153), whereas receptor associates with DNA with a half-life of seconds (152). It appears that FRAP is best suited for providing spatial information of receptor mobility. ChIP assays appear to capture the average receptor scenario at a given time, though dynamics may be considerably faster. Recruitment of proteasome components, chromatin-remodeling complexes, and heat shock proteins appear to facilitate this rapid cycling (153). ChIP is therefore best suited for measuring the percentage of promoter associated with a particular protein.

Although ligand is critical for establishing receptor-coregulator interactions, ligand is insufficient for maintaining stable interactions over time (153, 157). There is an active process of complex disassembly and removal from the promoter, suggesting that receptor cycling at a promoter is a mechanism to constantly probe for changes in cellular hormone levels (151, 152, 158). Based on these findings, it is possible that ligand-bound ERα is highly mobile, translocates rapidly, and cycles on and off promoters on the order of seconds. Accumulation of receptors at responsive promoters may be progressively stabilized by successive recruitment of coactivators to the growing complex.

*ERα Signaling Through Growth Factor Crosstalk Pathways*

The underlying complexity of signaling cascades lies in the network of intracellular proteins that sequentially link a cell surface receptor to a nuclear transcriptional regulator. ERα can signal through epidermal growth factor receptor (EGFR), analogous to the dependence of a wide variety of G-protein-coupled receptors on the EGF receptor to effect signaling (159). Activated EGFR and other growth factors further stimulate MAPK and other kinase pathways resulting in additional cell response

(160). Studies using the cell-impermeable BSA-conjugated-17β-estradiol reveal estrogen activation of MAPK through membrane-bound ERs (161) and accumulation of second messenger molecules. E2-binding can also directly influence non-genomic receptor activity by inducing receptor phosphorylation, which can, in turn, activate other growth signaling pathways. Steroid-independent phosphorylation of ERα by HER-2 ligands can also promote cell proliferation (162).

*Coactivators*

The transcriptional activity of ERα is regulated by coactivators, corepressors, and chromatin remodeling complexes. By definition, coactivators are considered to interact directly with the steroid receptor and enhance transcription (163). A concise list of all ERα interacting proteins are listed on the Human Reference Protein Database (http://hprd.org/interactions?protein=00589&isoform_id=00589_1&isoform_name=). A summary of coactivators recruited to ERα and DNA in the presence of E2 have also been diagrammed and described using ChIP and FRAP technology (152, 153). ERα interacts with the TATA-box–binding protein and RNA polymerase II, along with numerous nuclear-receptor coregulatory proteins to form a transcription initiation complex (5); the complexity of proteins provides increased stability and transcriptional specificity. In response to E2 binding, residues of helix 12 within the ERα ligand binding domain interact with the LXXLL motif (NR-box motif), which is present in many nuclear receptor coactivators (164). ERα transcription is thereby enhanced by coactivators, namely the p160-family (SRC 1-3), SWI/SNF complexes, CREB-binding protein (CBP), p300/CBP-associated factor (pCAF), and TRAP/DRIP/SMCC (163). The SRC family of

proteins and p300/CBP appear to have the greatest capacity to increase the transcriptional activity of ER (165, 166).

The expression levels of ERα and many coactivators are significantly higher in intraductal carcinomas than those in normal breast tissue (167). The p160 coactivator AIB1 (Amplified In Breast cancer 1, or SRC3) is frequently amplified in breast tumors and correlates with ERα and PR expression, tumor size, (168), and tamoxifen resistance (169). AIB1 is also is necessary and sufficient to induce E2-mediated receptor turnover (170), revealing a dual role for AIB1 in activating receptor transcription and facilitating receptor turnover. Cyclin D1, which is frequently amplified in breast cancer, directly binds with ERα in a CDK-independent manner (171) and serves as a bridging factor between ERα and SRCs, providing an indirect mechanism to regulate breast cancer cellular growth (172).

Regulation of ERα gene expression involves complex interactions with many coregulators. Coactivators use a variety of mechanisms to enhance ERα-mediated transcription. Among them, SRC1 possesses HAT activity as well as transcription factor acetyl transferase activity (FAT) (173). Multiprotein coactivator-containing complexes often contain CARM1, which has histone methyl-transferase (HMT) activity (174). The SWI/SNF coactivator has ATPase activity and can alter DNA nucleosome arrangement by physically open the repressive chromatin structure to allow easier access to DNA (175). The coactivator p300 serves multiple functions, including a bridge and scaffold for further coactivator recruitment, as a HAT to acetylate histone tails of nucleosomes, thus favoring chromatin remodeling and activation of basal-and enhancer-regulated transcription (176). ERα acetylation at lysines K266/268 are also mediated by p300 (30).

Several observations have been made that associate proteasome activity with ERα transactivation. Several ERα coregulatory proteins are also components of the ubiquitin/proteasome degradation pathway. These are SUG1/TRIP1 (177), RSP5/RPF1 (178, 179), E6-AP (180), and Ubc9 (181). RSP5 and E6-AP are both ubiquitin ligase proteins and stimulate receptor-dependent gene activation (180). In addition to proteasome-mediated degradation of ERα , other cofactors (SRC-1, TIFII, RAC3 and CBP) that associate with ERα are also degraded through proteasome action (182). Oddly, E6-AP ubiquitin ligase activity is not required for its coactivator function, suggesting that E6-AP may serve to recruit proteasome components to clear transcriptional complexes, and not necessarily be involved in degradation of ERα (180). The SUMO ligases PIAS1 and PIAS3 also serve as receptor coactivators (32) by sumoylation of ERα at lysines K266/268. Sumoylation enhances receptor transcriptional activity, suggesting that sumoylation directly or indirectly activates transcription. Either sumoylation itself is an activator of receptor transcription, or alternatively, sumoylation blocks further receptor modification (by acetylation or ubiquitination), and thereby functions indirectly to maintain receptor transcriptional competency.

Another association is revealed in studies that showed the proteasome inhibitors MG132 and lactacystin abrogated transactivation by ERα (182), implying that proteasome degradation is necessary for ERα-mediated transcription. However, more recent evidence indicates that proteasome inhibition by MG132 treatment prolongs ERα transactivation; despite receptor ubiquitination, degradation is not required for receptor transactivation, and ubiquitinated receptors remain transcriptionally active (183). FRAP

assays demonstrates that, in contrast to unliganded ERα which is highly mobile, the mobility of ERα is severely impaired by treatment with MG132 (155). Immobilized receptors may therefore retain transcriptional activity, and receptor degradation may facilitate promoter clearance and thereby temporally limit receptor transactivation (184).

*Corepressors*

Corepressors decrease receptor-mediated transcription by interacting with DNA-bound receptors. Corepressors recruit a complex of proteins having histone deaceylase (HDAC) activity in order to condense chromatin and silence gene expression (163). OHT antagonizes receptor transactivation by repositioning ERα helix 12 in a conformation that blocks coactivator recruitment (35). ERα activity is also decreased by the recruitment of corepressors, including NCoR, to the ERα-ERE complex. NCoR associates with the receptor as part of a complex that contains Sin3 and histone deacetylase (HDAC) complexes (185, 186). This NCoR-associated complex is responsible for histone modification and transcriptional silencing. REA (Repressor of Estrogen Action) (187) and SMRT (Silencing Mediator for Retinoid and Thyroid hormone receptor) (188) function in similar manners also recruit HDACs. NCoR is required for the antagonist activity of OHT; low tumor expression of NCoR has been associated with poor response to OHT (189). TAF-1β differs from NCoR1 in that, rather than relying on the activities of its associated proteins, TAF-1β itself is able to repress histone acetylation (190).

The ubiquitin-like NEDD8 (Neural precursor cell Expressed, Developmentally Down-regulated 8), is also linked to ERα transcription (191, 192). The NEDD8-

activating function of Uba3 is required for Uba3-mediated repression of ER transactivation. Fan *et al.* later showed that Uba3-mediated inhibition of ERα transactivation function is due to increased receptor protein turnover (192). Loss of Uba3 neddylation activity resulted in a loss of ICI-induced receptor turnover. As neddylation has only been shown to occur on the cullin family of proteins, the NEDD8 pathway likely stimulate ERα ubiquitination and degradation through neddylation of a cullin within an SCF-based E3-ubiquitin ligase complex, thereby activation the E3 ubiquitin ligase (192).

## ASPECTS OF ESTROGEN RECEPTOR REGULATION

*Receptor synthesis*

ERα regulation is a key component of normal cell function. ERα-mediated transcription is coupled to receptor and ERα mRNA downregulation in a classic autoregulatory negative-feedback manner (193-202). The degree of cellular response to hormones is dictated by the level of ERα expressed in these cells (183, 203-205). The cellular response to E2 is also due in part to distinct expression profiles of ERα in individual tissues (205). Regulation of cellular ERα levels is therefore crucial to maintenance of normal cell function, and aberrant ERα signaling is a driving force in the development and progression of breast and other estrogen-responsive cancers (206, 207).

Regulation of ERα synthesis occurs at several levels. E2 can upregulate and downregulate ERα mRNA expression level, depending on the tissue type (199, 208-210), allowing for regulation of ERα protein levels at the level of mRNA synthesis. Additional mRNA regulation occurs at the level of mRNA stability. The 3' UTR of ERα is unusually long, having twice the length (4.3 kb) of the coding region (2 kb). Some

regions of the 3’ UTR show extensive homology between species, including regions rich in AUUUA sequences, known to destabilize mRNA (211-213). The 3' untranslated region of the human ERα gene post-transcriptionally reduces mRNA levels (214) and mediates rapid messenger ribonucleic acid turnover (213). Specific segments of the ERα 3' UTR were later shown to be responsible for the destabilization of ERα mRNA (215). Utilizing miRNA microarray technology, a number of miRNA have been shown to be differentially expressed in breast cancer tissues. Some miRNAs are up-regulated in breast cancer *vs*. normal breast tissue, and a smaller cohort of miRNAs is up-regulated in ERα-negative *vs*. ERα-positive tumors (216). miR-206, which is elevated in ERα-negative breast cancer downregulates the expression of ERα by binding two sites in the ERα mRNA 3' UTR (216), revealing a novel mechanism for the posttranscriptional regulation of ERα.

*Post-translational Modification*

Regulation of ERα protein stability and activity is mediated in part by a number of post-translational modifications. ERα is covalently modified by phosphorylation, acetylation, ubiquitinylation, and sumoylation, providing a rheostat system in which to switch ERα protein between different functional states (217). ERα is phosphorylated at serine, threonine and tyrosine residues (reviewed in (218)). A threonine residue has also been shown to be modified by O-linked N-acetylglucosamine (*O*-GlcNAc) (219). The ε-amino group of lysine residues are substrates for acetylation, neddylation, sumoylation, methylation, and ubiqutination (220), although ERα has only been shown to be targeted for acetylation (30, 31, 221), ubiquitination (222), and sumoylation (32). A cysteine residue of ERα has been shown to be palmitoylated, anchoring ERα to the plasma

membrane (52), allowing for rapid E2 signaling at the cell membrane. Different modifications are mutually exclusive, thus leading to their potential competition. Competition exists between sites and further complicates the role of post-translational modification on receptor function (33, 220). Competition between post-translational modifications at receptor residues is likely to occur, further modulating receptor protein function or stability (223-225). The growing list of ERα post-translational modifications have been annotated on the Human Protein Reference Database http://hprd.org/interactions?protein=00589&isoform_id=00589_1&isoform_name=).

*Phosphorylation*

Steroid receptor phosphorylation has been the longest studied receptor modification, and a wealth of literature reviews are available (141, 218, 226). All steroid receptors, including ERα, are phosphorylated after binding to their respective ligands (141), and in response to growth factors or cytokines (160, 227-229). Depending on the ERα residue that is phosphorylated, as well as the kinase pathway that mediates receptor modification, phosphorylation can either increase or decrease receptor transcriptional activity and/or regulate receptor stability (230). The role of phosphorylation in regulating receptor stability is detailed in the *signals for receptor ubiquitination* section.

S118, and less so S104 and S106 are the main residues modified after ligand binding, resulting in enhanced E2-mediated transactivation (231-234). S104 and S106 are targets of cyclin A2-CDK2 (235). S118, and also S167, are phosphorylated through MAPK pathway and CDK7 (228, 236). Activation of the Akt pathway also stimulates S167 phosphorylation (237), increasing the binding affinity of liganded ERα to an ERE

(229). S305 phosphorylation has been shown to block K303 acetylation and mutation of S305 resulted in a receptor that mimics the ERα-K303R hypersensitive phenotype (33). ERα dimerization and DNA binding are both enhanced when phosphorylation occurs at S236 (protein kinase A-mediated) (236, 238) or at Y537 (p60c-src/p56lck-mediated) (239, 240). Both S118 and S167 phosphorylation have been suggested as positive markers for responsiveness to endocrine therapy in breast tumors, presumably as phosphorylation at these sites signifies that ERα pathways are being activated (241, 242). A recent paper has reported that constitutive phosphorylation of S118 may be responsible for protecting ERα from proteasomal degradation during chronic estrogen exposure (243).

*Acetylation*

The role of acetylation in steroid receptor function has only recently been appreciated. Similar to phosphorylation, ERα acetylation regulates receptor transcriptional activity (244). Acetylation of lysine residues neutralizes the positive charge on the lysine residue, effectively modifying receptor-DNA and receptor-protein interactions. The cofactor p300 but not P/CAF, both of which have intrinsic acetylase activity, was found to directly acetylate lysine residues at position 302/303 at the boundary between the hinge region and the LBD (31). Mutagenesis of these residues to either neutral (K to A, Q or T) or to arginine (R), in the presence of exogenous p300, resulted in an increased E2-induced transactivation capacity of the mutant ERα (31), suggesting that ERα acetylation may be one mechanism by which receptor sensitivity to hormone is to decreased (31). Furthermore, it has been suggested that ERα is unable to

be acetylated due to mutation, transcription may be potentiated by an increase in the time that transcriptionally competent ERα resides on a responsive promoter, allowing further increase in receptor transactivation (190) . One study has shown that over 30% of the premalignant breast tumors posses an ERα mutant in which a somatic DNA mutation resulted in a K303R mutated receptor (245) that was hypersensitive to E2 and enhanced breast cancer cell proliferation due to increased affinity for the coactivator TIF2 (245). Loss of this specific acetylation site was suggested as one possible mechanism that promotes or accelerates the development of cancer from premalignant breast lesions. Several studies dispute the relative frequency of the mutation in US population-based studies (246, 247), and the mutation was not detected in a cohort of Japanese breast cancer samples (248). A more precise estimation of K303R mutation in breast cancer has yet to be determined, although small sampling size in the initial report may have overestimated the frequency in breast hyperplasias examined.

Contrary to original reports that placed ERα acetylation at K302/303, Kim *et al.* has reported that ERα is acetylated by p300 at lysines 266/ 268 (30). Acetylation of K266 and K268 stimulated ligand-dependent activity in reporter gene assays (30). The seemingly opposing actions of acetylation, enhancing transactivation but reducing E2 sensitivity, may be resolved if acetylation is thought of as fine-tuning mechanism for ERα activity. Acetylation may enhance E2-induced transcriptional activity while simultaneously decreasing receptor sensitivity to ligand, providing an immediate positive response to ligand while dampening prolonged E2 stimulation. PKA-mediated phosphorylation of ERα S305 has been shown to prevent K303 acetylation, suggesting

that kinase-activation of ERα limits acetylation-activation and prevents overstimulation through dual post-translational modification (33).

The acetylation of ERalpha by p300 is reversed by native cellular deacetylases, including trichostatin A-sensitive enzymes (i.e. class I and II deacetylases) and nicotinamide adenine dinucleotide-dependent/nicotinamide-sensitive enzymes (i.e. class III deacetylases, such as sirtuin 1) (30). Template-activating factor β (TAF1β) also inhibits ERα acetylation and subsequent transactivation by blocking receptor acetylation or by increasing non-acetylated ERα interaction with responsive promoters (190). pp32 has also been shown to block receptor acetylation, in part by promoting apo-ERα binding to DNA, and elevating non-productive receptor-promoter cycling (190, 249). In addition to receptor acetylation, numerous ERα-interacting proteins are acetylated, further complicating the role of acetylation in ERα function (reviewed in (250)).

*Sumoylation*

Sumo is structurally related to ubiquitin (251), though its attachment is typically monomeric. Sumoylation does not trigger protein degradation (251); rather, it alters several different protein functions including subcellular localization, DNA-binding, and transcriptional regulation (252). Similarly to ubiquitinylation, sumoylation is a three-step process involving an E1 activating-enzyme (Aos/Uba2), an E2 conjugation enzyme (Ubc9) and an E3 ligase belonging to one of three classes (253). Sumo is covalently attached to target protein lysines residues embedded in the consensus ψKxE motif, where ψ is a large hydrophobic residue (I, V, or L), K is the lysine to which sumo is conjugated, X is any amino acid, and E is glutamic acid {Sampson, 2001 #3637}. Although ERα

does not have the consensus ψKxE motif, it is still sumoylated by the E3 ligases PIAS1 and PIAS3 (32). ERα has been shown to be sumoylated in the receptor hinge region, at K266 and K268 and possibly K299, K302, and K303. Sumoylation is strictly ligand-dependent (32). Together with Ubc9, PIAS1/3 act as ERα coactivators, however this role is independent of their enzymatic activity (32, 254). Elimination of the sumo acceptor sites leads to reduced ERα activity (32), although the exact role sumo plays in this is obscured by the fact that ERα is also acetylated at K266 and K268 (30). Competition may therefore exist between receptor acetylation and sumoylation. Both acetylation and sumoylation are reversible modifications. Several SUMO-specific proteases have been discovered and reverts target proteins to their unmodified state (255); dynamic interchange of ERα post-translational modifications may add another dimension of receptor regulation precision.

*Neddylation*

NEDD8, also an ubiquitin-like protein (256) , is conjugated (neddylation) in a similar manner as ubiquitination, and involves the action of amyloid precursor protein-binding protein (APP-BP1)/Uba3, a heterodimeric E1-like enzyme, and Ubc12, an E2-like enzyme (257). Whether a ligase is required for neddylation is unknown. To date, the only established function of Uba3 is to activate NEDD8, which is required for neddylation of cullin family members (258, 259). Cullins are essential components of the SCF (Skp1-cullin-F-box protein) group of E3 ubiquitin ligases (260-262). Cullin neddylation plays a crucial role in stimulating the ligase activity of cullin-based ubiquitin ligase complexes (192, 256, 263-265). Regulation of cullin-based ligase activity by Uba3

is the limiting factor in neddylation-associated suppression of receptor activity (191), and is required for the recruitment of a SCF E3 ligase complex to ERα in the presence of ICI (192). Functional neddylation activity of Uba3 is therefore required for ICI-induced degradation of ERα and is essential for the antiproliferative activity of ICI (192). Downregulation of NEDD8 pathway components may be one mechanism for ERα-positive breast cancers to become resistant to ICI (192). Direct neddylation of steroid receptors has not been shown.

## DISSECTION OF ESTROGEN RECEPTOR DEGRADATION PATHWAYS

The selective degradation of many short-lived proteins in eukaryotic cells is carried out by the ubiquitin-proteasome pathway. Proteins are targeted for degradation by covalent ligation to ubiquitin, a highly conserved 76 amino-acid residue protein. Ubiquitin was first discovered in 1975 (266), with subsequent discovery of the multi-step ubiquitin activation (E1), conjugation (E2) and ligation (E3) enzyme system (267) (reviewed in (268)). Ubiquitin-mediated degradation of regulatory proteins plays important roles in the control of numerous processes, including cell-cycle progression, signal transduction, transcriptional regulation, receptor down-regulation, and endocytosis, immune response, development, and apoptosis. Abnormalities in ubiquitin-mediated processes have been shown to cause pathological conditions, including Alzheimer's disease and cancer (268). It is worth noting that Aaron Ciechanover, Avram Hershko and Irwin Rose were awarded the Nobel Prize for Chemistry in 2004 for their the pioneering discoveries that describe the ubiquitin-proteasome pathway.

*E1-E2-E3 ubiquitin attachment*

The 76 ubiquitin protein is covalently linked to proteins targeted for degradation, marking them for recognition by the 26S proteasome (269, 270). Ubiquitin protein ligation requires the sequential action of at least three enzymes. Usually there is a single E1, but there are many species of E2s and multiple families of E3s or E3 multiprotein complexes. Extensive homology exists between ubiquitin and ubiquitin-like proteins such as NEDD8, RUB1, and SUMO. Activation and conjugation of these homologous proteins follow similar E1-E2-E3 pathways (reviewed in (218)). Ubiquitin is activated by conjugation to the ubiquitin activating enzyme (E1 or Uba). The activated ubiquitin is subsequently transferred to a ubiquitin-conjugating enzyme (E2 or Ubc). The E2 proteins catalyze substrate ubiquitination in conjunction with an ubiquitin-protein ligase (E3 or Ubl). E3s are enzymes that bind, directly or indirectly, to specific protein substrates and promotes the transfer of ubiquitin to an ε-amino group of a substrate protein lysine residue (***Figure R5***).

