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"Analysis of Antagonist-liganded Estrogen Receptor Alpha Interactomes: New Insights on Antiestrogen Activity in Human Breast Cancer Cells"

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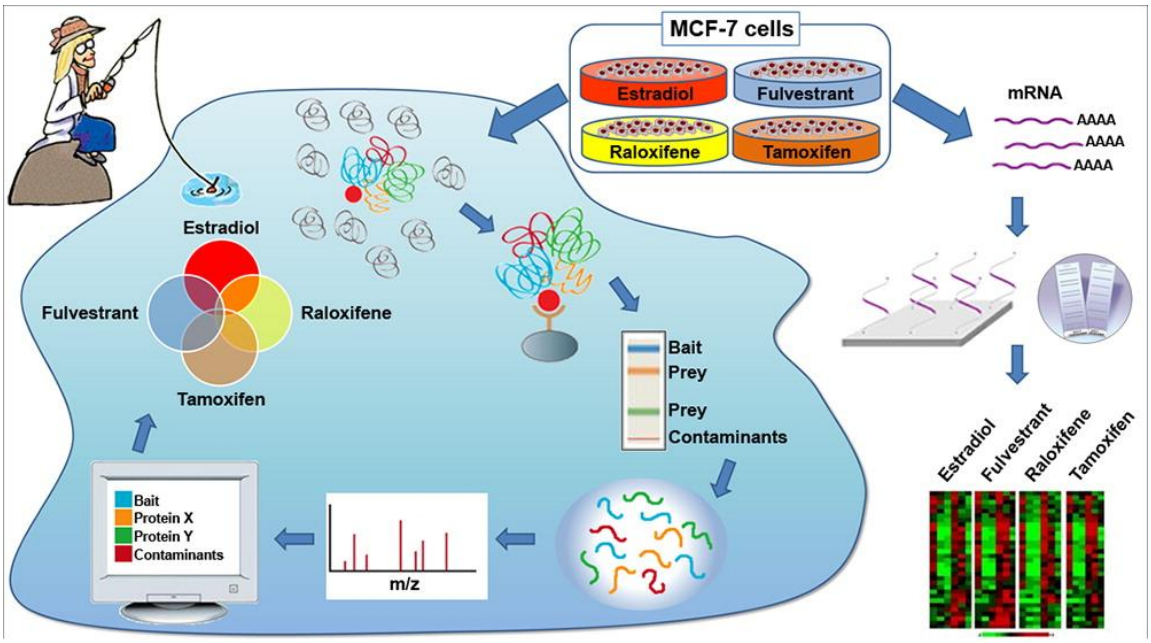
ABSTRACT

Estrogen receptor alpha (ERα) is a ligand activated transcription factor that controls key cellular pathways via protein−protein interactions involving multiple components of transcriptional coregulator and signal transduction complexes. Natural and synthetic ERα ligands are classified as agonists (17β-estradiol/E2), selective estrogen receptor modulators (SERMs: Tamoxifen/Tam and Raloxifene/ Ral), and pure antagonists (ICI 182,780-Fulvestrant/ ICI), according to the response they elicit in hormone responsive cells. Crystallographic analyses reveal ligand dependent ERα conformations, characterized by specific surface docking sites for functional protein−protein interactions, whose identification is needed to understand antiestrogen effects on estrogen target tissues, in particular breast cancer (BC). Tandem affinity purification (TAP) coupled to mass spectrometry was applied here to map nuclear ERα interactomes dependent upon different classes of ligands in hormone-responsive BC cells. Comparative analyses of agonist (E2)- vs antagonist (Tam, Ral or ICI)-bound ERα interacting proteins reveal significant differences among ER ligands that relate with their biological activity, identifying novel functional partners of antiestrogen−ERα complexes in human BC cell nuclei. In particular, the E2-dependent nuclear ERα interactome is different and more complex than those elicited by Tam, Ral, or ICI, which, in turn, are significantly divergent from each other, a result that provides clues to explain the pharmacological specificities of these compounds.


MCF-7 cells
Estradiol
Fulvestrant
Raloxifene
Tamoxifen
mRNA
AAAA
AAAA
AAAA
Estradiol
Fulvestrant
Raloxifene
Tamoxifen
Bait
Prey
Prey
Contaminants
Bait
Protein X
Protein Y
Contaminants
m/z
Estradiol
Fulvestrant
Raloxifene
Tamoxifen

1. BACKGROUND

1.1 Breast cancer: statistics and risk factors

Breast cancer (BC) is the most common cancer diagnosed in women worldwide [1]. As a disease that will personally affect the health of one in the ten women in the western world [2], BC poses a significant clinical problem and is a major public health issue, in the European Union a woman is diagnosed with BC every 2 minutes [3]. It was estimated that, in 2008, 332,800 diagnoses of BC were made in the EU, with death occurring in 27% of those diagnosed (89,800 deaths) [3].

Almost all BCs originate in the glandular epithelium lining the ducts and ductules of the breast. A higher incidence of ductal carcinoma *in situ* (DCIS), which are non-invasive tumors, has been reported in the last 20 years, which is partly due to increased availability of BC screening. However, the majority of primary BCs have breached the epithelium and invaded into the surrounding stroma by the time of diagnosis (invasive carcinoma). Approximately 25% of all BC diagnoses in the United States are represented by DCIS [4] DCIS is a non-invasive condition; however it is characterized by malignant cells that proliferate into a mass in the breast, and it has been reported by various researchers that between 50% and almost 100% of cases will progress to an invasive phenotype if left untreated [5].