E3 ubiquitin ligases can be classified into three major structurally distinct types: N-end rule E3s, E3s containing the HECT (Homology to E6-AP C-Terminus) domain, and E3s with the RING (Really Interesting New Gene) finger, including its derivatives, the U-box and the PHD (reviewed in (271)). The HECT domain family includes E6-AP (E6-associated protein), and is responsible for the ubiquitinylation and degradation of p53 (272) and is a coactivator for ERα (180). Most HECT-domain proteins are likely E3 enzymes or parts of multiprotein complexes that contain E3-like activities. The RING

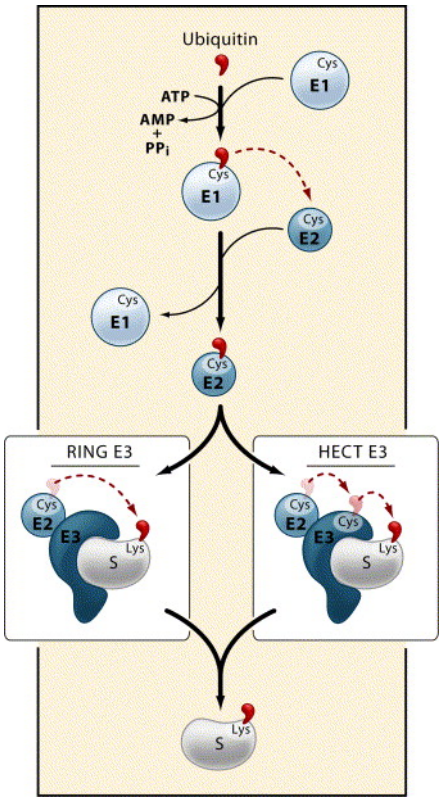



**Figure R5. Outline of the Ubiquitin-Conjugation Pathway** Activation of the ubiquitin C-terminus by E1 proceeds in two steps: adenylation (*not shown*) followed by attack by a cysteine side chain to form a thioester bond between the E1 and ubiquitin. Ubiquitin is then passed to a cysteine of an E2. Ligation of the ubiquitin to a substrate (S) follows either directly with the aid of a RING-bearing E3 or after an intermediate transthiolation to a cysteine side chain of a HECT-domain E3. Both types of E3 interact with their substrates, many of which acquire a polyubiquitin chain rather than just a single ubiquitin. Adapted from Hochstrasser *et al.* Cell 2006 (3).

finger domain binds two zinc atoms using pairs of cysteine residues, creating a cross-braced finger-like structure (273). The PHD (plant homeodomain) and U-box E3s are related to RING finger E3s in that the PHD domain is a variant of the RING finger, whereas U-box E3s adopt a RING structure, but lack conserved Zn-coordinating residues, (274). The U-box E3 ligase CHIP promotes ERα turnover (275, 276), and is discussed in detail in the ERα basal turnover section. RING E3s ubiquitin ligases comprise a heterotetrameric group of proteins consisting of Skp1-Cullin-RING-F-box (SCF) protein complex (256, 277). Together, these proteins coordinate the RING E3-mediated transfer of ubiquitin from the E2 to the protein target, utilizing the F-box as a substrate specificity recognition molecule (256).

After the linkage of ubiquitin to the substrate protein, a polyubiquitin chain is usually formed, in which the C-terminus of each ubiquitin unit is ligated to the previous ubiquitin. Synthesis of long ubiquitin chains is mediated by E3 ligases and in some cases by additional polyubiquitin ligases called E4s (278). p300 contains E4-like activity and facilitates polyubiquitination of p53 following MDM2-mediated monoubiquitination (279). It remains unknown whether p300, as an ERα coactivator, is involved in receptor polyubiquitination.

*The 26S Proteasome*

Proteins ligated to polyubiquitin chains are degraded by the ~1500kDa 26S proteasome complex (reviewed in (269). The 26S generates peptide fragments and free amino acids through the use of three distinct proteolytic activities: trypsin-like, chymotrypsin-like, and post-glutamyl peptidyl hydrolytic (PGPH) activities (280). The

19S subunits act as caps at the entry and exit sites for the 26S proteasome (*Figure R6*). These caps recognize ubiquitinated proteins and contain enzymes that depolymerize the ubiquitin chain and as well as enzymes to unfold the substrate and facilitate its entry into the 20S particle (281). The 19S subunit also isolates the active sites within the 20S particle, restricting substrate entry and delaying or preventing nonspecific destruction of cytosolic proteins. One regulatory protein component of the 19S subunit, SUG1, interacts directly with ERα and stimulates receptor ubiquitination and turnover (282), providing a direct link between ERα and the proteasome.

*Ubiquitin-C-Terminal Hydrolases and Isopeptidases*

The uniqueness of ubiquitin and its ability to direct protein function and degradation necessitates mechanisms that guarantee its efficiency and allow for ubiquitin removal if needed. Many deubiquitinating enzymes (DUBs) exist, suggesting this large number of hydrolases allow for specific functions, such as the recognition of different types of ubiquitin conjugates, or as as proof-reading enzymes, altering the rate of mono-or-polyubiquitination and allowing for reversal of protein fate at various stages of the process (283). DUBs are classed into two distinct families: ubiquitin C-terminal hydrolases (UCHs) and the ubiquitin-specific proteases (USPs/UBPs). UCHs are relatively small enzymes (20-30 kDa) that catalyze the removal of peptides and small molecules from the C-terminus of ubiquitin. Most UCHs cannot generate monomeric ubiquitin from protein conjugates or disassemble poly-ubiquitin chains. USPs can process ubiquitin precursors, remove ubiquitin from protein conjugates and disassemble ubiquitin chains. Most recently the JAMM isopeptidases, otubains and ataxin-3/josephin have also been identified as ubiquitin-specific proteases (268). Proteases also exist for ubiquitin-

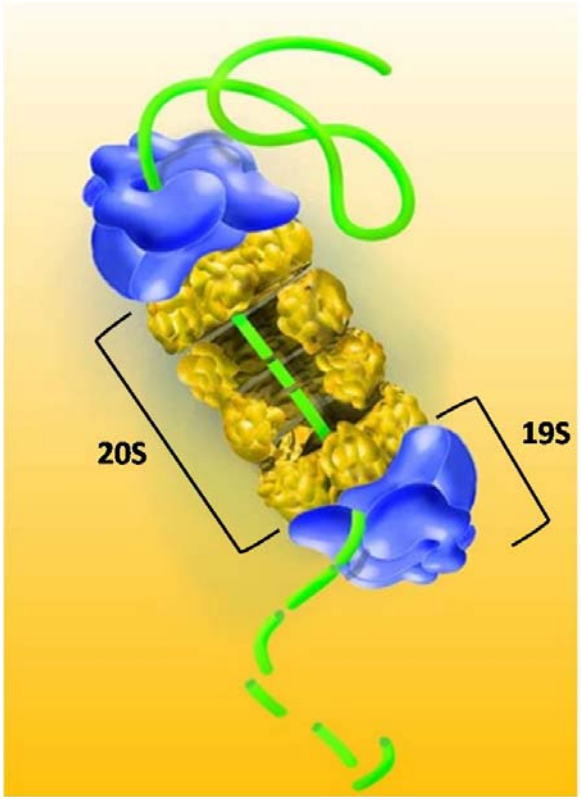



**Figure R6. The 26S proteasome.** The 26S proteasome is composed of a 20S core proteasome subunit and is capped on each end by a 19S regulatory subunit. The ATP-dependent protease domains are contained within the 20S core. The 19S subunits perform regulatory functions, recognizing ubiquitinated proteins and facilitating protein unfolding and proteasomal entry. Image of the proteasome courtesy of Stockholm University, Department of Molecular Biology and Functional Genomics (http://www.molbio.su.se./)

like proteins: SUMO-specific proteases (SENPs) (255), NEDD8-specific proteases (NEDP1, COP9 signalosome) (284) and ISG15-specific proteases (UBP43) (285); common regulatory mechanisms may therefore exist between ubiquitin and ubiquitin-like pathways. DUB-mediated ubiquitin hydrolysis of ERα lysines remains an unexplored field.

*Inhibitors*

Specific inhibitors of the proteasome have proved to be important research tools, allowing dissection of the structure and function of the proteasome as well as elucidating the ERα degradation pathways that converge on the 26S proteasome. Peptide-aldehyde inhibitors, including Cbz-leu-leu-leucinal (MG132) are potent inhibitors of the chymotrypsin-like activity of the proteasome, while lactacystin strongly inhibits both trypsin- and chymotrypsin-like activities of the complex (286). A recently developed derivative of the calpain inhibitors, carboxybenzyl-Leu-Leu-Leu-vinyl sulfone (Z-L3VS) inhibits efficiently and specifically all three activities of the proteasome (287). These inhibitors are eliciting appreciable interest because of their potential applications in medicine, especially in cancer therapy. Bortezomib (originally PS-341 and marketed as Velcade by Millennium Pharmaceuticals) is the first therapeutic proteasome inhibitor to be tested in humans (288). It is approved in the U.S. for treating relapsed multiple myeloma and mantle cell lymphoma. Systemic proteasome inhibition was anticipated to be quite toxic, but Bortezomib has only been limited by increased peripheral neuropathy and myelosuppression in patients. The ability of these inhibitors to induce apoptosis

without severe side effects is likely due to the apparent selectivity towards rapidly dividing, transformed cells (289).

## DISTINCT ERα DEGRADATION PATHWAYS

Distinct mechanisms that convey proteins to the proteasome, including ubiquitination and neddylation, have been identified as key regulatory processes involved in ERα turnover (218). Receptor levels are maintained by distinct degradation pathways that ultimately converge on the ubiquitin-proteasome pathway (182, 192, 205, 290-295). The engagement of one pathway over another depends on the nature of the stimulus (155, 230, 293, 294). The protein partners, pathways, and the potential roles of ERα lysines 302/303 in ERα turnover has been summarized in a model (*Figure 9*). Although seemingly energy wasteful, receptor turnover allows for precise control over receptor-mediated transcription, as the overall magnitude of response to estrogen is dependent on the cellular level of ERα (128, 203, 205). All steroid receptors are subjected to ubiquitination, and several of the enzymes involved in receptor ubiquitination have been identified. In most cases however, the exact site of modification has proven difficult to identify, due to the instability, low-abundance, and relative insolubility of polyubiquitinated proteins.

The decline in receptor number is clearly part of an adaptive response that allows cells to alter sensitivity to external stimuli depending on environmental conditions. The mechanisms responsible for partitioning receptors between these pathways are yet to be elucidated, but tilts in this balance have the potential to alter the availability of functional receptors.

*Proteolysis of unliganded receptor*

In the absence of ligand, ERα is a short-lived protein (half-life of 4-5 hours) and undergoes constant degradation (296). ERα folding involves progressive dynamic association with folding machinery, heat shock proteins, and chaperones in a "foldosome" (297). Through this network, ERα is either processed forward into transcriptionally competent protein or regressed and targeted for degradation. ERα must be protected during the folding process to mask the DNA-binding domain of the receptor, shield the hydrophobic ligand binding pocket, and reduce unliganded receptor activity (298, 299). An equilibrium exists between proteasome-mediated receptor degradation and heat shock proteins (hsp)-mediated receptor folding (300). Receptors associate dynamically with the foldosome, and through cycles of hsp binding and release, ERα is able to adopt a native conformation and achieve ligand binding competency (301). Progression through the foldosome requires ATP hydrolysis mediated by ATPase activity of Hsps and other foldosome components (297). During each of the release or ATP-ADP exchange states, receptors are vulnerable to potential degradation as misfolded proteins. ER is progressively transferred from hsp40 to hsp70 and associated cochaperones Hsc-70-interacting protein (Hip) and Hsp organizing protein (Hop). The U-box E3-ubiquitin ligase CHIP (Carboxy-terminus of Hsc-70-Interacting Protein) is recruited to the Hsp90/Hsc70 complex and is responsible for basal and geldanamycin (GA)-induced receptor ubiquitination (275, 276). CHIP antagonizes receptor maturation by binding to hsp90 through a tetratricopeptide repeat domain and therefore competitively inhibits the binding of other tetratricopeptide repeat-containing cochaperones such as Hop and p23

(302). In disrupting hsp90 interactions with cochaperones, CHIP effectively alters the composition of the chaperone complex. The cochaperone Bag1 is found in early receptor-chaperone complexes and has been shown to link Hsp70 client proteins to the proteasome through its N-terminal ubiquitin-like domain (303, 304). Bag1 may therefore escort hsp70 client proteins within proximity of the proteasome and, through induction of nucleotide exchange, release substrate directly to the 26S proteasome for degradation (305). Bag1 interacts with many steroid hormone receptors, including the androgen (306), estrogen (276), glucocorticoid (307) and retinoic acid receptors (308). Both CHIP and Bag1 have been copurified with the unliganded ERα along with hsp 40, hsp70, hsc70, and hsp90 (275, 276). CHIP has also been found to interact directly with Bag1 (309), suggesting that CHIP and Bag1 may cooperate to increase the turnover rate of the unliganded ERα, by increasing receptor ubiquitination and proteasome delivery (275). Protein synthesis inhibitors cycloheximide and puromycin do not affect the turnover rate of unliganded ERα, revealing that all cochaperone proteins that mediate apo-receptor turnover are present in breast cancer cells (294). Hsp90 inhibitors such as GA (275) and ritonavir (310) enhance apo-ERα turnover. The GA analog 17-allylaminogeldanamycin (17-AAG) has been proposed as chemotherapy in a variety of pathologies including breast cancer, and as such, is currently in phase I clinical trials (311).

*Ligand-induced ERα degradation*

Downregulation of receptor in response to ligand was one of the earliest functional readouts of steroid hormone action (1, 312). Proteasome-mediated ligand-induced downregulation is highly conserved among most nuclear receptors, including

ERα (290-292), PR (313), GR (314), TR (315), RAR (522), RXR (316), MR (317), and PPAR (318), although downregulation in response to ligand is not a generalized response among nuclear receptors since AR (319) and VDR are upregulated by ligand (320). E2-induced receptor degradation is coupled with transcription and requires new protein synthesis (155, 170, 182, 230, 293-295, 321), suggesting that synthesis of regulatory proteins, perhaps an E3 ligase or F-box protein, is required in order to initiate E2-induced turnover. Contrary to breast cancer cells, CHX does not block E2-induced ERα turnover in PR1 pituitary lactotrope cells (290), revealing alternative and tissue-specific mechanisms for ligand-induced receptor turnover.

Upon ligand binding, ERα undergoes a conformational change that leads to the dissociation from a Hsp90-based complex and effectively completes the folding process (35). ERα is then capable of dimerization and translocation to the nuclear compartment. Ligand-induced receptor disassociation from CHIP directs ERα toward alternative degradation pathways (275, 276, 293, 294, 322). In the presence of ligand the turnover rate of ERα can be increased or decreased, depending upon the nature of the ligand, thus modulating receptor protein levels (155, 230, 293, 294). ERα is preferentially ubiquitinated in the presence of E2 (222), and ICI (182, 291). Agonists induce degradation with a potency that is directly related to their binding affinity, though the efficiency of degradation can vary (323). Partial agonists, such as tamoxifen, and antagonists fail to or only weakly induce proteolysis thereby stabilizing ERα (323). Thyroid hormone and protein kinase K activators (Forskolin, 8-bromocAMP) also block receptor degradation and subsequently increase ERα protein levels (324-326).

*Proteasome-related Coactivators*

Aside from receptor degradation, studies of nuclear receptors have revealed multiple points of overlap between the proteasome and receptors. Polyubiquitination and degradation of ERα and other nuclear receptors by the ubiquitin–proteasome system is important for regulating nuclear receptor levels and their transcriptional activities (152, 182, 222, 276, 291). Several components of the ubiquitin-proteasome degradation system, such as PSMC5 (SUG/TRIP1) (177), RSP5/RPF1 (179), UBCH7 (327) and CHIP (275, 276), have been reported to interact with a number of nuclear receptors, including ERα. Recently, sumoylation has been identified as another mechanism that regulates the transcriptional activity of ERα and involves UBC9, PIAS1 and PIAS3 (32, 254).

The E3 enzyme E6-AP, which has been implicated as the cause of Angelman syndrome (328, 329) is a coactivator for ERα (180) along with its conjugating enzyme UbcH7(327), and another E3 ligase mouse double minute 2 (MDM2) (330). The stability of ERα is correlated with the ability of receptor to recruit these E3 ligases to target gene promoters; it appears these coactivators may facilitate stalled transcriptional complexes or removal of pre-initiation complexes thereby enhancing transcription (331). UBCH7 has been identified as a coactivator for several steroid hormone receptors, including ER, PR, AR, GR, and RAR (327), suggesting this mechanism may be conserved among nuclear receptors. The ubiquitination of proteins by the E6-AP E3 enzyme depends on ubiquitin conjugation by UbcH7 (327), although interestingly, the ubiquitin ligase activity of E6-AP is not required for its ERα coactivator function (180).

Suppressor for Gal1 (SUG1), a subunit of the regulatory 19S cap of the proteasome, directly interacts with ERα and ERβ in a ligand-dependent manner and is

recruited simultaneously with elongation factors to the pS2 promoter (152, 153, 332), suggesting the proteolytic component of the 26S proteasome may be recruited to remove stalled RNA polymerase II at the transcriptional stop sites terminating transcription (333). SUG1 stimulates receptor ubiquitination and turnover, thus impairing its transactivating potential (282).

*Signals for Receptor Ubiquitination*

The ERα N-terminal B and C domains, as well as the C-terminal E domain are required for ligand-induced downregulation (182, 318, 331, 334). These two regions encode the AF1 and AF2 domains, respectively, and neither is sufficient to induce turnover on its own. Therefore, one model for signaling degradation might involve ligand-facilitated interactions between the AF1 and AF2 that when joined, create a binding surface for either a ligase or other accessory proteins that is recognized by the ubiquitination machinery. Perhaps folding at the hinge-region exposes the hinge to such machinery. Alternatively, AF1 and AF2 may serve distinct purposes, such as recognition *vs.* recruitment. The overall implication of the dual requirement for B and E domains is that, unlike transcription where AF1 and ASF2 can function as independent modules, the signal(s) for ubiquitin/proteasome-mediated degradation are coordinated by distal regions of the receptor necessitating the full-length receptor. Deletion of the last 61 amino acids of ERα, which includes helix 12 residues, abolishes ligand-mediated downregulation of the receptor, as do point mutations in the ligand binding domain that impair coactivator binding (182). The truncated ERα mutant 1-282 does not contain the LBD/AF2 region and therefore is not degraded after E2 treatment, but is stabilized by MG132, suggesting

that the AF1 domain contains lysine residues that may be ubiquitinated and facilitate unliganded receptor degradation (182).

Phosphorylation events, especially within PEST domains often trigger a given protein to be degraded by the ubiquitin-proteasome pathway (268, 335). A PEST sequence exists in the carboxyl-terminal F domain of ERα (219, 336). Although phosphorylation of the ERα PEST has not been shown, ERα from murine, bovine, and human sources are all modified by O-linked N-acetylglucosamine (*O*-GlcNAc) at Thr-575 within the PEST sequence (219, 336). The PEST sequence for ERα does not appear to regulate receptor turnover (43), and no data exists if *O*-GlcNAc modification alters receptor stability.

The primary target for ligand-induced phosphorylation of ERα is S118, and mutation of this site results in loss of ERα proteolysis, consistent with a possible role for phosphorylation in signaling receptor turnover (323) though later reports by the same group suggested this was not always the case (331). Phosphorylation can either stabilize or destabilize ERα, depending on the kinase and the amino acid residue that is phosphorylated. PKC and ERK7 destabilize ERα (230, 337), while PKA stabilizes ERα (230, 326). Kinase inhibition has also been shown to prevent E2-induced down-regulation of ERα (338), further suggesting that phosphorylation regulates ligand-induced receptor turnover.

BRCA1 mutations are a major contributor for familial breast cancer (339). BRCA1 is a DNA repair enzyme, and when complexed with BARD1, has ubiquitin ligase activity (340). Recently, monoubiquitination of ERα K302 by BRCA1/BARD1 was reported, and reduced receptor transcriptional activity (34). BRCA1 interacts with

RAP80, a deubiquitination enzyme (341), possibly revealing the first deubiquitination enzyme involved in ERα regulation.

*Antiestrogen-induced ERα Turnover*

The SERD class of antiestrogens blocks ERα activity by inhibiting newly synthesized receptors from translocating to the nucleus, blocking receptor-coactivator interaction, and rapidly inducing polyubiquitination and downregulation (140, 155, 182, 198, 230, 291, 342-344). FRAP experiments reveal rapid ERα mobility in the absence of ligand, which is slowed by E2 and OHT, and completely immobilized by ICI (113-115, 152, 155) by receptor sequestration in the nuclear matrix with cytokeratins 8 and 18 (114). Unlike E2-induced turnover, protein synthesis inhibitors (cycloheximide, puromycin) do not inhibit receptor downregulation by pure antiestrogens (292, 295, 345).

Full antagonists like ICI are potent receptor downregulators (323). ICI is structurally a steroidal compound with an extremely long, flexible extending side chain. The current consensus among agonist and antagonist studies is that the degradation signal is in part conferred by the conformation of helix 12 in the ligand-binding domain. Helix 12 serves as a structural probe for ligand binding, and determines receptor fate (36, 346-350). The bulky ICI side-chain prevents helix 12 from closing over the ligand binding pocket resulting in exposure of hydrophobic residues that are normally packed inside the LBD and protected from the surrounding solvent. Exposed hydrophobic patches on the protein surface are known targeting signals for protein degradation (351). Further structural and molecular determinants for distinguishing SERM and SERD action have been probed using derivatives of tamoxifen and ICI (352), revealing the requirement of

hydrogen bonding between ligand functional groups and helix 12 to maintain helix positioning (352). ERα-E542A, a helix 12 receptor mutant, is not degraded in the presence of E2 and has limited degradation in response to ICI 182,780 (349), likely due to loss of a hydrogen bonding residue. Alternatively, as ERα, but not ERβ, is degraded in the presence of ICI (344), ligand binding pocket mutations may also alter ERα in a way that mimics the ERβ ligand-binding pocket. The activating enzyme of NEDD8 (Uba3) inhibits steroid receptor function (191), and was later shown to be required for proteasome-mediated degradation of ERα and essential for the antiproliferative activity of ICI 182,780 in ERα-positive breast cancer cells (192)

*PROTAC: Selective Enhancement of ER Degradation*

E3 ubiquitin ligases and F-box proteins determine the specificity of proteasome-mediated protein degradation. Unfortunately in many cases, including ERα, the E3 or F-box proteins for turnover are not known. One clever remedy is to alter the tropism of a known E3-ligase away from its conventional target and towards a protein of interest. This approach has evolved into the development of a heterobifunctional small molecule called Protein Targeting Chimeric Molecules (PROTAC) that serves as a bridge to link a target protein to an ubiquitin ligase. Such an approach was used to design an E3 ligase that targets ERα or AR (353) and other proteins of interest (354). PROTACs have been developed using the the IκBα phosphopeptide linked to either estradiol (E2) or dihydroxytestosterone (DHT) and were used to to recruit ER or AR to the $SCF^{\beta\text{-}TRCP}$ complex to accelerate their ubiquitination and degradation (353). This approach remains a promising method to direct receptor turnover *in vitro*.

*Novel E3 ligases*

Novel RING E3 ligases are being identified that are closely linked to human breast cancer and are coregulated with ERα. BRCA1/BARD1 binds and monoubiquitinates the ERα hinge (34). In apparent opposition, RAP80, which contains ubiquitin interacting motifs and binds the hinge-region of ERα (355) in a ligand-dependent manner, reduces ERα ubiquitination and stabilizes the receptor. RAP80 is also known to interact with BRCA1 (341). The balance between BRCA1and RAP80 interaction with ERα may be one mechanism that regulates the degree of receptor ubiquitination. Perhaps inhibitors of RAP80 may enhance BRCA1-mediated ERα ubiquitination. Similarly, knockdown of Siah2, an E3-ubiquitin ligase that degrades the corepressor NCoR (356) may also suppress ERα function. Alternatively, upregulation or activation of estrogen-responsive E3 ligases E6-AP (180), Rsp5/Rpf1 (179), and EEP (357) may be useful methods for downregulating ERα.

Although extensive research has gone into the discovery of ERα ubiquitination processes, little is known if/when ERα is deubiquitinated. As inhibition of deubiquitination can promote proteolysis, discovery of a deubiquitin enzyme for ERα may lead to novel drug targets. Inhibiting this enzyme would function similarly to a SERD drug. Broad spectrum inhibitors of DUB enzymes exist, including N-ethyl maleimide (NEM), as well as aldehyde and vinyl sulfone derivatives of ubiquitin. To date, these compounds have not been compared to current drugs that increase receptor ubiquitination.

Continued investigation into the mechanisms by which ERα is degraded will bring a greater understanding to the role this receptor plays in breast cancer proliferation and cancer progression. It is therefore not so much the endpoint as it is the process by which receptors are marked for proteolysis that will have the broadest impact on receptor activity. Further evaluation of the sequence requirement for proteasome-mediated degradation of ERα may reveal post-translational modifications, cellular compartmentalization, DNA binding, or specific protein interactions, that may contribute to ERα fate. The components of the ubiquitination machinery responsible for transferring ubiquitin to receptors are not definitively established, and it remains unknown which of the 29 receptor lysine residues are sites for ubiquitination. Pinpointing the ubiquitinated residues may reveal novel receptor interacting proteins and may provide additional methods to repress ERα action in breast cancer.

# REGULATION OF ERα UBIQUITINATION AND PROTEASOME-MEDIATED RECEPTOR DEGRADATION

## ABSTRACT

Cellular levels of estrogen receptor-alpha (ERα) protein are regulated primarily by the ubiquitin-proteasome pathway. Dynamic interactions between ERα and the protein degradation machinery facilitate the downregulation process by targeting receptor lysine residues for polyubiquitination. To date, the lysines that control receptor degradation have not been identified. Two receptor lysines, K302 and K303, located in the hinge-region of ERα, serve multiple regulatory functions, and we examined whether these might also regulate receptor polyubiquitination, turnover, and receptor-protein interactions. We utilized ERα-negative breast cancer C4-12 cells to generate cells stably expressing wild-type (wt) ERα or ERα with lysine-to-alanine substitutions at K302 and K303 ("ERα-AA"). In the unliganded state, ERα-AA displayed rapid polyubiquitination and enhanced basal turnover, as compared to wtERα, due to its elevated association with the ubiquitin ligase CHIP and the proteasome-associated cochaperone Bag1. Treatment of C4-12 cells with either 17β-estradiol (E2) or the pure anti-estrogen ICI 182,780 (ICI) induced rapid degradation of wtERα via the ubiquitin-proteasome pathway; however, in the presence of these ligands, ERα-AA was less efficiently degraded. Furthermore, ERα-AA was resistant to ICI-induced polyubiquitination, suggesting that these lysines are polyubiquitinated in response to the antiestrogen and demonstrate a novel role for these two lysines in the mechanism of action of ICI-induced receptor downregulation. The reduced stability of ERα-AA in the unliganded state and the increased stability of ERα-

AA in the liganded state were concordant with reporter gene assays demonstrating that ERα-AA has lower basal activity but higher E2-inducibility than wtERα. These data provide the first evidence that K302/303 protect ERα from basal degradation and are necessary for efficient E2 and ICI-induced turnover in breast cancer cells.

## INTRODUCTION

The female sex-steroid hormone 17β-estradiol (E2) is essential for the normal growth and development of the reproductive system and the breast, and its action is mediated primarily by the estrogen receptor alpha (ERα) (358). Aberrant E2 signaling through ERα has been strongly associated with disease development and the progression of breast cancer (206, 207). Thus, appropriate ERα levels and subsequent E2-responses are precisely controlled, in part, by receptor turnover (183, 203-205).

Cellular levels of ERα are maintained by distinct receptor degradation pathways that ultimately converge on the ubiquitin-26S proteasome system (182, 205, 290-295). Although both basal and ligand-induced ERα degradation are mediated by these proteolysis pathways (155, 230, 293, 294, 322), regulation of receptor degradation at the molecular level is highly dependent upon the physiological state and nature of the cellular stimulus. For example, in the unliganded state (*i.e.*, basal receptor turnover), ERα is targeted for degradation by dynamic interactions with heat shock proteins, cochaperones, and the ubiquitin ligase CHIP (Carboxyl terminus of Hsc70-interacting protein) (275, 276). In the presence of E2, hormone-bound receptors are targeted for degradation through a transcription-coupled pathway requiring new protein synthesis (thus blocked by the protein synthesis inhibitor cycloheximide) (292). However, neither transcriptional

activity nor new protein synthesis are needed for degradation of ERα when bound by a class of drugs called selective estrogen receptor downregulators or SERDs (ICI 182,780 or fulvestrant; Faslodex®) (152, 155, 293). Furthermore, drugs that inhibit Hsp90 function (*e.g.,* geldanamycin; GA) induce ERα downregulation by altering receptor-Hsp90 interactions (275, 359, 360), in an ubiquitin ligase (CHIP)-dependent manner (275, 276). In contrast, by dissociating receptor-chaperone complexes, selective estrogen receptor modulators (SERMs; e.g., tamoxifen; OHT) stabilize and protect ERα from both basal turnover and GA-induced degradation pathways (275, 294, 324).

ERα protein turnover results from the formation of polyubiquitin chains on receptor lysines and its subsequent proteasomal degradation through the distinct degradation pathways described above. However, of the 29 lysines found within ERα, none have been identified as residues targeted for polyubiquitination, and thus mediating receptor turnover. Two possible candidates for receptor polyubiquitination are lysines K302 and K303, found within the hinge-region of ERα. Lysines 302 and 303 have multiple regulatory functions, including receptor coactivator binding (33, 245, 361, 362), and in the presence of E2, K302 is monoubiquitinated by BRCA1/BARD1, a ubiquitin ligase (34) The impact of K302 monoubiquitination on ERα stability is unknown, though these data reveal the availability of these hinge-region lysines for posttranslational modification, and we hypothesize that they may be suitable targets for polyubiquitination.

ERα matures into a form capable of ligand binding and transactivation via progression through dynamic receptor-cochaperone interactions (363). Several molecular chaperones, including Hsp70 and Hsp90, mediate ERα progression through this "foldosome" (297) by facilitating ERα interaction with cochaperones, including CHIP,

Bag1 and p23 (Ptges3) (275, 276, 301, 364). ERα hinge-region lysines 302 and 303 reside between the DNA-binding and ligand-binding domains and are within the receptor surface area that interacts with Hsp90 (364, 365). Therefore, mutation of these residues may alter ERα-Hsp90-cochaperone interactions, resulting in altered receptor progression through the foldosome. We and others have shown that the cochaperone CHIP is an E3-ubiquitin ligase required for basal ERα ubiquitination and proteasomal degradation (275, 276). We have also reported that geldanamycin (GA) increases ERα-Hsp90 association with CHIP, enhancing receptor degradation in the absence of ligand (275). The cochaperones Bag1 and p23 have also been found in Hsp90-ERα complexes (276); however, their precise role in receptor turnover remains unknown. Bag1 is found in early receptor-chaperone complexes and has been shown to link Hsp70 client proteins to the proteasome through its N-terminal ubiquitin-like domain (303, 304), suggesting that Bag1 may promote receptor degradation. Conversely, p23 is found in mature receptor-Hsp complexes and has been found to enhance basal and ligand-induced receptor transactivation (366). In addition, p23 competes with CHIP for receptor association (302), suggesting that p23 may exert a stabilizing effect on ERα. These previously defined actions suggest that Bag1, and/or p23, may play a functional role in mediating receptor turnover.

In the present study, we utilized the ERα-negative breast cancer cell line C4-12 to stably express either wild-type (wt) ERα or ERα with lysine-to-alanine substitutions at K302 and K303 ("ERα-AA") to investigate the role of these lysines in ERα polyubiquitination, turnover, and receptor-cochaperone interactions. We demonstrate that lysines K302 and K303, by impeding receptor-CHIP and Bag1 interactions, and

promoting p23 interactions, protect unliganded ERα from basal turnover. Additionally, in the presence of ligand, these two lysine residues control proteasome-mediated turnover and promote ICI-induced receptor polyubiquitination/degradation, thus revealing a novel role for these lysines in regulating receptor turnover.

## RESULTS

### Expression of wtERα and ERα-AA in C4-12 cells

Site-directed mutagenesis was performed on wtERα in pcDNA. Lysines at positions 302 and 303 were mutated to alanines to create ERα-K302A,K303A (ERα-AA; *Fig. 1A*). Stable cell lines harboring wtERα or ERα-AA were established using C4-12 cells, an ERα-negative subline of MCF7 breast cancer cells (367). Two wtERα and three ERα-AA clones were chosen that had similar ERα expression levels. ERα expression level among the clones was two-fold higher than MCF7 cells (*Fig. 1B*). ERα mRNA levels in wtERα and ERα-AA clones were two and four-fold higher than MCF7 cells, respectively (*Fig. 1C*). The discrepancy between ERα-AA mRNA expression and protein levels was due to elevated basal ERα-AA degradation (described later).

### Lysines 302/303 reduce basal turnover of unliganded ERα

As apo-(unliganded) ERα is a short-lived protein and undergoes constant degradation (290, 291, 296), we investigated the effect of hinge-region lysine mutations on stability of the unliganded receptor.

C4-12 cells stably expressing wtERα or ERα-AA were treated with the protein synthesis inhibitor cycloheximide (CHX) and apo-receptor degradation was then monitored by

Fig. 1A

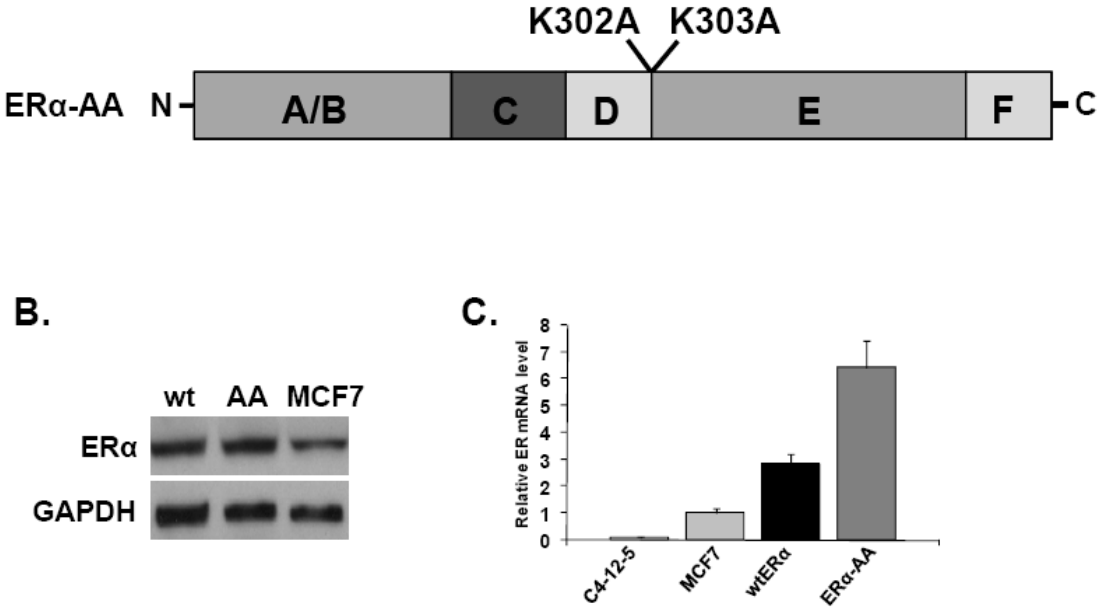



**Figure 1. Expression of wtERα and ERα-AA in C4-12 cells. A)** Schematic showing the mutation of hinge-region lysines to generate ERα-AA. Lysines 302 and 303 reside in the hinge-region between the DNA-binding domain (letter D) and the ligand-binding domain (letter E). **B)** Representative ERα protein levels from two wtERα clones and three ERα-AA clones. GAPDH was used as SDS-PAGE loading control. ERα negative breast cancer C4-12 cells were transfected with 1μg of pcDNA-ERα (wtERα) or ERα-K302A,K303A (ERα-AA) using Lipofectamine/PLUS and then treated with 800μg/ml G418 daily until single colonies were visible. Multiple clones from each transfection were selected and expanded. **C)** RT-qPCR analysis of ERα mRNA levels. EF1α mRNA level was used as internal control.

immunoblot. Levels of wtERα decreased in a time-dependent manner, displaying a half-life of 3.85 ± 0.3 h (*Fig. 2A, upper panel and Fig. 2C*), in agreement with previous reports using CHX and other methods (43, 295, 296, 368, 369). In comparison, increased ($p<0.01$) basal turnover of the mutant receptor ERα-AA was observed, and the half-life of ERα-AA was 1.04 ± 0.3 h (*Fig. 2A, lower panel and 2C*). These results demonstrate that lysines 302/303 regulate ERα stability in the unliganded state by limiting basal receptor turnover.

In the absence of ligand, apo-ERα is degraded by associating with heat shock proteins, including Hsp90, and the E3-ubiquitin ligase CHIP (275, 276). The Hsp90 inhibitor geldanamycin (GA), by blocking ATP binding to Hsp90 (311, 370-372), enhances ERα association with CHIP and increases receptor turnover (275, 359, 360). As lysines 302 and 303 reside in the Hsp90-interacting domain of ERα (364, 365), we investigated the effect of hinge region lysine mutation on GA-induced ERα turnover. Treatment of C4-12 cells stably expressing wtERα with GA displayed an increased ($p<0.01$) receptor turnover rate; the half-life of wtERα decreased from 3.85 ± 0.3 h to 1.40 ± 0.3 h (*Fig. 2B, upper panel and Fig. 2C*). GA treatment did not further increase ERα-AA basal turnover rate; receptor half-life was unchanged in the absence or presence of GA (1.04 ± 0.3 h vs. 0.92 ± 0.3 h, respectively; *Fig. 2B, bottom panel and Fig. 2C*). To summarize these findings, CHX and CHX/GA data are plotted together in Figure 2C. As shown, GA-induced wtERα turnover occurred at the same rate as ERα-AA basal and GA-induced turnover, suggesting that loss of lysines 302/303 and Hsp90 inhibition by GA share a common molecular mechanism to promote ERα degradation.

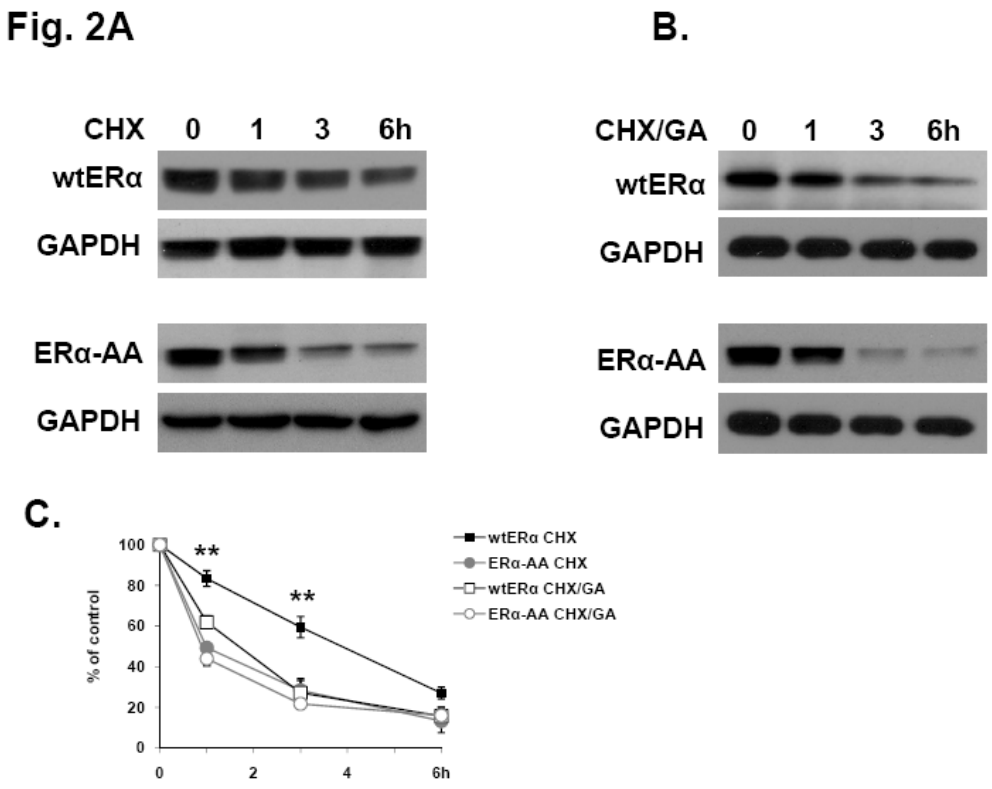



**Figure 2. Lysines 302/303 reduce basal turnover of unliganded ERα.** C4-12 cells stably expressing wtERα or ERα-AA were treated with **A)** CHX (25μg/ml) for 30 min before cell harvest at the indicated time points. **B)** CHX (25μg/ml) for 30 min, followed by GA (1μM) for the indicated times. Experiments were performed in duplicate and repeated twice using two wtERα-expressing clones and three ERα-AA-expressing clones. GAPDH was used as SDS-PAGE loading control. **C)** Turnover rates of ERα and ERα-AA in the absence or presence of GA. The band density of the exposed film was evaluated with ImageJ software. Relative ERα levels (*vs.* untreated cells) are shown as means ± SE. **p<0.01.

**Lysines 302/303 protect unliganded ERα from polyubiquitination**

Rapid ERα-AA protein turnover was observed in the absence of ligand (*Fig. 2*) and ERα-AA mRNA levels were increased in ERα-AA clones that had equal levels of protein as wtERα (*Fig. 1C*), suggesting that loss of lysines 302/303 resulted in a destabilized receptor in the absence of ligand. As ERα turnover is mediated by the ubiquitin-proteasome pathway (182, 290-292), we investigated the role of K302 and K303 in ubiquitination of ERα. Polyubiquitination assays in ERα-negative HeLa cells were performed as we have described previously (275). Briefly, cells were transfected with equal amounts of wtERα or ERα-AA in addition to a hemagglutanin-tagged ubiquitin (HA-ubiquitin) or vector control plasmid. Transfected cells were then treated with the proteasome inhibitor MG132 and allowed to accumulate polyubiquitinated proteins. Following MG132 treatment, immunoprecipitation was carried out using an ERα-specific antibody. Proteins were then resolved by SDS-PAGE and the presence of ubiquitinated receptor was detected by immunoblotting with an HA antibody (polyubiquitinated species were detected as a high-molecular-weight ladder on the membrane). In the absence of MG132, wtERα polyubiquitination levels remained low (*Fig.3*, *lane 1*), but subsequently increased after MG132 treatment (*lane 3*). In contrast, in the absence of MG132, total immunoprecipitated (*lower panel*) and polyubiquitinated forms of ERα-AA species were notably more abundant than untreated wtERα (*lane 5*). In addition, MG132 treatment resulted in greater accumulation of polyubiquitinated forms of ERα-AA and total ERα-AA protein compared to wtERα (*Fig. 3, lane 6).* While it appeared that the mutant receptors may be more polyubiquitinated than wtERα, using these methods it was

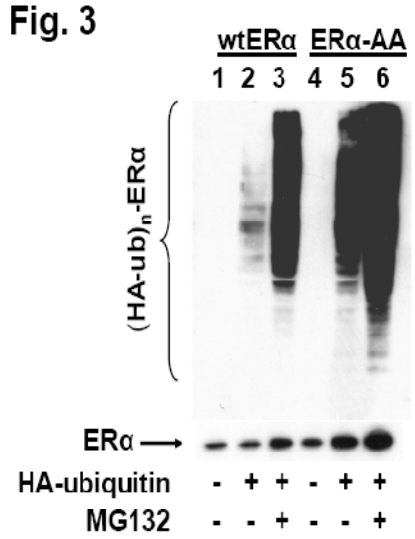



**Figure 3. Lysines 302/303 protect unliganded ERα from polyubiquitination.** HeLa cells were transfected with equal amounts (250ng) of wtERα or ERα-AA along with 1μg HA-ubiquitin, using LipofectAMINE/PLUS. 24 h later, the cells were treated with DMSO or MG132 for 5h, and then ERα immunoprecipitated with anti-ERα antibody. Precipitated proteins were then resolved by SDS-PAGE and Western blot performed using an HA antibody. Levels of immunoprecipitated ERα were also determined by probing with anti-ERα antibody (*bottom panel*).

not possible to quantify the degree of polyubiquitination per receptor. The mutant receptor displayed enhanced basal turnover rate and possibly enhanced basal polyubiquitination, indicating that lysine residues 302 and 303 may protect ERα from basal degradation by limiting apo-receptor ubiquitination .

**K302 and K303 reduce ERα association CHIP and Bag1 complexes**

We and others have previously shown that GA increases the association of ERα-Hsp90 complexes with the E3-ubiquitin ligase CHIP, increasing receptor degradation (275 , 276). As apo-ERα-AA had a rapid basal turnover rate that was not further increased by GA (*Fig. 2B*), we investigated the possibility that lysine mutations mimic the effects of GA by enhancing ERα-AA association with CHIP. In addition, we wished to examine ERα interactions with the Bag1 cochaperone. Bag1 links Hsp70 to the proteasome (303) and has also been detected in ERα complexes (276), but its precise role in ERα turnover has not been previously explored. Finally, as GA inhibits p23 interaction with Hsp90 (373) and also blocks p23-mediated enhancement of receptor transactivation (373, 374), we examined receptor-p23 interactions to determine whether p23 plays a stabilizing role on ERα. To investigate the role of K302/303 in Hsp90, CHIP, Bag1 and p23 interactions with the receptor protein, and to determine if alterations in receptor-cochaperone interactions contributed to basal turnover of ERα-AA, we performed coimmunoprecipitation assays and analyzed Hsp90-cochaperone-receptor complexes in the presence or absence of GA. Complexes were immunoprecipitated with an ERα-specific antibody and complexed proteins then identified by immunoblot, as shown in Figure 4A. Relative receptor-cochaperone levels for CHIP, Bag1, and p23 are

shown in Figure 4B. In untreated C4-12 cells stably expressing either wtERα or ERα-AA, comparable levels of ERα, Hsp90, CHIP, Bag1, and p23 proteins were observed (*Fig. 4A, lanes 1 & 6*). Prior to treatment, wtERα coimmunoprecipitated with Hsp90, CHIP, the cytosolic form of Bag1 (36kDa), and p23 (*Fig. 4A, upper panel, lane 2*). ERα-AA also coimmunoprecipitated with these cochaperones, but association of ERα-AA with Bag1 and CHIP appeared to be enhanced, and only a weak association of ERα-AA with p23 was observed (*Fig. 4A, lower panel, lane 7*). Overall levels of immunoprecipitated Hsp90 did not change throughout the duration of the experiment for both wtERα and ERα-AA (*Fig. 4A*), suggesting that changes in receptor turnover were not simply due to changes in ERα-Hsp90 interaction. GA treatment resulted in an increase in association of CHIP and Bag1 with wtERα, with a concomitant decrease in p23 association (*Fig. 4A, lane 3; Fig. 4B*). While GA treatment further enhanced the interaction of ERα-AA with CHIP and Bag1, the p23 association was completely abolished (*Fig. 4A, lanes 8 & 9; 4B*). These data suggest that K302/303 stabilize ERα by facilitating receptor progression through the foldosome, decreasing interaction with CHIP and Bag1, and increasing interaction with p23.

**Depletion of CHIP and Bag1 reduces ERα turnover, while p23 knockdown increases receptor turnover**

Previously, we have demonstrated that knockdown of CHIP via siRNA abolished basal and GA-induced ERα downregulation in both HeLa and MCF7 cells (275). To further establish a role for CHIP, Bag1, and p23 in regulating ERα turnover, we used siRNA to investigate turnover of wtERα and ERα-AA in the absence of these cochaperones. CHIP associated more strongly with rapidly-degraded ERα-AA (*Fig. 4),*

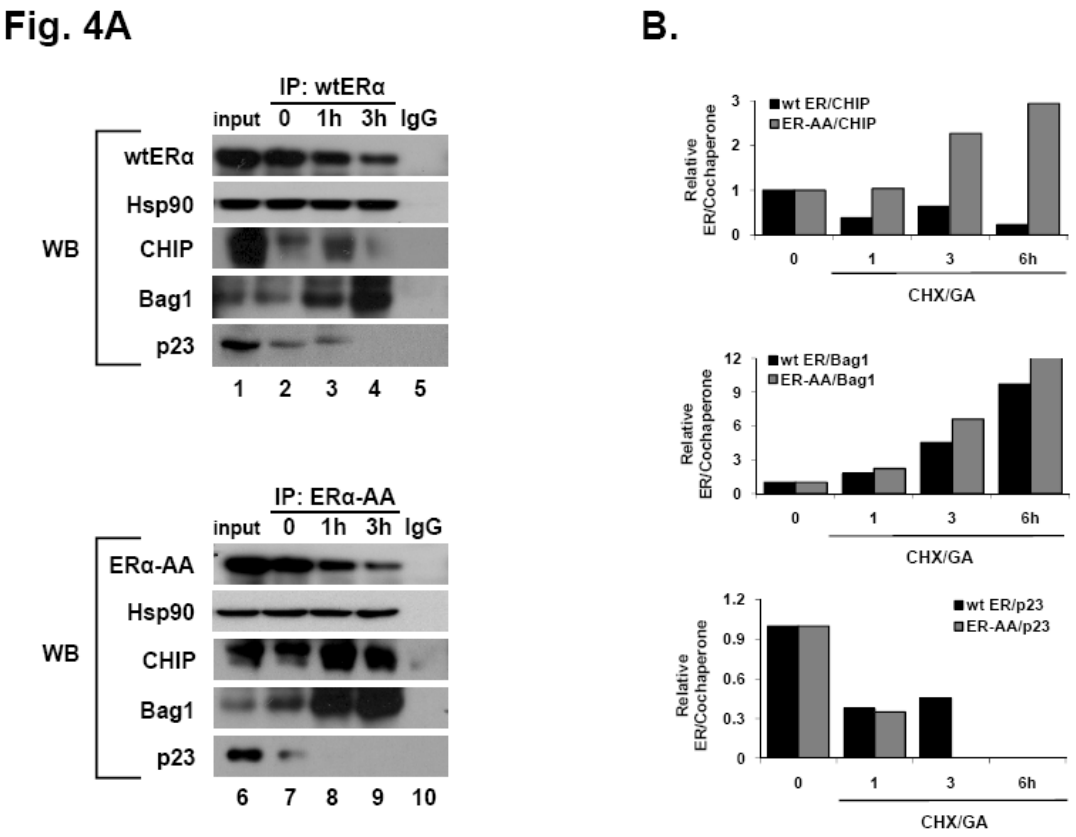



**Figure 4. K302 and K303 reduce ERα association with CHIP and Bag1 complexes.**

**A)** C4-12 ERα stable cells were pretreated with CHX (25μg/ml), followed by GA (1μM) for 0-6h. Proteins were harvested and receptor-cochaperone complexes immunoprecipitated with anti-ER or control IgG antibodies. Proteins were resolved by SDS-PAGE and Western blot performed using specific antibodies against ER, p23, Bag1, and CHIP. **B)** Relative cochaperone/ER levels for CHIP, Bag1, and p23. Cochaperone/ER ratios were normalized to cochaperone/wtERα levels for untreated cells.

and ERα-AA was also resistant to GA-induced degradation (*Fig. 2*), suggesting that ERα-AA degradation is CHIP-dependent. siRNA against CHIP was performed in HeLa cells as we have described previously (275). HeLa cells were transfected with equal amounts of wtERα or ERα-AA plasmid, with or without the CHIPi vector, and cells were then treated with CHX followed by vehicle or GA). Empty vector and mock transfection had no effect on CHIP levels (*Fig. 5A, top panel*). In addition, basal and GA-induced downregulation in the vector control cells were not different than that of cells treated in Figure 2 and were used as controls for CHIPi assays. Expression of CHIPi decreased the level of CHIP by over 60% (*Fig. 5A, top panel*), and this was sufficient to completely block basal turnover of both wtERα and ERα-AA (*Fig. 5A, middle panel*). CHIPi also blocked GA-induced turnover of wtERα and ERα-AA (*Fig. 5A, bottom panel*), thus confirming that CHIP mediates basal and GA-induced turnover of wtERα and ERα-AA.

Similar to CHIP, association of Bag1 was stronger with ERα-AA than wtERα. As this protein has been shown to link Hsp70 to the proteasome (303, 304), C4-12 cells were transfected with siRNA against Bag1 or scrambled (Sc) siRNA to investigate whether Bag1 is involved in ERα turnover. Scrambled siRNA or mock transfection had no effect on Bag1 levels (*Fig. 5B, top panel),* and basal and GA-induced ERα downregulation was not different from untransfected cells (see *Fig. 2C*). Scrambled siRNA was therefore used as a control for Bag1 siRNA treatment. Knockdown of Bag1 in C4-12 stable clones delayed basal turnover of wtERα, increasing ($p< 0.01$) its half-life from 3.14 ± 0.3h to >6 h (*Fig. 5B, middle panel*). Bag1 knockdown also delayed ERα-AA basal degradation, increasing ($p< 0.01$) the mutant receptor half-life from 1.2 ± 0.3 h (*see Fig. 4A*) to 3.4 ± 0.2 h (*Fig. 5B, middle panel*). GA-induced downregulation of both wtERα and ERα-AA

Fig. 5A

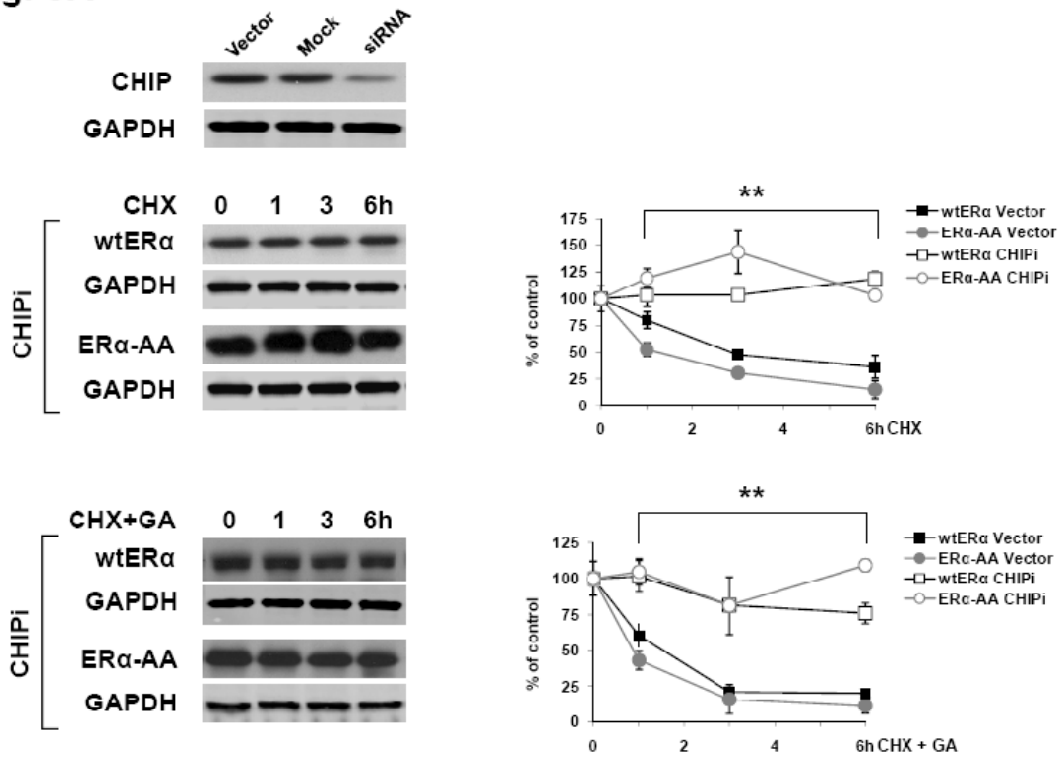



**Figure 5A. Depletion of CHIP and Bag1 reduces ERα turnover, while p23 knockdown increases receptor turnover.** For siRNA CHIP experiments, HeLa cells were transfected with equal amounts of wtERα or ERα-AA along with CHIPi, pcDNA empty vector, or transfection reagent only (Mock). 24 h later, cells were pretreated with CHX followed by vehicle or GA (1μM) for the time periods indicated. Western blots were then performed against CHIP and ERα, using specific antibodies.

Fig. 5B

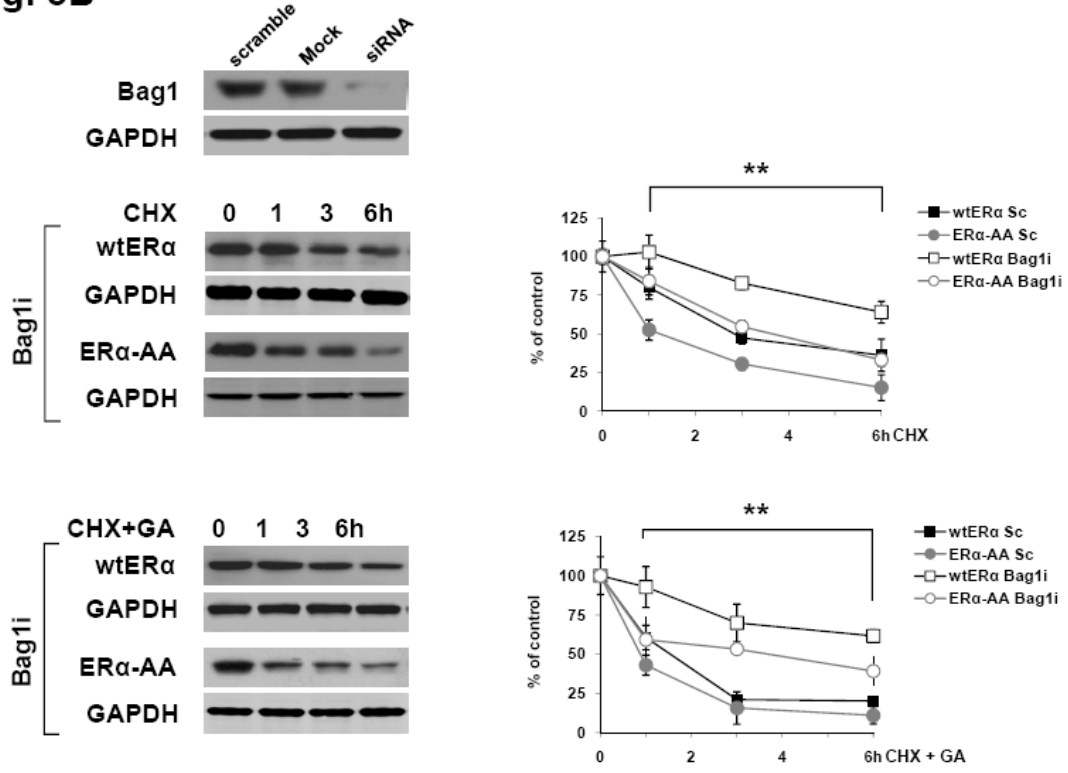



**Figure 5B)** siRNA against Bag1 (Bag1i, 50nM) and control scrambled oligo (Sc, 100nM) were transfected into C4-12 stable cells using the siRNA transfection reagent Dharmafect1 for 3 d. 72 h after transfection, cells were pretreated with CHX, followed by vehicle or GA for the indicated time periods. Protein levels were examined by Western blot using specific antibodies. The band density of exposed film was evaluated with ImageJ software. Sc (scrambled siRNA) and mock (M; Dharmafect1 only) were specificity and transfection controls. GAPDH was used as SDS-PAGE loading control. Experiments were performed in duplicate and repeated twice using two wtERα and three ERα-AA clones. **$p<0.01$.

Fig. 5C

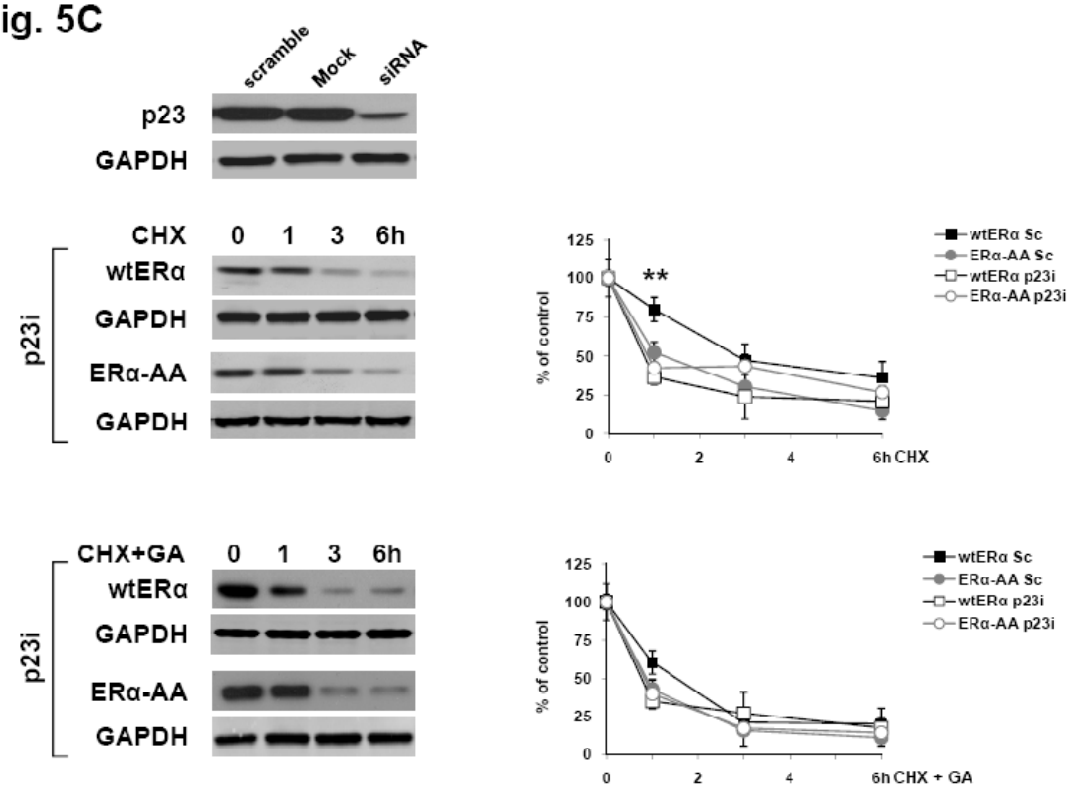



**Figure 5C)** siRNA against p23 (p23i, 100nM), and control scrambled oligo (Sc, 100nM) were transfected into C4-12 stable cells using the siRNA transfection reagent Dharmafect1 for 3 d. 72 h after transfection, cells were pretreated with CHX, followed by vehicle or GA for the indicated time periods. Protein levels were examined by Western blot using specific antibodies. The band density of exposed film was evaluated with ImageJ software. Sc (scrambled siRNA) and mock (M; Dharmafect1 only) were specificity and transfection controls. GAPDH was used as SDS-PAGE loading control. Experiments were performed in duplicate and repeated twice using two wtERα and three ERα-AA clones. **p<0.01.

was delayed by Bag1 knockdown; wtERα half-life was increased ($p < 0.01$) from 1.40 ± 0.3 h to >6 h, and ERα-AA half-life increased ($p < 0.01$) from 0.92 ± 0.3 h to 3.3 ± 0.2 h (*Fig. 5B, lower panel*; *and see Fig. 2C*), thus confirming that Bag1 promotes both basal and GA-induced receptor turnover.

The cochaperone p23 is associated with mature Hsp90 complexes and enhances ERα transactivation (365, 366). ERα-AA bound less strongly to p23 than wtERα; consequently, we transfected C4-12 cells with siRNA against p23 or scrambled siRNA to assess whether loss of p23 would destabilize ERα and enhance its turnover. Scrambled siRNA or mock transfection had no effect on p23 levels (*Fig. 5C, top panel),* and basal and GA-induced ERα downregulation in cells transfected with scrambled siRNA or mock transfection were not different from untransfected cells (see *Fig. 2C*). Scrambled siRNA was therefore used as a control for p23 siRNA treatment. Knockdown of p23 enhanced wtERα turnover in CHX-treated cells; receptor half-life decreased from 3.1 ± 0.3 h to 0.85 ± 0.1 h (*Fig. 5C, middle panel*). Moreover, GA-induced turnover of wtERα was also increased after p23 knockdown ($p < 0.01$), with its half-life decreased from 1.5 ± 0.2 h to 0.75 ± 0.2 h (*Fig. 5C, lower panel*). However, p23 knockdown had no effect on basal or GA-induced turnover of ERα-AA, as the half-life of ERα-AA was similar in the presence or absence of p23 siRNA (1.04 ± 0.3 h *vs.* 0.94 ± 0.1 h; *Fig. 5C, middle panel*). This was not unexpected, as low levels of p23 were detected in ERα-AA-immunoprecipitated complexes (*Fig. 4A*). These results demonstrate that p23 exerts a stabilizing effect on ERα. Together, these data suggest that CHIP and Bag1 promote, while p23 inhibits, basal and GA-induced ERα degradation. Furthermore, K302/303

appear to be important for the association of ERα with these cochaperones during basal and GA-induced receptor turnover, by decreasing receptor association with the degradation-promoting cochaperones CHIP and Bag1, while increasing association with the stabilizing cochaperone p23.

**Hinge-region lysines promote ligand-induced receptor turnover**

Ligand binding dissociates ERα from Hsp90 complex and directs ERα toward alternative degradation pathways (293, 294, 322). To investigate the effect of hinge-region lysine mutations on ligand-mediated receptor turnover, C4-12 cells were treated with various ligands, and changes in ERα stability were monitored by immunoblot. As shown, E2 induced ERα downregulation following transcriptional activation, decreasing wtERα protein level in a time-dependent manner (*Fig. 6A, upper panel*), while impairing degradation of ERα-AA under the same experimental conditions (*Fig. 6A, lower panel*). ICI, which directly targets ERα for degradation (152, 155, 192, 293), similarly reduced wtERα levels to less than 50% by 1 h (*Fig. 6B, upper panel*); this same level of reduction in ERα-AA levels was not seen until 3 h after ICI treatment (*Fig. 6B, lower panel*). CHX pretreatment did not significantly affect ICI-induced downregulation of either receptor (*Fig. 6C*), confirming that the slower decline of ERα-AA protein in the presence of ICI was due to impaired receptor degradation rather than elevated synthesis of the mutant ER. These data suggest two possible roles for ligand action on ERα-AA: 1) Ligand binding may rescue ERα-AA from interaction with CHIP and Bag1, protecting it from the rapid basal turnover observed in Figure 2B; or 2) ERα-AA may be less sensitive to ligand-induced degradation.

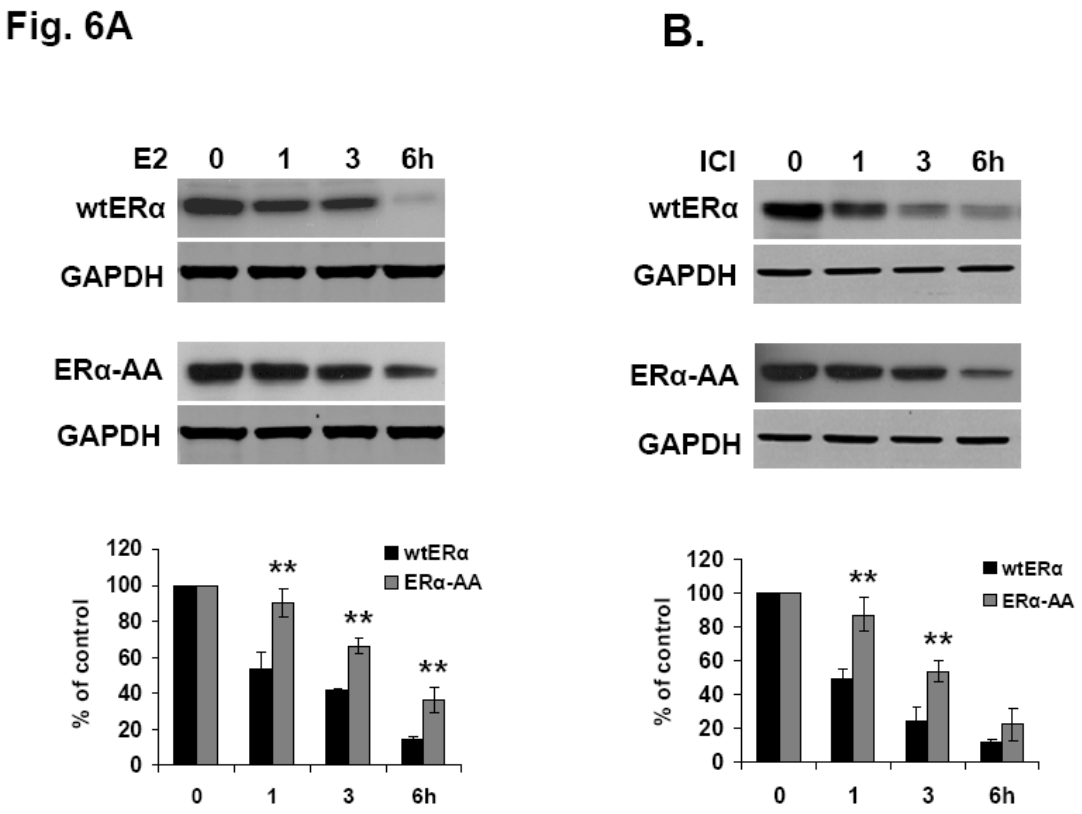



**Figure 6. Hinge-region lysines promote ligand-induced receptor turnover.** C4-12 ERα stable cells treated with **A)** E2 (10nM), or **B)** ICI (100nM) for the indicated times**.** Experiments were performed in duplicate and repeated twice using two ERα and three ERα-AA clones. GAPDH was used as SDS-PAGE loading control. The band density of exposed film was evaluated with ImageJ software. Relative ERα levels (*vs.* untreated cells) are shown in the corresponding graph as the mean ± SE. ** $p<0.01$.

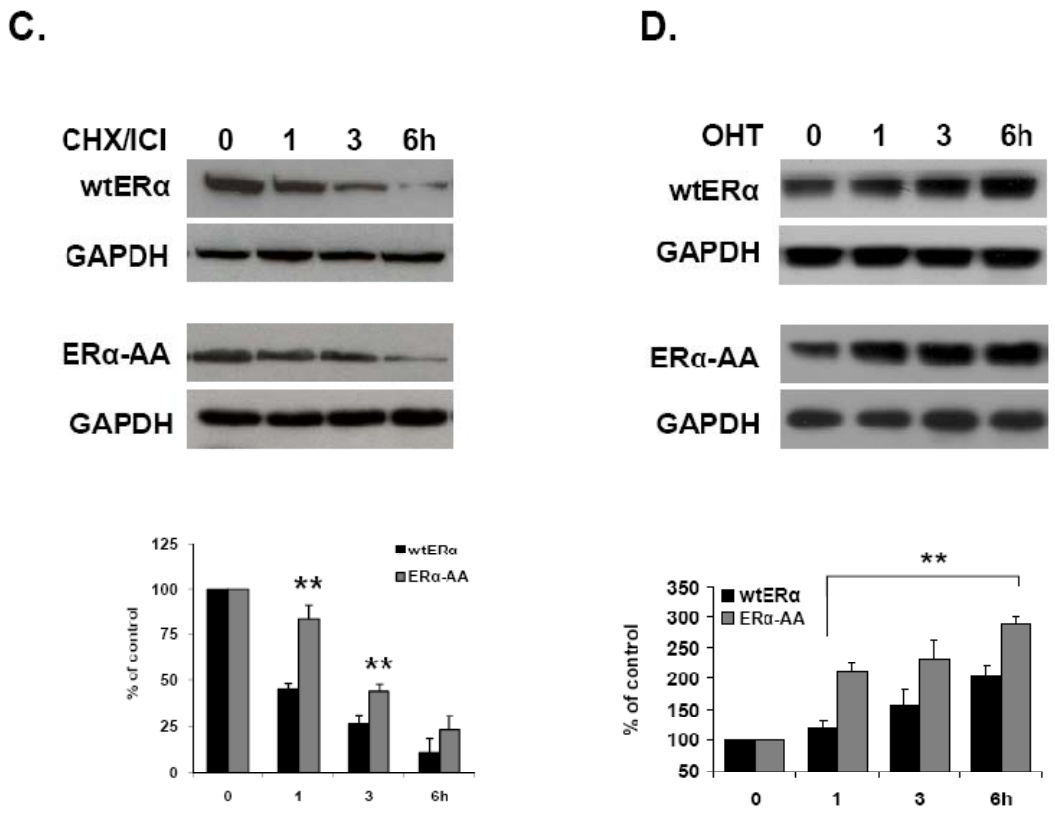



**Figure 6. Hinge-region lysines promote ligand-induced receptor turnover.** C4-12 ERα stable cells treated with **C)** CHX (25ug/ml 30' followed by 100nM ICI) or **D)** OHT (100nM) for the indicated times**.** Experiments were performed in duplicate and repeated twice using two ERα and three ERα-AA clones. GAPDH was used as SDS-PAGE loading control. The band density of exposed film was evaluated with ImageJ software. Relative ERα levels (*vs.* untreated cells) are shown in the corresponding graph as the mean ± SE. ** $p<0.01$.

By dissociating receptor-chaperone complexes, 4-hydroxytamoxifen (OHT) stabilizes ERα and protects receptors from basal turnover (275, 294, 324). As expected, wtERα was stabilized by OHT (*Fig. 6D, upper panel*), and OHT caused ERα-AA levels to accumulate (*Fig. 6D, lower panel*), suggesting that OHT was able to antagonize rapid ERα-AA basal turnover, further implicating lysines 302/303 in protecting ERα from basal turnover.

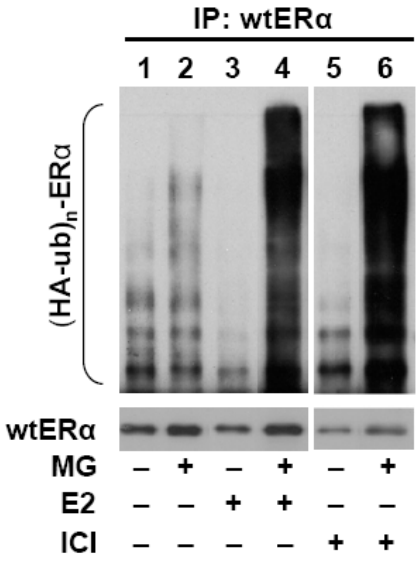
**Hinge-region lysines promote ligand-induced receptor polyubiquitination**

It is well known that both E2 and ICI stimulate receptor ubiquitination (192, 222, 291, 375) and its subsequent degradation by the 26S proteasome (291). Consequently, our observation that these ligands were unable to efficiently degrade ERα-AA indicated that ERα-AA may be resistant to polyubiquitination. To investigate this possibility, we measured the ubiquitination of ERα and ERα-AA after inhibiting the proteasome with MG132 and stimulating receptor ubiquitination with E2 or ICI. ERα-negative HeLa cells were transiently transfected with equal amounts of wtERα or ERα-AA expression constructs, along with the HA-ubiquitin construct. Cells were then pretreated with DMSO or MG132 before treatment with DMSO, E2, or ICI. Subsequently, ERα was immunoprecipitated with an ERα-specific antibody and HA-polyubiquitinated species of ERα were detected as a high-molecular-weight ladder on the membrane. As shown, MG132 treatment of cells containing wtERα resulted in accumulation of polyubiquitinated receptor forms (*Fig. 7A, lane 1 vs. 2*). After E2 or ICI treatment, similar levels of ubiquitinated wtERα were observed, presumably due to proteasomal degradation of ubiquitinated receptors (*Fig. 7A, lane 3 & 5*). As expected, proteasome

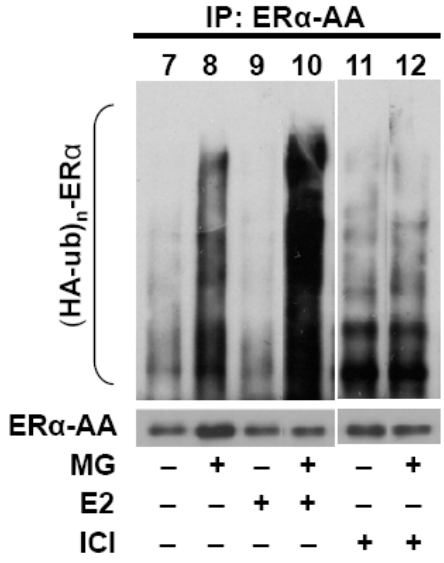
inhibition with MG132, prior to E2 or ICI treatment, resulted in the accumulation of polyubiquitinated wtERα (*Fig. 7A, lanes 4 & 6)*.

In ERα-AA transfected cells, MG132 treatment also resulted in accumulation of polyubiquitinated receptor forms, but to a greater extent than cells transfected with wtERα (*Fig. 7, compare lanes 1-2 vs. 7-8*). In the absence of MG132, E2 treatment did not further increase ERα-AA ubiquitination (*Fig. 7B, lanes 9*); however, MG132 pretreatment increased polyubiquitinated ERα-AA in the presence of E2 (*Fig. 7B, lanes 10*). Ubiquitination levels of ERα-AA and wtERα in the presence of E2 were similar (*Fig. 7, lane 3 vs. 9*). ICI induced modest polyubiquitination of ERα-AA in the absence of MG132 (*Fig. 7B, lane 11*), although these levels were not different than ICI-induced polyubiquitination in the presence of MG132 (*Fig. 7B, lane 11 vs. 12*). Importantly, ICI-treated ERα-AA protein levels were slightly higher than ICI-treated wtERα levels, but less polyubiquitination occurred in ERα-AA cells (*Fig 7. lane 12)*. An ERα mutant with lysine-to-arginine substitutions, ERα-K302R/K303R (ERα-RR), shared a similar ubiquitination profile to that of ERα-AA. In contrast to wtERα, both mutant receptors were heavily ubiquitinated in the absence of ligand and no further ubiquitination was observed in response to ICI treatment (*Fig. 7C*). These results indicate that K302/303 may be direct targets for polyubiquitination in the presence of ICI. We therefore report a previously undescribed role for these hinge-region lysines in mediating receptor polyubiquitination induced by the pure antiestrogen.

Fig. 7A




B.

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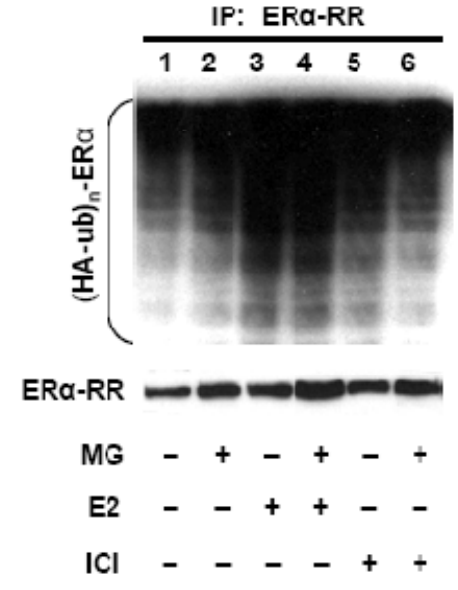


**Figure 7. Hinge-region lysines promote ligand-induced receptor polyubiquitination.** HeLa cells were transfected with equal amounts (250ng) of **A)** wtERα **B)** ERα-AA or **C)** ERαK302R/K303R, along with 1μg HA-ubiquitin using LipofectAMINE/PLUS. Transfected cells were pretreated with vehicle (DMSO) or MG132 (25μM) for 1 h, followed by DMSO, E2 (10nM) or ICI (100nM) for 4 h. ERα was then immunoprecipitated with anti-ERα antibody. Precipitated proteins were resolved by SDS-PAGE and Western blot performed with an HA antibody. Levels of immunoprecipitated ERα were also determined by probing with an anti-ERα antibody (*lower panel*).

**K302 and K303 contribute to ERα target gene transactivation**

While E2 binding increases ERα transactivation, apo-ERα is also capable of eliciting basal transcriptional activity (151). Mutating K302 and K303 resulted in rapid ERα turnover in the absence of ligand (*Fig. 2*), but increased receptor stability in the presence of E2 (*Fig. 6*). It was therefore of interest to examine whether these two hinge region lysines play a functional role in ERα transactivation in the presence and absence of E2. To examine transcriptional competency of ERα-AA, basal and E2-induced receptor activity was examined utilizing an E2-responsive chloramphenicol acetyltransferase construct (ERE-CAT (183)). C4-12 stable cell lines were transiently transfected with an ERE-CAT reporter and treated with E2. The absolute CAT levels in untreated (DMSO) ERα-AA-expressing cells exhibited lower ($p<0.01$) transcriptional output than cells expressing wtERα (0.15±0.02 *vs.* 1.62±0.09 pg/mg lysate; *Fig. 8A*). E2 treatment elicited a response in both cell lines, but CAT-expression remained lower ($p<0.01$) in ERα-AA-expressing cells *vs*. wtERα-expressing cells (0.98 ±0.06 *vs.* 2.67±0.06 pg/mg lysate), suggesting an overall reduction in ERα-AA mediated transcriptional activity. Normalized CAT values (untreated CAT levels set to one; *Fig. 8B*) revealed that E2-induced fold changes in CAT levels were higher ($p<0.01$) for ERα-AA compared to wtERα (11.12±2.58 fold *vs.* 2.11±0.19 fold), suggesting that mutation of K302/303 results in overall lower transcriptional activity, with enhanced E2-inducibility.

In a more physiologically relevant context, we investigated the expression of the endogenous E2-responsive gene cathepsin D in C4-12 cells. Induction of cathepsin D levels by E2 was observed in both wtERα and ERα-AA C4-12 cells (*Fig. 8C)*. Similar to CAT assays, at all time points examined, absolute levels of cathepsin D mRNA were

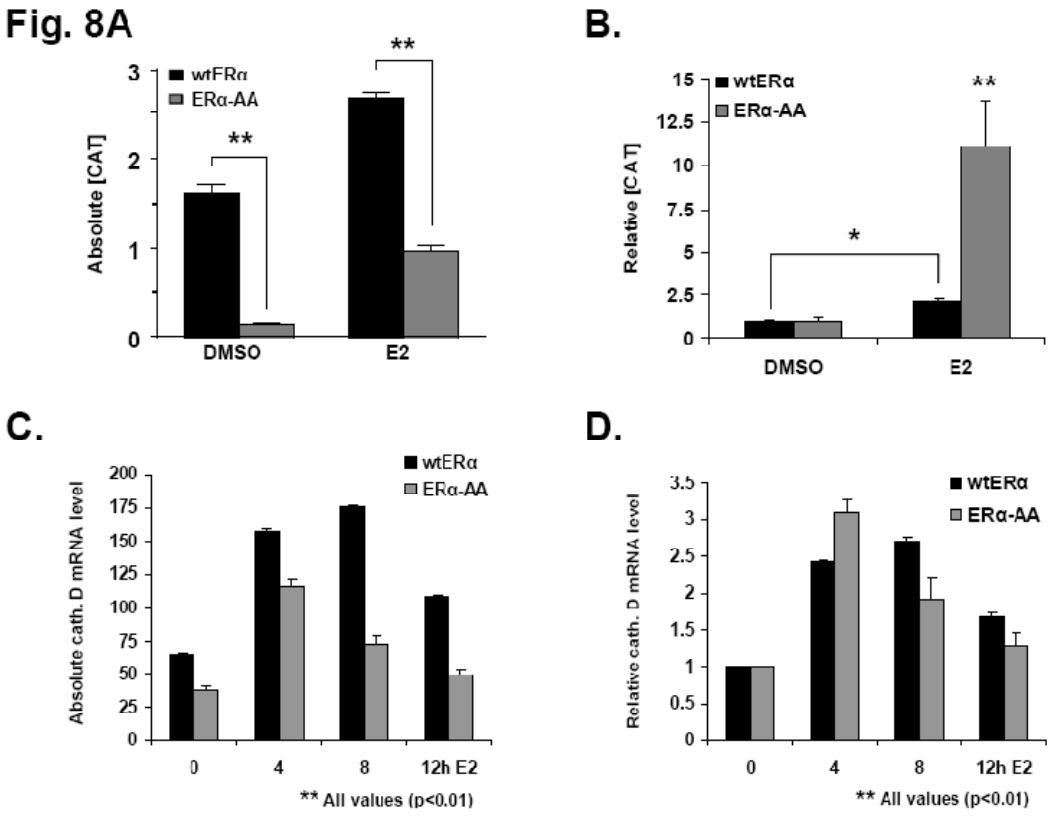


**Figure 8. A) K302 and K303 contribute to ERα target gene transactivation.** A) Estrogen-responsive CAT assays were performed in C4-12 ERα stable cells. Cells were transfected with 250 ng ERE-Vit-CAT and then treated with DMSO or E2 (10nM) for 48 h**.** Cells were then lysed and total protein (100μg) from each treatment group used to determine CAT levels. **B)** Relative levels of CAT expressed as fold-change of E2-induced gene expression by setting untreated levels to 1. The E2-induced transactivation for ERα-AA was significantly higher than for wtERα. Results were expressed as mean ± SE from three independent experiments. ** $p<0.01$. **C)** Transactivation of cathepsin D expression by ERα-AA and wtERα. Induction of the E2- responsive gene cathepsin D was determined by RT-qPCR analysis after E2 treatment (10 nM) of C4-12 cells for the indicated time periods. Cathepsin D levels were normalized with EF1α. **D)** Relative levels of cathepsin D mRNA were expressed as fold-change of E2-induced gene expression by setting untreated levels to 1. Results are the mean ± SE from three independent experiments. **$p<0.01$.

lower ($p<0.01$) in cells expressing ERα-AA (*Fig. 8C*). However, when normalized (untreated mRNA level set to 1), the fold-change of E2-induced cathepsin D mRNA expression was greater ($p<0.01$) in cells expressing ERα-AA (*Fig. 8D*). The basal and E2-induced expression levels of cathepsin D mRNA in wtERα C4-12 cells were comparable to that in ERα-positive MCF7 cells (*Fig. 8 E-G*), while cathepsin D mRNA level in the parental ERα-negative C4-12 cells was not affected by E2 treatment (*data not shown*).

The ERα hinge-region contains the receptor nuclear localization sequences (376). To determine whether the low transcription activity of ERα-AA is caused by altered cellular localization, we examined nuclear translocation of wtERα and ERα-AA. In the absence of ligand, ERα-AA was found equally distributed between cytosolic and nuclear fractions, while the majority of wtERα protein was found in the nuclear fraction (*Fig 8I*). This result is in agreement with coimmunoprecipitation data which revealed elevated association of ERα-AA with cytosolic cochaperones *(Fig 4*). Both receptors efficiently translocated to the nucleus in response to E2 treatment, suggesting that the decreased ERα-AA transcription activity in response to E2 is not due to impaired nuclear localization.

E2-induced cathepsin D expression was also examined after siRNA knockdown of cochaperones to determine the relative contribution of each cochaperones to receptor transcriptional activity. CHIP knockdown increased basal and E2-induced cathepsin D mRNA levels in both wtERα and ERα-AA expressing cells (*Fig. 8E-G*). Notably, CHIP knockdown had a greater effect on ERα-AA-mediated gene expression. CHIPi increased basal and E2-induced wtERα activity by 2-fold, while increased basal and E2-induced

Fig. 8E

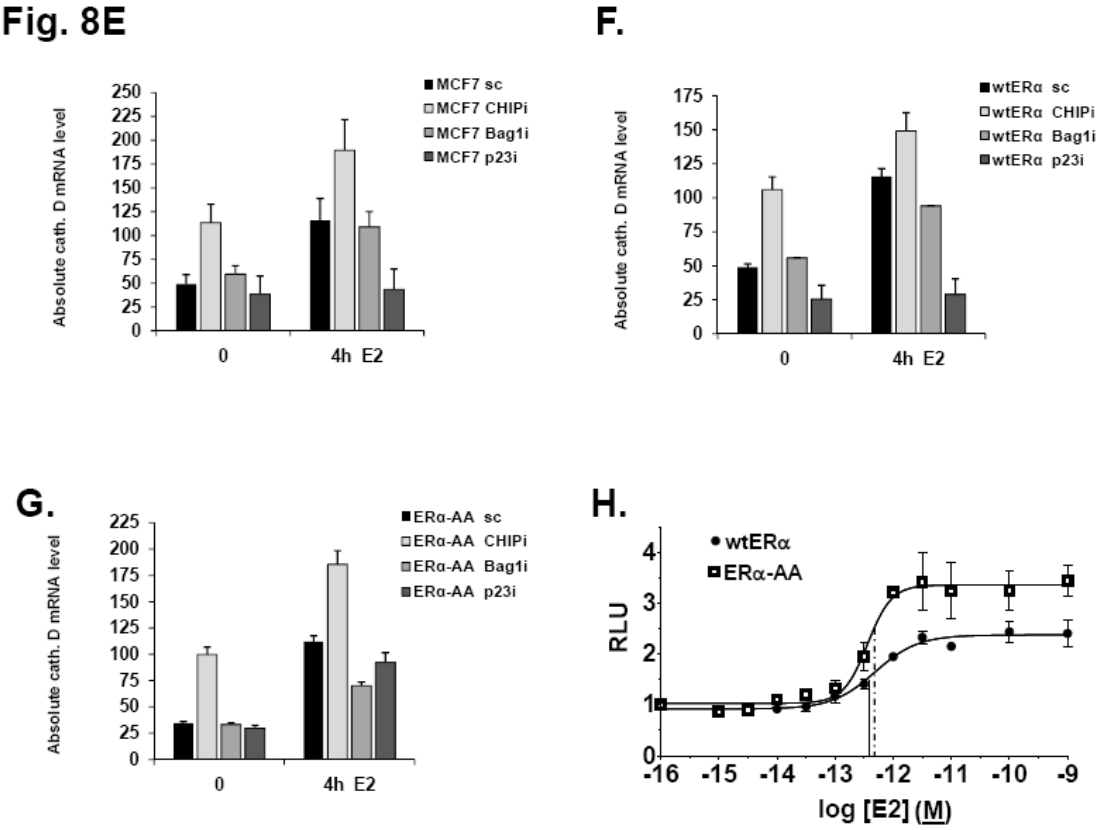


**Figure 8E-G) Cochaperone knockdown alters ERα-mediated transcriptional output.** MCF7 cells (F), wtERα C4-12 cells (G) or ERα-AA C4-12 cells (H) were transfected with siRNA against CHIP, Bag1, and p23 for 2d. E2-induction of cathepsin D was determined by RT-qPCR analysis after 4h E2 treatment (10 nM). Cathepsin D levels were normalized with EF1α and expressed as mean ± SE of results from two MCF7 replicates, two wtERα clones, or three ERα-AA clones. Scrambled oligo (Sc) was included as control. **H) Lysines 302/303 do not modulate E2-sensitivity.** ERE-Luciferase assays were performed in C4-12 ERα stable cells. Cells were transfected with 2x-ERE-ps2-luc and then treated with increasing dose of E2 (0-10nM) for 12 h**.** Luciferase activity was measured and normalized to cotransfected CMV-β-gal, and compared to vehicle (DMSO) treated cells. Values are expressed as mean ± SE of three independent experiments, each performed in triplicate. The $EC_{50}$ values were calculated using 95% confidence function of Prism software. The $EC_{50}$ values are shown as dashed lines and solid lines for wtERα and ERα-AA, respectively. **I) Nuclear localization of wtERα and ERα-AA.** C4-12 cells were pretreated with vehicle (DMSO) or E2 (10nM) for 20 minutes. Whole cell fractions (W) or nuclear extracts fractions (N) were isolated and Western blot was performed for ERα. GAPDH was used as an SDS-PAGE loading control.

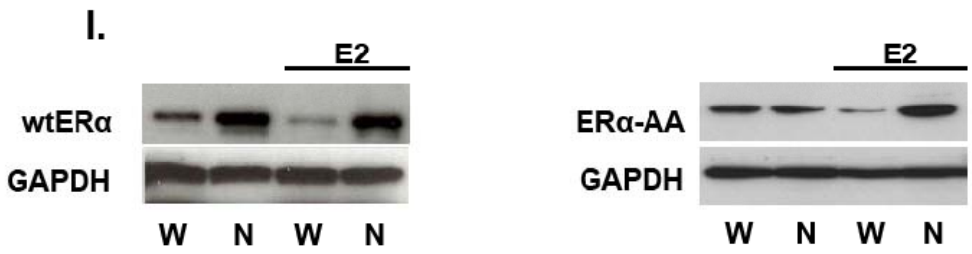


**Figure 8I) Nuclear localization of wtERα and ERα-AA.** C4-12 cells were pretreated with vehicle (DMSO) or E2 (10nM) for 20 minutes. Whole cell fractions (W) or nuclear extracts fractions (N) were isolated and Western blot was performed for ERα. GAPDH was used as an SDS-PAGE loading control.

ERα-AA activity by 3-fold. The enhanced interaction between ERα-AA and CHIP is clearly involved in decreasing the transcriptional capacity of the mutant receptor. Bag1 knockdown did not significantly alter cathepsin D expression mediated by either ERα-AA or wt-ERα. Knockdown of p23 significantly decreased both basal and E2-induced cathepsin D levels in wtERα-expressing cells, but not in ERα-AA cells, in agreement with a previous report that p23 enhances receptor activity (377). It is not surprising that knockdown of p23 had no effect on ERα-AA-mediated cathepsin D expression, as ERα-AA does not significantly interact with p23 (*Fig. 4*).

In both CAT and cathepsin D assays, mutation of K302 and K303 resulted in lower transcriptional output in the presence and absence of ligand, suggesting that these residues are critical for full ERα transcriptional competence. The effect of hinge region mutation on ERα sensitivity to E2 has recently been investigated, with disparate findings (245, 378). The discordant reports on this issue appear be due to experiments using different cellular environments. To shed light on this issue, we examined the sensitivity of ERα-AA to E2 in the previously unexplored C4-12 cellular background. C4-12 ERα clones were transfected with the estrogen-responsive luciferase reporter 2x-ERE-pS2-luc (183), then treated with E2 (range $10^{-16}$ to $10^{-9}$ M). A dose-responsive increase in luciferase activity was observed for both wtERα and ERα-AA transfected cells after E2 treatment (*Fig. 8H*). Sigmoidal curve-fit analysis was then used to determine the concentration of E2 inducing 50% maximum luciferase activity (E2 $EC_{50}$). There was no significant difference in sensitivity of wtERα and ERα-AA to E2: $EC_{50}$ was $10^{-12.315}$ M *vs.* $10^{-12.44}$ M for wtERα *vs.* ERα-AA, respectively (*Fig. 8H*; *indicated by dashed and solid vertical lines on the X-axis*). Taken together, these results demonstrate a role for

K302/303 in promoting both basal and E2-induced transactivation, without altering hormone sensitivity.

## DISCUSSION

Protein turnover and degradation pathways, which ultimately converge on the ubiquitin-26S proteasome system (182, 205, 290-295), are the predominant mechanisms for regulating cellular levels of ERα (352, 375). Distinct mechanisms that downregulate ERα and other steroid hormone nuclear receptors promote lysine polyubiquitination and subsequent proteasome-mediated receptor degradation (379). However, none of the 29 ERα lysine residues have been identified as direct polyubiquitination sites that stimulate ERα turnover. While previous studies have suggested that ERα lysines K302 and K303, found within the hinge-region, can serve multiple regulatory functions (245, 362), the role of these two lysines in receptor turnover has not been established. In the present study, we focused on how K302 and K303 control ERα ubiquitination and turnover. By mutating these two lysines, we demonstrate that K302 and K303 promote ERα stability in the unliganded state, allow for efficient receptor turnover in response to E2, and finally promote polyubiquitination and turnover in response to the antiestrogen ICI. The potential roles of lysines 302/303 in ERα degradation pathways have been summarized in a model in Figure 9.

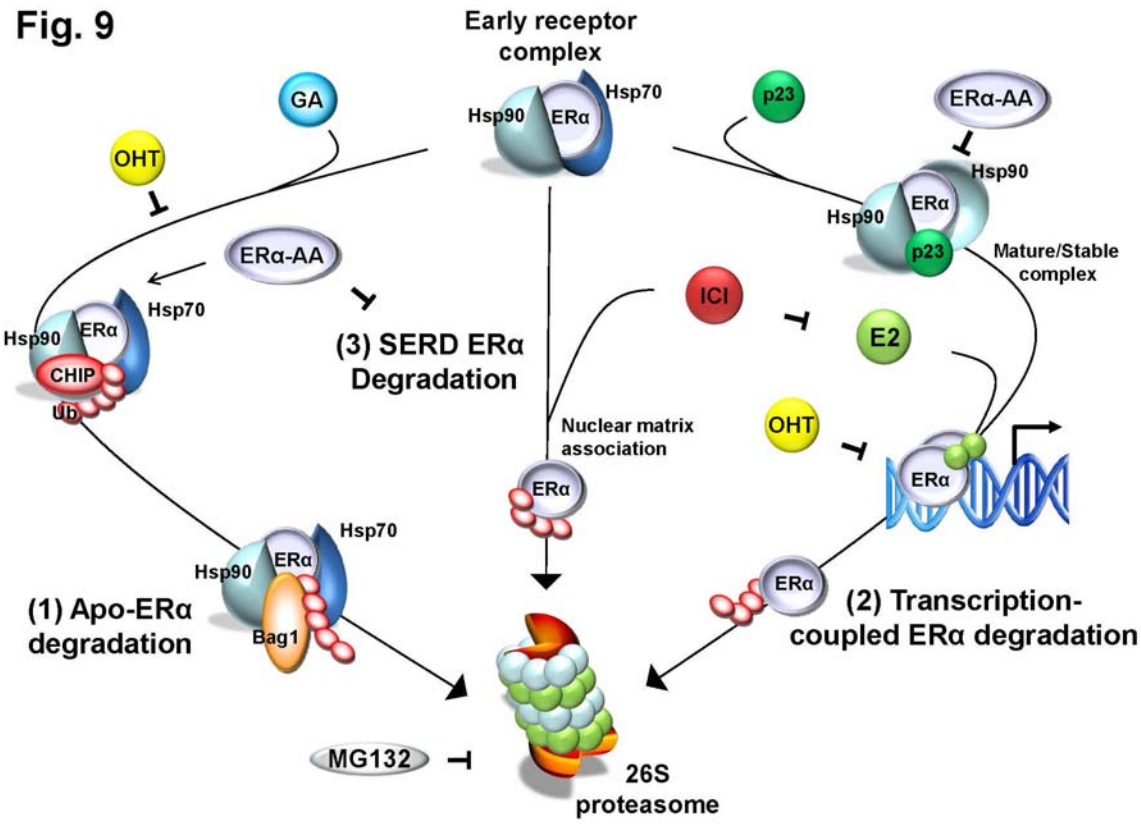


**Figure 9. Lysines 302/303 protect ERα from basal turnover and promote E2 and SERD-induced degradation.** ERα is degraded via three distinct downregulatory pathways that converge on the 26S proteasome. **(1) Basal Turnover** (left pathway). ERα protein folding and maturation begins in a multi-protein Hsp70/90 chaperone complex (shown as a simplified complex at top). In the absence of ligand, ERα is ubiquitinated by CHIP, and ubiquitinated receptors are recognized by Bag1 and delivered to the proteasome. Apo-ERα turnover (basal turnover) is enhanced by both GA and lysine 302/303 mutation, through increased complex association with CHIP and Bag1. **(2) Transcription-coupled ERα turnover** (right pathway). ERα is dynamically maintained in a mature receptor complex that includes p23. Upon binding of E2, ERα disassociates from the Hsp90 complex and is thus protected from CHIP-mediated degradation. Activation of E2-responsive target genes results in receptor ubiquitination and degradation in a transcription-coupled manner. OHT stabilizes receptor-DNA complexes and blocks both basal and transcription-coupled turnover. ERα-AA blocks transcription and transcription-coupled turnover by limiting p23-mediated receptor maturation. **(3) SERD-mediated ERα degradation** (middle pathway). The antiestrogen ICI stimulates ERα release from Hsp complexes and blocks receptor transactivation, sequestering ERα in the nuclear matrix, and triggering rapid receptor ubiquitination and degradation. Lysines 302/303 are required for ICI-induced polyubiquitination and turnover. As ERα degradation is dependent on the 26S proteasome, the proteasome inhibitor MG132 blocks all receptor turnover pathways.

It is possible that lysine mutations resulted in a misfolded, unstable receptor. In the present study, it is not possible to determine whether ERα-AA is misfolded, as the mutant receptor favors the CHIP/proteasome-dependent pathway, which degrades both mature and misfolded receptors (275, 276). However, we do not believe that ERα-AA was misfolded, as crystal structures are not possible in this region due to the flexible nature of the hinge region (36), suggesting mutation does not disrupt secondary protein structures. Additionally, Fuqua *et al*. reported that a similar mutant, ERα-K303R, bound E2 and OHT with the same affinity as wtERα (245).

ERα lysines 302/303, located within the Hsp90/ER interface (364, 365), may influence receptor stability by altering receptor-Hsp90-cochaperone interactions. Though both receptors associated similarly with Hsp90, coimmunoprecipitation analysis revealed an increased interaction of ERα-AA with CHIP and decreased interaction with p23, compared to wtERα. We have previously shown that Hsp90 inhibitor geldanamycin (GA) induces receptor association with CHIP and dissociation from p23, resulting in receptor ubiquitination and degradation by the 26S proteasome (275). These observations suggest that loss of lysine 302/303 and GA may promote receptor degradation through the same CHIP-mediated protein degradation pathway. In support of this notion, both ERα-AA turnover and GA induced wtERα could be blocked by OHT (*Fig. 6*), which interrupts ERα interaction with HSP90/cochaperones.

The cochaperone Bag1 has been also found to associate with ERα (276), but to date, an association between Bag1 and ERα turnover has not been established. Bag1 functions as a nucleotide exchange factor (305) that may destabilize protein-Hsp complexes and promote delivery of ubiquitinated client proteins to the proteasome. The

glucocorticoid receptor (GR), another Hsp70/90 client, is also ubiquitinated by CHIP (302), and following CHIP-mediated ubiquitination, Bag1 delivers GR to the proteasome (309). Similar to the results of the present study with ERα and CHIP, mutations in the Hsp90-interacting residues of GR likewise resulted in altered GR-Hsp90-cochaperone dynamics and receptors that were immune to GA-induced turnover (374, 380, 381). Therefore, the mechanism by which ERα is delivered to the proteasome is likely similar to Bag1-mediated delivery of GR. Our results further suggest that Bag1 promotes basal and GA-induced receptor degradation, as ERα-AA-Bag1 association increased following GA treatment, while Bag1 siRNA delayed receptor turnover (*Figs. 4 and 5*). As with GR, Bag1 may again cooperate with CHIP, delivering polyubiquitinated ERα to the proteasome through its proteasome-recognition domain (303, 309). CHIP and Bag1 cooperation may therefore represent a common basal turnover mechanism for nuclear receptor degradation.

While CHIP and Bag1 are found in early receptor complexes, the ERα cochaperone p23 is found in late/mature receptor complexes (366). p23 has been shown to enhance both basal and ligand-induced ERα transactivation (377) and also to compete with CHIP for Hsp90 binding (302). We found that wtERα was rapidly degraded upon p23 knockdown (*Fig. 5C*), suggesting that p23 exerts a stabilizing effect on the receptor. In addition, ERα-AA preferentially associated with CHIP and Bag1 and also had less affinity for p23 (*Fig. 4*). Knockdown of p23 expression decreased wtERα-mediated cathepsin D gene expression, but not ERα-AA. In contrast, CHIP knockdown had a greater effect on ERα-AA-mediated gene expression (*Fig. 8E-G*). These results suggest

that p23 positively regulates ERα activity by stabilizing receptors, while CHIP limits ERα function by promoting receptor degradation.

Taken together, these data indicate that lysines 302/303 may encourage receptor association with p23, facilitating progression of ERα through the foldosome and increasing receptor transactivation potential. Numerous mutations that stabilize ERα in the presence of ligand also block E2-mediated receptor transactivation (4). Indeed, ERα-AA was stabilized in the presence of E2 and the mutant receptor was less transcriptionally competent than wtERα (*Fig. 8*). Alterations in the hinge-region may reduce basal ERα-AA-mediated transactivation due to disruption of an ERα prototypical nuclear localization sequence (pNLS) located between K299 and K303 (376). We observed increased cytosolic retention of unstimulated ERα-AA, which may contribute to the low basal transcription activity observed in ERα-AA cells and the elevated ERα-AA interaction with cytosolic CHIP. Elevated basal ERα-AA degradation may also explain the discrepancy between ERα-AA mRNA expression and protein levels. In untreated cell, the level of ERα-AA mRNA in the clones was twice that of wtERα (*Fig. 1*). As the half-life of apo-ERα-AA was significantly less than wtERα (*Fig. 2*), and ERα-AA displayed elevated polyubiquitination in the absence of ligand (*Fig. 3*), it is likely that ERα-AA clones maintained similar protein levels as wtERα clones due to rapid ERα-AA protein degradation.

Upon ligand binding, nuclear receptors dissociate from Hsp90-CHIP complexes and are directed toward alternative downregulatory pathways (294, 322, 324). Treatment with E2 moves ERα toward a transcription-coupled degradation pathway (379). Concordantly, we observed increased polyubiquitination and turnover of wtERα after E2.

In contrast to the wild type receptor, ERα-AA was stabilized by E2. While ERα-AA was more stable than wtERα following E2 treatment, E2-induced polyubiquitination of ERα-AA did not appear to be different from wtERα. The stabilization of ERα-AA by E2, without decreased polyubiquitination, may be due to its protection from rapid basal turnover observed in the unliganded condition (*Fig. 2*). Alternatively, K302/303 may be required for efficient E2-induced turnover, either by interacting with degradation machinery directly or by serving as sites of posttranslational modification that recruit degradation machinery. In fact, K302/303 have been reported to be sites for acetylation (31) and sumoylation (32), in addition to K302 monoubiquitination (34), so it is possible that altered receptor stability was due to loss of a posttranslational modification site. However, a recent report has shown ERα to be acetylated at lysines 266/268 and specifically not at lysines 302/303 (30). As lysines 266/268 are also sumoylation sites (32), E2-induced monoubiquitination of lysines 302/303 by BRCA1/BARD1 (34) remains the likely signal for initiating E2-induced polyubiquitination and receptor turnover.

In contrast to E2 treatment, K302 and K303 appeared to play a significant role in ICI-induced receptor polyubiquitination (*Fig. 7*). ERα-AA was more stable than wtERα upon ICI treatment and the mutant receptor had markedly diminished polyubiquitination. As further ERα-AA polyubiquitination did not occur in the presence of the antiestrogen, this raises the strong possibility that lysines 302/303 are ICI-induced polyubiquitination targets. This is the first report to identify lysines whose mutation results in altered ICI-induced receptor ubiquitination, providing insight into the mechanism of ICI action. Both ERα-AA and ERα-RR were resistant to ICI-induced polyubiquitination. Lysine

mutation to alanine removes positive charges, while lysine mutation to arginine preserves positive charges. As both ERα-AA and ERα-RR have a similar ubiquitination profile, we suggest that the charge of the residues is not responsible for directing receptor ubiquitination. Rather, it may be the loss of post-translational modifications of these lysines that is responsible for the decrease in ICI-induced polyubiquitination, raising the possibility that lysines 302/303 are preferential ubiquitination sites in response to ICI. Furthermore, it has been recently shown that K302 is monoubiquitinated in the presence of ligand (34); it is very likely that additional K302 polyubiquitin attachment could occur in the presence of ICI, thus facilitating receptor degradation.

In conclusion, we propose that lysines 302/303 regulate basal ERα turnover pathways by preventing interaction with the cochaperones CHIP and Bag1 in the absence of ligand. We also report that K302/303 appear to function as polyubiquitination sites in the presence of ICI. These results reveal that K302/303 play a multifaceted role in regulating receptor stability and also highlight a previously undescribed role for these hinge-region lysines in the mechanism of ICI action. Using mass spectrometry, we are investigating which of the 29 ERα lysines are ubiquitinated during receptor degradation and attempting to identify the specific ubiquitin ligase(s) involved in these processes. It is well established that deregulation of ERα stability occurs in breast cancer cells. Consequently, understanding the role of receptor lysines in ERα turnover will aid in understanding the mechanisms of antiestrogen therapies and may also facilitate the development of novel ERα downregulators.

## MATERIALS AND METHODS

*Materials*

The following antibodies and reagents were used in this study: anti-ERα (HC-20; Santa Cruz Biotechnology, Inc.); anti-glyceraldehyde phosphate dehydrogenase (GAPDH) (Chemicon International); Anti-HA (Roche); anti-p23 (Abcam) anti-CHIP and anti-Bag1 (Affinity Bioreagents), anti-Hsp90 (Stressgen). SuperSignal West Pico chemiluminescent substrate (Pierce); protease inhibitor cocktail set III (PIC III) and Protein-G agarose beads (Calbiochem-Novabiochem); LipofectAMINE/PLUS reagents, G418, and cell culture reagents (Invitrogen); TrueBlot anti-mouse IgG beads (eBioscience). FuGENE6 and CAT-ELISA kit (Roche Applied Science); ICI 182,780 (Tocris Cookson Ltd.); CHX, E2, GA, MG132, and OHT (Sigma); passive lysis buffer and luciferase assay system (Promega); fetal bovine serum and dextran-coated charcoal-stripped FBS (Hyclone Laboratories, Inc.); cell culture supplementary reagents (Life Technologies, Inc.). siRNA and DharmaFect1 transfection reagent (Dharmacon).

*Plasmid Constructs*

pcDNA-ERα and pcDNA-ERα-K302A,K303A constructs were kindly provided by Dr. H. Nakshatri (Indiana University School of Medicine). ERα lysines 302 and 303 within the pcDNA plasmid were changed to alanines by site-directed mutagenesis using the QuikChange$^{TM}$ site-directed mutagenesis kit (Stratagene) to generate ERα-K302A,K303A. ERE-Vit-CAT, 2x-ERE-pS2-Luc, CMV-β-gal, HA-ubiquitin, and pBS/U6/CHIPi have been described previously (275).

*Cell Line\s*

The human cervical carcinoma HeLa cell line and the breast cancer cell lines MCF7 and the ERα-negative MCF7-derived C4-12 cells (generously provided by Dr. W. Welshons, University of Missouri) are routinely maintained in our laboratory and have been described previously (23). Before all experiments involving transient transfection and/or hormone treatment, cells were cultured in hormone-free medium (phenol red-free minimum Eagle's medium (MEM) with 3% charcoal-stripped fetal bovine serum) for 2 d.

*Stable Transfection of ERα*

C4-12 cells were transfected with ERα constructs using LipofectAMINE/PLUS reagent and exposed to antibiotic (G418; 0.8mg/ml) for 3 weeks. Multiple single G418-resistant clones were selected, expanded, and ERα levels were determined by immunoblot.

*Protein extraction, coimmunoprecipitation and immunoblot*

Soluble cell lysates were prepared in ER extraction lysis buffer (50mM Tris, pH 7.4, 150mM NaCl, 5mM EDTA, 1% Triton X-100, ATPase inhibitors [1mM $Na_3O_4V$, 25mM NaF, 20μM $MoNa_2O_4$], and PIC III. Receptor-chaperone complexes were immunoprecipitated with an ERα antibody (HC-20; Santa Cruz). Complexes were pelleted with anti-rabbit IgG agarose beads (TrueBlot; eBiosciences). Beads were washed in Tris-buffered saline (TBS) with ATPase inhibitors and 1mM PMSF. Samples were boiled in 2xSDS loading buffer and proteins resolved by SDS-PAGE. Western blot was performed using antibodies specific for ERα, Hsp90, CHIP, Bag1, and p23. To prepare nuclear extracts, cells were resuspended in hypotonic buffer (20mM HEPES,

0.5mM $MgCl_2$, 0.5mM DTT, 5mM KCl, 2mM $CaCl_2$, 8.55% sucrose, 1mM PMSF) and cell membranes disrupted with in a Dounce homogenizer on ice (30 strokes using pestle B). Fractured cells were centrifuged at 2500rpm for 10' at 4ºC. Nuclei pellets were washed 2 times with hypotonic buffer and nuclear extracts were prepared with ER extraction lysis buffer.

*Polyubiquitination assays*

HeLa cells were transiently transfected with ERα or ERα-AA and HA-tagged ubiquitin for 24h using LipofectAMINE/PLUS, according to manufacturer's guidelines. Cells were pretreated with 25μM MG132 for 1h to block proteasome activity. Cells were then treated with DMSO, E2 (10nM), or ICI (100nM) for 4h. Following treatment, cells were lysed in ER extraction buffer. 500μg of lysate was precleared with protein G-agarose for 30 min at 4°C and immunoprecipitated using anti-ERα antibody or IgG at 4°C overnight followed by addition of 30μl of protein G-agarose beads for 30 min. Beads were briefly centrifuged, washed 3 times with TBS with 0.1M PMSF, and resuspended in 2xSDS loading buffer. Proteins were separated by electrophoresis and transferred to PVDF membrane. Blots were probed for ubiquitinated ERα using ant-HA antibody.

*RNA interference (siRNA)*

siRNA transfection reagent Dharmafect1 and SMARTpool siRNA targeting Bag1, p23, and scrambled siRNA were purchased from Dharmacon. Bag1 and p23 siRNA were transfected into C4-12 cells according to the manufacturer's protocol. At 24 and 48 h, media was changed. Seventy-two hours after transfection, cells were pretreated with

DMSO or CHX (25μg/ml) then treated with or without GA (1μM) for the indicated times. Cells were lysed and Western blotting performed using specific antibodies. CHIP siRNA was generated by transfection of pBS/U6/CHIPi plasmid into HeLa cells using LipofectAMINE/PLUS; pcDNA vector was used as non-targeting control, as described previously (275). Mock transfection was transfection reagent only.

*Estrogen-responsive reporter gene assays*

For luciferase assays, C4-12 cells were transfected with 250ng 2xERE-ps2-Luc using Fugene. Twenty-four hours later, media was changed and cells treated with E2 ($10^{-16}$ to $10^{-9}$ M) for 12 h. At the end of the experiment, cell lysates were prepared for reporter enzyme assays. Luciferase activity was determined using the Promega Luciferase Assay System. Luciferase activity was normalized to β-gal activity as determined by the Galacto-Light Plus chemiluminescent reporter assay (Tropix Inc.). For estrogen-responsive chloramphenicol acetyltransferase (CAT) assays, C4-12 cells were transfected with 250ng ERE-CAT for 24 h using Fugene. Media was then changed and cells treated with vehicle (DMSO) or E2 (10nM) for 48 h. Cell lysates were prepared and protein quantified using the Bio-Rad BCA Protein Assay Kit. 100μg total protein from each treatment group was used to determine CAT levels with the colorimetric Roche CAT ELISA kit. ERα expression, determined by immunoblot of vehicle treated cells, was quantified and used to adjust CAT levels to account for any slight difference in stable clone ERα expression level and eliminate any possibility that elevated CAT levels were due to elevated ERα expression in a clone.

*Quantitative Real-Time RT-qPCR*

Total RNA was prepared by RNAeasy Mini Kit (Qiagen), according to the protocol provided by the manufacturer. RNA (2μg) was reverse-transcribed in a total volume of 25μl containing 400U Molony murine leukemia virus reverse transcriptase (New England Biolabs), 400ng random hexamers (Promega), 80U Ribonuclease inhibitor and 1mM deoxynucleotide triphosphates. The resulting cDNA was used in subsequent RT-qPCR performed in 20μl Roche LightCycler Mix with 5pmol forward and reverse primers for cathepsin D forward primer, 5'-GTACATGATCCCCTGTGAGAAGGT-3'; reverse primer, 5'-GGGACAGCTTGTAGCCTTTGC-3' (183) and TaqMan primers for EF1α forward primer 5'-CTGAACCATCCAGGCCAAAT-3'; reverse primer 5'-GCCGTGTGGCAATCCAAT-3' and EF1α TaqMan probe 5'-FAM-AGCGCCGGCTATGCCCCTG-TAMRA-3'. The relative concentration of mRNA was calculated using the ΔΔCt method according to Relative Quantitation of Gene Expression (Applied Biosystems) with EF1α mRNA as an internal control.

*Quantification and Statistical Analysis*

Films were quantified with ImageJ software (http://rsb.info.nih.gov/ij/). Statistical analyses were performed using Prism software. P-values were determined by Student's t-test and ANOVA. $EC_{50}$ values were calculated using sigmoidal dose-response curve-fit analysis.

# IDENTIFICATION OF ESTROGEN RECEPTOR ALPHA UBIQUITINATION SITES BY MASS SPECTROMETRY


## ABSTRACT

Transcriptional activity and stability of estrogen receptor alpha (ER) are tightly regulated by several mechanisms, including post-translational modifications. For example, serine, threonine, and tyrosine residues can be targeted for phosphorylation, while lysine residues are substrates for acetylation and ubiquitination. The ER lysine residue(s) targeted for ubiquitination have not yet been identified. Mass spectrophotometry (MS) is a powerful technique capable of detecting increases in the molecular weight of proteins due to the addition of various post-translational modifications (PTMs). The purpose of this study was to utilize MS to span the ER protein sequence and reveal amino acid (AA) residues targeted for phosphorylation, acetylation, and ubiquitination. ERα peptide (Panvera, Carlsbad, CA) was resolved by SDS-PAGE and visualized with Coomassie blue staining. A 66kDa band corresponding to ERα was extracted, reduced with dithiothreitol and alkylated with iodoacetamide. ERα was then digested overnight with trypsin. The resulting peptides were subjected to liquid chromatography followed by MS using an UltiMate-nano liquid chromatograph (LC Packings, Sunnyvale, CA) coupled to a Finnigan LCQ Deca XP MAX LC/MS (Thermo, Franklin, MA). Resulting sequences were analyzed using MASCOT, and fragments meeting the MASCOT 95% confidence score were considered as ERα with a PTM. Several phosphorylated ER fragments were identified, as indicated by an 80Da increase in molecular mass. Peptides containing AAs S167, S282, S559, and T563 were phosphorylated. Of these AA residues, only S167 has

been previously reported to be phosphorylated; the other phosphorylated AAs may be unique to this insect cell-expressed form of the receptor (the Panvera ERα peptide is expressed in SF9 cells). Acetylation at the N-terminus of ERα, as indicated by a 42Da mass increase, was also observed. Previous reports have shown ER to be preferentially acetylated at lysines 266 and 268 or lysines 302 and 303, suggesting N-terminal acetylation may also be related to SF9 cell expression. Using the MS approach, ubiquitinated lysine residues were not detected, perhaps due to the inherent instability and insolubility of ubiquitinated proteins. In conclusion, this is the first report using MS to identify alternative phosphorylation and acetylation sites on ERα. MS is a direct approach for visualizing PTMs and may be useful for identifying additional modifications on ERα and other members of the nuclear receptor superfamily.

**METHODS:**

Instrumentation

Ultimate nano-LC (LC Packings)

LCQ Deca XP (Thermo Finnigan)

Sample Preparation

The complexity of MCF7 proteome was reduced as follows to enrich for ERα. Whole cell extracts were prepared from MCF7 cells previously serum starved in 3% csFBS for 3 days, pretreated with MG132 (25uM) for 1 hour, and treated with or without 17β-estradiol (10nM) or Fulvestrant (ICI 182, 780; 100nM) for 4 hours. 5 x 1mg protein from each treatment group was diluted at least 10-fold in TBS with PMSF (10μM). Samples were precleared with normal rabbit IgG and immunoprecipitated with ERα antibody (HC-20, Santa Cruz) followed by protein-G-agarose beads. ERα protein was resolved by SDS-PAGE and visualized with Coomassie stain. Gel slices were excised, reduced with DTT, alkylated with iodoacetmide, and digested with pepsin or trypsin (proteomics grade; Sigma). From this digest, samples were dried and reconstituted in 10ul of 0.1% formic acid in preparation for LC-MS/MS.

To enrich for ubiquitinated ERα, immunoprecipitated samples were further purified through a ubiquitin enrichment kit (Pierce) before SDS-PAGE. Commercially available column-purified ERα (Panvera/Invitrogen) was also analyzed by LC-MS/MS to determine the maximum possible sequence coverage for this protein.

Electrospray Conditions:

Trypsin digests were resolved on a 60 minute reversed-phase gradient using an Ultimate nano-LC coupled to a LCQ-Deca XP mass spectrometer.

Mass spectrometry Analysis

MS data were submitted to Mascot (http://www.matrixscience.com)

And searched against human proteins to obtain peptide sequence, identification, and post-translational modifications. Variable modifications of acetylation (K, protein N-term), carbamido methyl (C), oxidation (M), phosphorylation (S, T, Y), and ubiquitination (K) were considered during the search. Peptides with Mascot scores above 30 were retained as true hits.

**Suggested improvements on purification techniques:**

*Improve protein extraction*

Further improvements are necessary to increase the yield and purity of ubiquitinated ERα. The difficulty thus far has been the isolation of sufficient ubiquitinated ER species in a manner that is conducive to immunopurification. Ubiquitinated ER is only partially soluble under conditions that are suitable for immunopurification.

Ubiquitinated ER is not efficiently extracted by gentle lysis buffers; ER extraction buffer (Triton X-100) and RIPA buffer (Triton + NP40 + 0.2% SDS) extract sufficient ubiquitinated ERα to detect by Western blot but not efficiently enough for high yield. I have had some success with strong lysis buffer containing 2% SDS. Although this lysis buffer is not suitable for immunoprecipitation, dilution of protein lysates to 0.2% SDS

using SDS-free buffers (using RIPA without SDS or ER extraction buffer) allows immunopurification of these SDS-extracted species. An alternative strategy is to utilize multiple detergents in concentrations that are still suitable for immunopurification (NP40, deoxycholate, IGEPAL, 0.2% SDS, Triton X-100, etc). The proper conditions must be determined empirically. Isolation of ER under stringent conditions followed by dilution or dialysis into buffer that maintains receptor solubility while allowing purification is a critical step during the optimizing of this process.

*Concentration of proteins*

ERα comprises only ~0.2% of cell protein (~30-60 fmol/mg total protein). Ubiquitinated receptor forms are even less abundant. Orders of magnitude of concentration and purification must be accomplished in order to acquire μg quantities of ubiquitinated ERα for mass spectrometry. Proteins may be concentrated to allow volume reduction and prepare samples for running on SDS-PAGE to separate purified proteins. Millipore columns with 20,000 kDa MW cutoff membranes are available as centrifugation columns in various sizes. An unfortunate consequence of using Triton X-100 detergents is the creation of detergent micelles when Triton X-100 concentration exceeds 5%. This limitation reduces the ability to concentrate samples that contain Triton X-100. Protein purification techniques such as acetone or TCA/TFA-mediated protein precipitation, FPLC, HPLC, ion-exchange, or other column approaches may be employed but have not been explored thus far.

Alternative purification strategies that do not rely on antibodies may circumvent immunopurification limitations. His-tagged-UBR7-conjugated ER may be purified on

nickel or cobalt (Talon) columns under much more stringent conditions (high salt, higher detergent) that would allow ubiquitinated ER to be purified under conditions where the receptors remain soluble. This would require transient transfection of His6-UBR7 into MCF7 cells by Fugene6 transfection. Stable transfected UBR7 cells have not been created; long-term blockade of ubiquitin chain formation is likely toxic to cells.

*Large scale purification*

Batch-style purification is necessary to optimize conditions before scale-up. However, scale-up by running numerous batches is time-consuming and less efficient than a column-based approach. A column of FLAG-beads or ER antibody + protein-G beads may be utilized. Elution using 3xFLAG or other methods that retain immunopurification capability would allow eluted ER proteins to be further purified on an ubiquitin bead-based column. Denaturing elution using SDS followed by concentration with Millipore Zip-tips or centrufigation columns would concentrate samples to small (µl) volumes that will fit into SDS-PAGE wells.

*Block deubiquitin enzyme activity*

The addition of deubiquitin enzyme (DUB) inhibitors such as NEM to MG132-treated cells may further enhance ubiquitinated forms of ERα. Alternatively, ubiquitin-aldehyde or ubiquitin-vinyl sulfone to protein extracts would also inhibit DUB activity. I have purchased NEM (Sigma) but have not compared the effect of DUB inhibition to MG132 action. Boston Biochem sells the ubiquitin derivatives.

## CONCLUSIONS

LC-MS/MS was capable of detecting 37 distinct ERα fragments which corresponds to 56.3% sequence coverage. Phosphorylation and acetylation were readily detectable with this method. Ubiquitin proteins were detected, as well as ubiquitinated species for abundant proteins such as cytokeratins. Optimization of the purification scheme may yield higher quality ERα protein. ERα may then be more fully investigated for PTMs after treatment with 17β-estradiol, Fulvestrant, and other ligands known to induce PTMs on ERα. LC-MS/MS is a promising tool for identifying the PTMs involved in ERα regulation.

ENRICHMENT PROCEDURE

1. MCF7 cells
2. WCE
3. Preclear
4. IP/enrich
5. SDS-PAGE
6. Band excision
7. Trypsin digest
8. LC separation
9. Mass spectrometry
10. MASCOT analysis

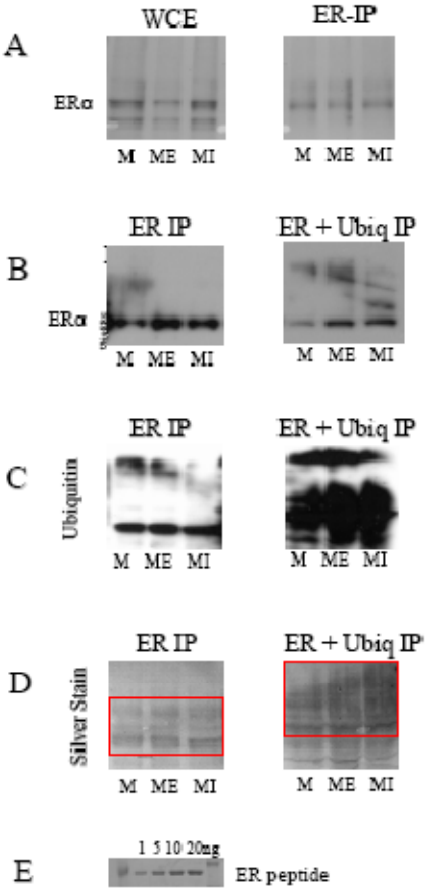


**Figure 10.** Purification of ERα.

**Panel A)** MCF7 cells serum starved for 3d in 3% csFBS were pretreated with MG132 (M, 25uM) for 1hr followed by treatment with E2 (ME, 10nM) or Fulvestrant (MI, 100nM) for 4hrs. ERα levels were detected by Western blot before and after immunoprecipitation with ERα antibody. Purification.

**Panel B)** Further enrichment of ubiquitinated species of ERα was achieved by ubiquitin affinity purification following ERα immunoprecipitation.

**Panel C)** Western blot for ERα after each. Ubiquitinated species of ERα were detected in ERα immunoprecipitated samples and ubiquitin enriched samples by Western blot using an anti-ubiquitin antibody (FK2; Biomol).

**Panel D)** ERα immunoprecipitated samples and ubiquitin enriched samples were resolved by SDS-PAGE and stained with silver stain before gel extraction.

**Panel E)** Purified ERα (baculovirus-infected, insect cell-derived; Panvera/Invitrogen) was also investigated by mass spectrometry

**Complete ERα Amino Acid Sequence:**

29 lysines

MTMTLHTKASGMALLHQIQGNELEPLNRPQLKIPLERPLGEVYLDSSKPAVYNY
PEGAAYEFNAAAAANAQVYGQTGLPYGPGSEAAAFGSNGLGGFPPLNSVSPSPL
MLLHPPPQLSPFLQPHGQQVPYYLENEPSGYTVREAGPPAFYRPNSDNRRQGGR
ERLASTNDKGSMAMESAKETRYCAVCNDYASGYHYGVWSCEGCKAFFKRSIQ
GHNDYMCPATNQCTIDKNRRKSCQACRLRKCYEVGMMKGGIRKDRRGGRML
KHKRQRDDGEGRGEVGSAGDMRAANLWPSPLMIKRSKKNSLALSLTADQMVS
ALLDAEPPILYSEYDPTRPFSEASMMGLLTNLADRELVHMINWAKRVPGFVDLT
LHDQVHLLECAWLEILMIGLVWRSMEHPGKLLFAPNLLLDRNQGKCVEGMVEI
FDMLLATSSRFRMMNLQGEEFVCLKSIILLNSGVYTFLSSTLKSLEEKDHIHRVL
DKITDTLIHLMAKAGLTLQQQHQRLAQLLLILSHIRHMSNKGMEHLYSMKCKN
VVPLYDLLLEMLDAHRLHAPTSRGGASVEETDQSHLATAGSTSSHSLQKYYITG
EAEGFPATV

**Complete ERβ amino acid sequence:**

30 lysines

MDIKNSPSSLNSPSSYNCSQSILPLEHGSIYIPSSYVDSHHEYPAMTFYSPAVMNYS
IPSNVTNLEGGPGRQTTSPNVLWPTPGHLSPLVVHRQLSHLYAEPQKSPWCEARS
LEHTLPVNRETLKRKVSGNRCASPVTGPGSKRDAHFCAVCSDYASGYHYGVWS
CEGCKAFFKRSIQGHNDYICPATNQCTIDKNRRKSCQACRLRKCYEVGMVKCG
SRRERCGYRLVRRQRSADEQLHCAGKAKRSGGHAPRVRELLLDALSPEQLVLTL
LEAEPPHVLISRPSAPFTEASMMMSLTKLADKELVHMISWAKKIPGFVELSLFDQ
VRLLESCWMEVLMMGLMWRSIDHPGKLIFAPDLVLDRDEGKCVEGILEIFDMLL
ATTSRFRELKLQHKEYLCVKAMILLNSSMYPLVTATQDADSSRKLAHLLNAVT
DALVWVIAKSGISSQQQSMRLANLLMLLSHVRHASNKGMEHLLNMKCKNVVP
VYDLLLEMLNAHVLRGCKSSITGSECSPAEDSKSKEGSQNPQSQ

Alignment: http://bioweb2.pasteur.fr/intro-en.html

Program: needle
Align_format: srspair
# Report_file: outfile.align

Aligned_sequences: 2
Matrix: EBLOSUM62
Gap_penalty: 1.0
Extend_penalty: 2.0

Length: 635
Identity: 297/635 (46.8%)
Similarity: 396/635 (62.4%)
Gaps: 145/635 (22.8%)
Score: 1538.0
Shared 14 (or 15) lysines

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ERα | 206 | 210 | 231 | 235 | 244 | 252 | 362 | 401 | 416 | 449 | 472* | 492 | 520 | 529 | 531 |
| | \| | \| | \| | \| | \| | \| | \| | \| | \| | \| | \| | \| | \| | \| | \| |
| ERβ | 170 | 174 | 195 | 199 | 208 | 216 | 314 | 353 | 368 | 401 | 443* | 471 | 480 | 482 | 504 |

**Figure 11. 14 lysine residues align perfectly between ERα and ERβ**
Summary of needle pairwise alignment performed in **Figure 11.** * is a potential 15th lysine pair that are one amino acid away from alignment. (http://bioweb2.pasteur.fr/intro-en.html)

| Query | Obs | Mr(exp) | Mr(cal) | Delta | Miss | Score | Rank | Peptide |
|---|---|---|---|---|---|---|---|---|
| 1282 | 550.9 | 1099.78 | 1098.55 | 1.23 | 0 | 46 | 1 | AANLWPSPLM |
| 1502 | 640.8 | 1279.58 | 1278.68 | 0.9 | 0 | 70 | 1 | AGLTLQQQHQR |
| 2695 | 887.34 | 2658.98 | 2658.38 | 0.6 | 0 | 75 | 1 | ASGMALLHQIQGNELEPLNRPQLK |
| 1131 | 1001.49 | 1000.48 | 999.39 | 1.1 | 0 | 58 | 1 | CYEVGMMK + Pyro-cmC (N-term camC) [-17.03] |
| 2003 | 844.44 | 1686.87 | 1686.63 | 0.24 | 0 | 48 | 1 | DDGEGRGEVGSAGDMR + Phospho (ST) |
| 2180 | 931.22 | 1860.43 | 1860.77 | -0.34 | 0 | 58 | 1 | DSSKPAVYNYPEGAAYE |
| 1432 | 610.52 | 1219.03 | 1217.58 | 1.45 | 0 | 63 | 1 | EAGPPAFYRPN |
| 1451 | 1240.6 | 1239.59 | 1239.64 | -0.05 | 0 | 47 | 1 | ELVHMINWAK |
| 2635 | 1293.41 | 2584.81 | 2584.2 | 0.61 | 0 | 92 | 1 | GGASVEETDQSHLATAGSTSSHSLQK |
| 2397 | 1051.23 | 2100.44 | 2099.98 | 0.46 | 0 | 70 | 1 | GQQVPYYLENEPSGYTVR |
| 2332 | 1007.87 | 2013.73 | 2014.06 | -0.33 | 0 | 69 | 1 | HQIQGNELEPLNRPQLK |
| 1174 | 512.61 | 1023.21 | 1022.58 | 0.64 | 0 | 41 | 1 | IPLERPLGE |
| 1045 | 463.74 | 925.47 | 924.53 | 0.95 | 0 | 53 | 1 | ITDTLIHL |
| 1951 | 821.43 | 1640.84 | 1639.75 | 1.09 | 0 | 93 | 1 | LASTNDKGSMAMESAK |
| 2423 | 1064.68 | 2127.35 | 2126.16 | 1.19 | 0 | 73 | 1 | LHQIQGNELEPLNRPQLK |
| 1514 | 643.84 | 1285.67 | 1284.74 | 0.93 | 0 | 48 | 1 | LLFAPNLLLDR |
| 2628 | 1288.51 | 2575.01 | 2574.25 | 0.76 | 0 | 61 | 1 | LQPHGQQVPYYLENEPSGYTVR |
| 1418 | 604.9 | 1207.78 | 1206.61 | 1.17 | 0 | 57 | 1 | PLGEVYLDSSK |
| 2179 | 930.95 | 1859.88 | 1858.99 | 0.89 | 0 | 53 | 1 | QIQGNELEPLNRPQLK + Pyro-glu (N-term Q) [-17.03] |
| 2539 | 1177.46 | 2352.9 | 2351.99 | 0.91 | 0 | 61 | 1 | SIQGHNDYMCPATNQCTIDK |
| 2675 | 875.41 | 2623.2 | 2623.1 | 0.11 | 0 | 54 | 1 | SIQGHNDYMCPATNQCTIDKNR |
| 1670 | 691.55 | 1381.08 | 1379.8 | 1.28 | 0 | 58 | 1 | VLDKITDTLIHL |
| 2023 | 571.34 | 1711 | 1710.93 | 0.07 | 0 | 49 | 1 | VLDKITDTLIHLMAK |
| 2482 | 1118.8 | 2235.58 | 2236.02 | -0.44 | 0 | 48 | 1 | VYLDSSKPAVYNYPEGAAYE |
| 1408 | 602.52 | 1203.03 | 1201.52 | 1.51 | 0 | 57 | 1 | YASGYHYGVW |
| 2386 | 1043.28 | 2084.55 | 2084.8 | -0.25 | 0 | 58 | 1 | YCAVCNDYASGYHYGVW |
| 2733 | 936.5 | 2806.46 | 2807.08 | -0.61 | 0 | 52 | 1 | YCAVCNDYASGYHYGVWSCEGCK |
| 1808 | 760.34 | 1518.66 | 1516.71 | 1.95 | 0 | 47 | 1 | YYITGEAEGFPATV |

| Query | Obs | Mr(exp) | Mr(cal) | Delta | Miss | Score | Rank | Peptide |
|---|---|---|---|---|---|---|---|---|
| 2111 | 747.89 | 2240.63 | 2239.82 | 0.81 | 6 | 22 | 1 | YSEYDPTRPFSEASMMGL + 2 Phospho (ST) |
| 971 | 576.76 | 1151.51 | 1150.67 | 0.84 | 3 | 27 | 1 | KIPLERPLGE |
| 1424 | 781.85 | 1561.68 | 1560.67 | 1.01 | 5 | 36 | 1 | YSEYDPTRPFSEA |
| 1413 | 1552.88 | 1551.87 | 1551.72 | 0.15 | 5 | 37 | 1 | QVYGQTGLPYGPGSE |
| 446 | 867.65 | 866.64 | 866.47 | 0.17 | 3 | 39 | 1 | LDAEPPIL |
| 1511 | 831.61 | 1661.2 | 1659.8 | 1.4 | 6 | 40 | 1 | SPFLQPHGQQVPYY |
| 852 | 540.73 | 1079.45 | 1078.61 | 0.84 | 4 | 45 | 1 | LEPLNRPQL |
| 1370 | 746.3 | 1490.59 | 1489.64 | 0.96 | 4 | 51 | 1 | YSEYDPTRPFSE |

| Query | Obs | Mr(exp) | Mr(cal) | Delta | Miss | Score | Rank | Peptide |
|---|---|---|---|---|---|---|---|---|
| 2032 | 671.03 | 1340.05 | 1339.73 | 0.32 | 0 | 33 | 1 | AANLWPSPLMIK |
| 2038 | 861.03 | 1720.04 | 1719.72 | 0.32 | 1 | 35 | 1 | LASTNDKGSMAMESAK + Phospho (ST) |
| 1913 | 643.38 | 1284.75 | 1283.76 | 0.99 | 0 | 52 | 1 | LLFAPNLLLDR |
| 2423 | 1064.68 | 2127.35 | 2126.16 | 1.19 | 0 | 55 | 1 | LHQIQGNELEPLNRPQLK |
| 1502 | 640.8 | 1279.58 | 1278.68 | 0.9 | 0 | 60 | 1 | AGLTLQQQHQR |
| 1951 | 821.43 | 1640.84 | 1639.75 | 1.09 | 1 | 80 | 1 | LASTNDKGSMAMESAK |

**Figure 12. MASCOT results from purified ERα (Panvera).** 47.4% coverage was achieved by trypsin digest (upper panel). 12.4% coverage was achieved by pepsin digest (middle panel). Total coverage was 56.3% . A total of 37 unique peptide fragments were identified by LC-MS/MS. MASCOT results from MCF7-extracted ERα (lower panel).


**Figure 13. Summarized sequence coverage for ERα.** 56.3% of ER (335/595 amino acids) was covered by trypsin digest. Yellow and green indicate two independent digests and analyses. Long spans of hydrophobic residues prevented extensive coverage. Long spans of hydrophobic residues and lack trypsin-sensitive residues, thus preventing more extensive covereage. Lysines are highlighted in red. S167 and K302/303 are bolded for orientation. * indicates PTM detected (phosphorylation of S167). A total of 63 peptide fragments were resolved by LC-MS/MS. (Top panel). *indicates PTM detected.

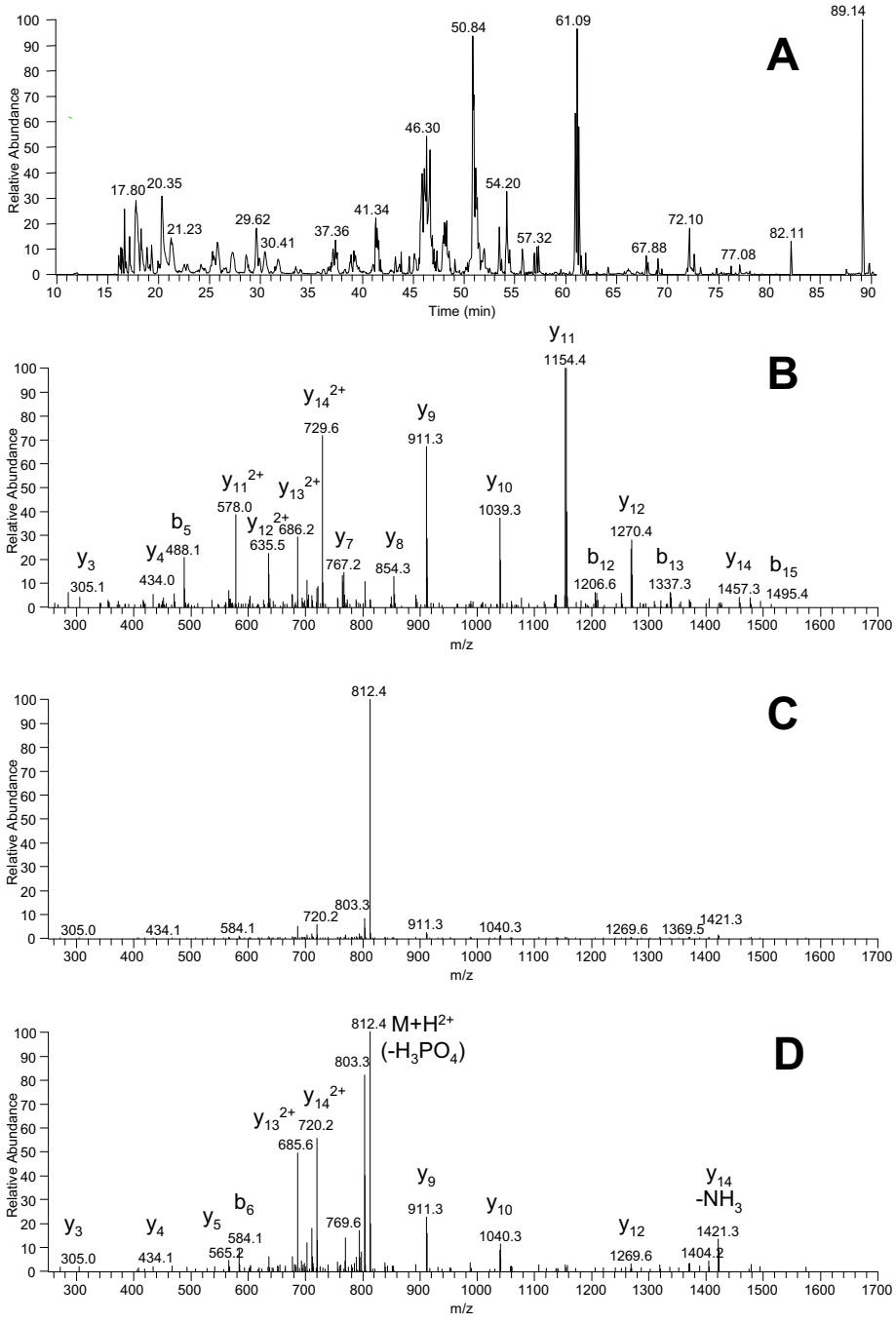


**Figure 14** A) LC-MS base peak chromatogram for trypsin digest of ESR1. B) MS/MS spectrum of precursor m/z 821.40 at retention time 19.10 minutes. Strong peaks are labeled with m/z values and fragment ions corresponding to the peptide LASTNDKGSMAMESAK. C) MS/MS spectrum of precursor m/z 861.00 at retention time 20.08 minutes. Peak at m/z 812.4 corresponds to the 98 Da neutral loss ion expected from a phosphoserine residue. D) same as C. with vertical scale expanded 10-fold to show sequence-specific fragment ions corresponding to LApSTNDKGSMAMESAK. Note that the $y_{14}^{2+}$ fragment appears 9 m/z lower in D. due to the loss of $H_3PO_4$ from the phosphoserine residue (18 Da less than an unmodified serine residue).

| MG132 | Score | Accession # | Name |
|---|---|---|---|
| K2C6A_HUMAN | 651 | P02538 | Keratin, type II cytoskeletal 6A Cytokeratin 6A CK 6A K6a keratin |
| K2C5_HUMAN | 445 | P13647 | Keratin, type II cytoskeletal 5 Cytokeratin 5 K5 CK 5 58 kDa cytokeratin |
| HS90A_HUMAN | 268 | P07900 | Heat shock protein HSP 90alpha HSP 86 |
| K1CO_HUMAN | 244 | P19012 | Keratin, type I cytoskeletal 15 Cytokeratin 15 K15 CK 15 |
| ALBU_HUMAN | 214 | P02768 | Serum albumin precursor |
| CH60_HUMAN | 206 | P10809 | 60 kDa heat shock protein, mitochondrial precursor Hsp60 60 kDa chaperonin CPN60 He |
| K2C4_HUMAN | 154 | P19013 | Keratin, type II cytoskeletal 4 Cytokeratin 4 K4 CK4 |
| FLNA_HUMAN | 152 | P21333 | Filamin A Alphafilamin Filamin 1 Endothelial actinbinding protein Actinbinding p |
| HS90B_HUMAN | 93 | P08238 | Heat shock protein HSP 90beta HSP 84 HSP 90 |
| K1CM_HUMAN | 86 | P13646 | Keratin, type I cytoskeletal 13 Cytokeratin 13 K13 CK 13 |
| TRAP1_HUMAN | 80 | Q12931 | Heat shock protein 75 kDa, mitochondrial precursor HSP 75 Tumor necrosis factor type 1 |
| ESR1_HUMAN | 69 | P03372 | Estrogen receptor ER Estradiol receptor ERalpha |
| K1CN_HUMAN | 65 | P02533 | Keratin, type I cytoskeletal 14 Cytokeratin14 CK14 Keratin14 K14 |
| UBIQ_HUMAN | 62 | P62988 | Ubiquitin |
| HNRPR_HUMA | 60 | O43390 | Heterogeneous nuclear ribonucleoprotein R hnRNP R |
| HA25_HUMAN | 53 | P01907 | HLA class II histocompatibility antigen, DQ5 alpha chain precursor DC1 alpha chain |
| OST5_HUMAN | 49 | Q8IZT8 | Heparan sulfate glucosamine 3Osulfotransferase 5 EC 2.8.2.23 Heparan sulfate Dglucos |
| S10A9_HUMAN | 48 | P06702 | Calgranulin B Migration inhibitory factorrelated protein 14 MRP14 P14 Leukocyte L |
| CEL_HUMAN | 46 | P19835 | Bilesaltactivated lipase precursor EC 3.1.1.3 EC 3.1.1.13 BAL Bilesaltstimulate |

| MG132 + E2 | Score | Accession # | Name |
|---|---|---|---|
| FAS_HUMAN | 1308 | P49327 | Fatty acid synthase EC 2.3.1.85 Includes Acylcarrierprotein Sacetyltransferase E |
| TKT_HUMAN | 300 | P29401 | Transketolase EC 2.2.1.1 TK |
| PRKDC_HUMA | 249 | P78527 | DNAdependent protein kinase catalytic subunit EC 2.7.1.37 DNAPKcs DNPK1 p460 |
| CO1A1_HUMAN | 216 | P02452 | Collagen alpha 1I chain precursor |
| HNRPU_HUMA | 185 | Q00839 | Heterogenous nuclear ribonucleoprotein U hnRNP U Scaffold attachment factor A SAFA |
| DHX9_HUMAN | 129 | Q08211 | ATPdependent RNA helicase A Nuclear DNA helicase II NDH II DEAHbox protein 9 |
| K22O_HUMAN | 121 | Q01546 | Keratin, type II cytoskeletal 2 oral Cytokeratin 2P K2P CK 2P |
| IQGA1_HUMAN | 103 | P46940 | Ras GTPaseactivatinglike protein IQGAP1 p195 |
| ESR1_HUMAN | 92 | P03372 | Estrogen receptor ER Estradiol receptor ERalpha |
| TOP2B_HUMAN | 81 | Q02880 | DNA topoisomerase 2beta EC 5.99.1.3 DNA topoisomerase II, beta isozyme |
| UBIQ_HUMAN | 81 | P62988 | Ubiquitin |
| SYV2_HUMAN | 60 | P26640 | ValyltRNA synthetase 2 EC 6.1.1.9 ValinetRNA ligase 2 ValRS 2 G7a |
| LPPRC_HUMAN | 59 | P42704 | 130 kDa leucinerich protein LRP 130 GP130 Leucinerich PPRmotif containing protein |
| TRY1_HUMAN | 47 | P07477 | Trypsin I precursor EC 3.4.21.4 Cationic trypsinogen |
| CO3A1_HUMAN | 45 | P02461 | Collagen alpha 1III chain precursor |

| MG132 + ICI | Score | Accession # | Name |
|---|---|---|---|
| ESR1_HUMAN | 2835 | P03372 | Estrogen receptor ER Estradiol receptor ERalpha |
| K1CJ_HUMAN | 1355 | P13645 | Keratin, type I cytoskeletal 10 Cytokeratin 10 K10 CK 10 |
| K2C1_HUMAN | 1036 | P04264 | Keratin, type II cytoskeletal 1 Cytokeratin1 CK1 Keratin1 K1 67 kDa cytokerati |
| K1CP_HUMAN | 556 | P08779 | Keratin, type I cytoskeletal 16 Cytokeratin 16 K16 CK 16 |
| K2C8_HUMAN | 322 | P05787 | Keratin, type II cytoskeletal 8 Cytokeratin 8 K8 CK 8 |
| EPIPL_HUMAN | 290 | P58107 | Epiplakin 450 kDa epidermal antigen |
| K1CS_HUMAN | 226 | P08727 | Keratin, type I cytoskeletal 19 Cytokeratin 19 K19 CK 19 |
| K2C6B_HUMAN | 181 | P04259 | Keratin, type II cytoskeletal 6B Cytokeratin 6B CK 6B K6b keratin |
| K1C9_HUMAN | 117 | P35527 | Keratin, type I cytoskeletal 9 Cytokeratin9 CK9 Keratin9 K9 |
| K2C3_HUMAN | 114 | P12035 | Keratin, type II cytoskeletal 3 Cytokeratin 3 K3 CK3 65 kDa cytokeratin |
| MYH9_HUMAN | 111 | P35579 | Myosin heavy chain, nonmuscle type A Cellular myosin heavy chain, type A Nonmuscle myos |
| K22E_HUMAN | 89 | P35908 | Keratin, type II cytoskeletal 2 epidermal Cytokeratin2e K2e CK 2e |
| K1CT_HUMAN | 70 | P35900 | Keratin, type I cytoskeletal 20 Cytokeratin 20 K20 CK 20 Protein IT |
| K2C6C_HUMAN | 67 | P48666 | Keratin, type II cytoskeletal 6C Cytokeratin6C CK 6C K6c keratin |
| UBIQ_HUMAN | 45 | P62988 | Ubiquitin |

**Figure 15. ER interacting proteins identified with mass spectrometry.**
Combined results from wtERα and ERα-AA from MG132, MG132 +E2, and MG132 + ICI ERα and ubiquitin were detected in all samples. Similar protein profiles were detected for both wtERα and ERα-AA complexes.

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# *CURRICULUM VITÆ*

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|---|---|
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EDUCATION/TRAINING

| INSTITUTION AND LOCATION | DEGREE *(if applicable)* | YEAR(s) | FIELD OF STUDY |
|---|---|---|---|
| Indiana University– Bloomington, IN | B.S. | 1997–2001 | Biology |
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## PROFESSIONAL EXPERIENCE

Graduate Research — Indiana University School of Medicine — *2001-present*

Thesis: Regulation of ERα Ubiquitination and Proteasome-mediated Receptor Degradation

Minor: Identification of Ovarian Specific Promoters for Use in Ovarian Cancer Gene Therapy

Graduate Teaching

Associate Instructor- undergraduate Anatomy Lab (*2 semesters*)
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Undergraduate Research

- Research Assistant *1997-2001*
  Assisted postdoctoral and graduate students with research projects
  Independent management of two transgenic (ERα-knockout, PR-knockout) mouse colonies
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- Tissue Interactions and Hormonal Responses in the Mouse Uterus *1998-2001*
  Examined tissue interactions and hormonal responses in the uterus
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- McNair Scholars Research Fellow *1999-2000*
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## PROFESSIONAL SOCIETIES

American Association for Cancer Research
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## HONORS AND AWARDS

| | |
|---|---|
| Elk's Foundation National Fellowship Undergraduate | *1999-2001* |
| McNair Scholar Fellowship– Indiana University | *1999-2001* |
| Associate Instructor Fellowship – IU School of Medicine | *2002-2003* |
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| Medical Science Graduate Student Fellowship Recipient– Indiana University | *2003-2004* |
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## PUBLICATIONS

- Bigsby RM, Caperell-Grant A, **Berry N**, Nephew K, Lubahn D.
  *Estrogen induces a systemic growth factor through an estrogen receptor-alpha-dependent mechanism.* Biology of Reproduction. 2004 Jan;70(1):178-83.
- **Berry NB**, Cho YM, Harrington MA, Williams SD, Foley JG, Nephew KP. *Transcriptional Targeting in Ovarian Cancer Cells Using the HE4 Promoter.* Gynecologic Oncology. 2004 Mar;92(3):896-904.
- **Berry NB**, Fan M, Nephew K. Estrogen Receptor alpha (ERα) Hinge-Region Lysines 302 and 303 Regulate Receptor Degradation by the Proteasome (*accepted; Molecular Endocrinology July 2008*)

## SCIENTIFIC PATENTS

"Transcriptional Targeting of Ovarian Cancer with the HE4 Promoter"
**N. Berry**, K. Nephew U.S. Patent Application # 05007 *Disclosure, August 2004*

- InvivoGen "pDRIVE-HE4" (San Diego, CA) *Marketed, December 2004*
- Gold Biotech "pHE4-luciferase" (St. Louis, MO) *Product Request, March 2006*

## PRESENTATIONS AT SCIENTIFIC CONFERENCES

1. Nephew KP, Huang T.H-M, Bigsby RM, Nam K, **Berry NB**, and Ahluwalia A. *Role of DNA methylation in progression of ovarian cancer*.
   Gordon Research Conference on Hormonal Carcinogenesis, Tilton, New Hampshire, August 1-6, 1999

   **Berry NB,** Nam K. *Differential Gene Expression Between Hey and HeyC2 Ovarian Cancer Cell Lines* National McNair Conference, SUNY Buffalo, New York, August 17, 1999 (*oral presentation*)

2. **Berry NB** *Identification of Ovarian-specific Promoters for Use in Gene Therapy* Midwest Regional Molecular Endocrinology Conference, Bloomington, Indiana, May 30-31, 2002

3. **Berry NB** Cho YM, Harrington MA, Williams SD, Foley JG, Nephew KP. *Transcriptional Targeting in Ovarian Cancer Cells Using the HE4 Promoter*
   American Association for Cancer Research Toronto, Ontario, Canada, May 4-9, 2003

4. **Berry NB**, Berndtson AK, Fan M, Nakshatri H, Nephew KP
   *Role of the estrogen receptor-alpha hinge region on receptor degradation and transcriptional activity*
   Midwest Regional Molecular Endocrinology Conference, Indianapolis, Indiana, May 19-21, 2004 (*oral presentation*)

5. **Berry NB**, Berndtson AK, Fan M, Nakshatri H, Nephew KP
   *Role of the ERα hinge region on receptor degradation and transcriptional activity in breast cancer*
   The Endocrine Society, New Orleans, Louisiana, June 16, 2004

6. **Berry NB**, Fan M, Nakshatri H, Nephew KP
   *Altered ubiquitination and degradation of an estrogen receptor-α hinge-region mutant*
   The Endocrine Society, San Diego, California, June 7-10, 2005

7. **Berry NB**, Nephew KP
   *Regulation of ERα transactivation and stability by hinge-region lysines*
   MCDB Seminar, Bloomington, Indiana, March 30, 2006 (*oral presentation*)

8. Hartman-Frey C, **Berry NB**, Nephew KP, Fan M
   *Deregulation of inhibitors of DNA binding in human breast cancer cells with aquired resistance to 4-hydroxytamoxifen and fulvestrant.*
   American Association for Cancer Research, Washington D.C., April 1-5 2006

9. Rawlinson, J **Berry NB**, Afrane M, , Nephew K
   *Design and Optimization of a Quanitative Method to Determine Herpes Simplex Virus Thymidine Kinase mRNA Expression* IU Medical Science Graduate Research Program April 28, 2006.

10. **Berry NB,** Arnold R, Nephew K
    *ERα Ubiquitination Site Determination Mass Spectrometry Determination*
    The Endocrine Society, Boston, Massachusetts, June 23-27, 2006

11. **Berry NB,** Fan M, Nephew K
    *ERα hinge-region lysines mediate receptor-cochaperone interactions, ubiquitination, and ERα turnover in breast cancer cells* American Association for Cancer Research Toronto, Los Angeles, CA, April 14-18, 2007(*oral presentation*)

12. **Berry NB,** Fan M, Nephew K
    *ERα hinge-region lysines mediate receptor-cochaperone interactions, ubiquitination, and ERα turnover in breast cancer cells* Midwest Regional Molecular Endocrinology Conference, Bloomington, Indiana, May 17-18, 2007 (*oral presentation*)