Hormones have long been implicated in the initiation and progression of several cancers, notably of the breast, endometrium and ovary in women, and of the prostate, and sometimes breast, in men [6]. The female hormone estrogen is a driving factors in BC [7]. Prolongated exposure to estrogen through usage of the contraceptive pill or postmenopausal hormone replacement therapy [8] are associated with greater incidence of BC. Early onset of menstruation or late menopause, both of which prolong the amount of time females are exposed to reproductive hormones, are also associated

with increased BC risk [9]. BC risk is reduced in women who are of a longer age at first pregnancy [10], while breast feeding is also associated with a protective effect against the development of BC [11]. This is thought to be due to the higher level of differentiation of breast tissue that occurs during pregnancy and lactation [12]. Breast tissue that is less differentiated tends to comprise a higher proportion of epithelial cells, which are more susceptible than other cell types to undergo neoplastic transformation [13] i.e. in women who have never been pregnant (nulliparous), or who are older when they experience their first full-term pregnancy [12]. There are a number of other risk factors to be taken into consideration, such as age at diagnosis, genetic mutation, previous BC, race, previous premalignant tumor biopsy, prior radiation treatment in the chest area, hormone replacement therapy, obesity, poor diet, failing to exercise, fail to breast feed and excessive alcohol intake [14].

1.2 Estrogens

Estrogens are steroid hormones that are synthesized from cholesterol. The most biologically potent and dominant estrogen in humans is 17β-estradiol (E2), but lower levels of the estrogens estrone (E1) and estriol are also present. Estrone is the most abundant estrogen in postmenopausal women. In premenopausal women, most of the estrogens are produced in the ovary, while in men and postmenopausal women it is produced by aromatization and androgens in peripheral tissue. Ovarian production of estrogens ceases at menopause, but the hormone is in continual supply at other sites postmenopausally, including in the breast, bones, the brain and the heart [15]. Tissues that have been reported to synthesize estrogen includes muscle, fat, liver and brain [16-19]. The great mass of muscle and fat could thereby be expected to be the main contributor to total peripheral estrogen

formation. Although aromatase activity and level of expression are low in skeletal muscle, such small activity can be compensated for by the bulk of the tissue in the body [20]. When estrogens are released into the circulation most of it is bound to plasma proteins and transported to target tissue. The steroid hormones are lipophilic and have a low molecular weight that enables them to enter the target cell by passive diffusion. Estrogens have a broad range of target tissues in the human body [21]. For example, estrogens are required for female sexual maturation and affects growth, differentiation and function of the female reproductive system. In addition, estrogens have important physiological effects on the growth, differentiation and function of hormone dependent tissues, including breast epithelium, uterus, vagina and ovaries. Moreover, estrogens preserve bone mineral density and reduce the risk for osteoporosis, protect the cardiovascular system by reducing cholesterol levels, and modulate cognitive functions and behavior. In skeleton, estrogens prevent bone resorption and estrogen replacement therapy are known to reduce osteoporosis in postmenopausal women [22]. In the nervous system estrogen has a numerous of different effects such as beneficial for learning and memory as well as controlling the hypothalamic-pituitary-gonadal axis [23, 24]. In the cardiovascular system estrogen exerts protective effects by influencing the vascular function with effects on vascular tone and blood flow and subsequently arterial blood pressure [25].

1.3 Estrogen receptors

In the early sixties the presence of an estrogen binding receptor was first reported by Jensen and Jacobsen [26]. This was isolated and cloned in the middle of the eighties by Green et al. (1986) and was for a long time believed to be the only existing estrogen receptor (ER) [27]. Then in 1996 an additional ER was discovered [28]. This new ER was named ERβ and

consequently the first ER was renamed ERα. The ERs belong to the nuclear receptor super family and share a common structure including five distinguishable domains. They are named A/B, C, D, E and F domains (Figure 1.1). The N-terminal A/B domain contains a transactivation function that activates transcription of target genes. This domain varies the most between ERα and ERβ. The C domain, the DNA-binding domain, is involved in specific DNA binding and receptor dimerization. This domain is highly similar between ERα and ERβ, which indicates that the target genes are the same for the two receptors. They share sequence homology within DNA binding domain and hormone recognition region, but have different transcriptional activation properties, suggesting that they each interact with unique sets of nuclear factors and play different roles in the regulation of gene expression [29- 31]. The D domain works as a flexible hinge between the DNA-binding domain and the E domain. The E domain is referred to as the ligand-binding domain. It is important for ligand binding, receptor dimerization and transcriptional activation. The function of the F domain is still poorly understood. There are two activation function sites in the ER, AF-1 and AF-2. The AF-1 is located in the N-terminal and AF-2 within the ligand binding domain of the receptor and induces ligand-dependent activation of transcription. They are believed to function by binding co-activators and bringing them to the promoter of the target gene. It can function autonomously and in the absence of estrogen. AF-1 is not well understood but seems to be weaker in ERβ than the AF-1 of ERα. To give full transcriptional response of an ER agonist, a synergism between the weaker AF-1 and the stronger hormone inducible AF-2 is required [32]. Several different spliced forms of ER have been reported, whether all are translated to protein and have any biological function is not established. Even though ERα and ERβ are highly homologous their alternative splicing pattern differs. Two splice variants of ERα have been shown to inhibit the wild-type receptor and might act as regulators of gene transcription [33].

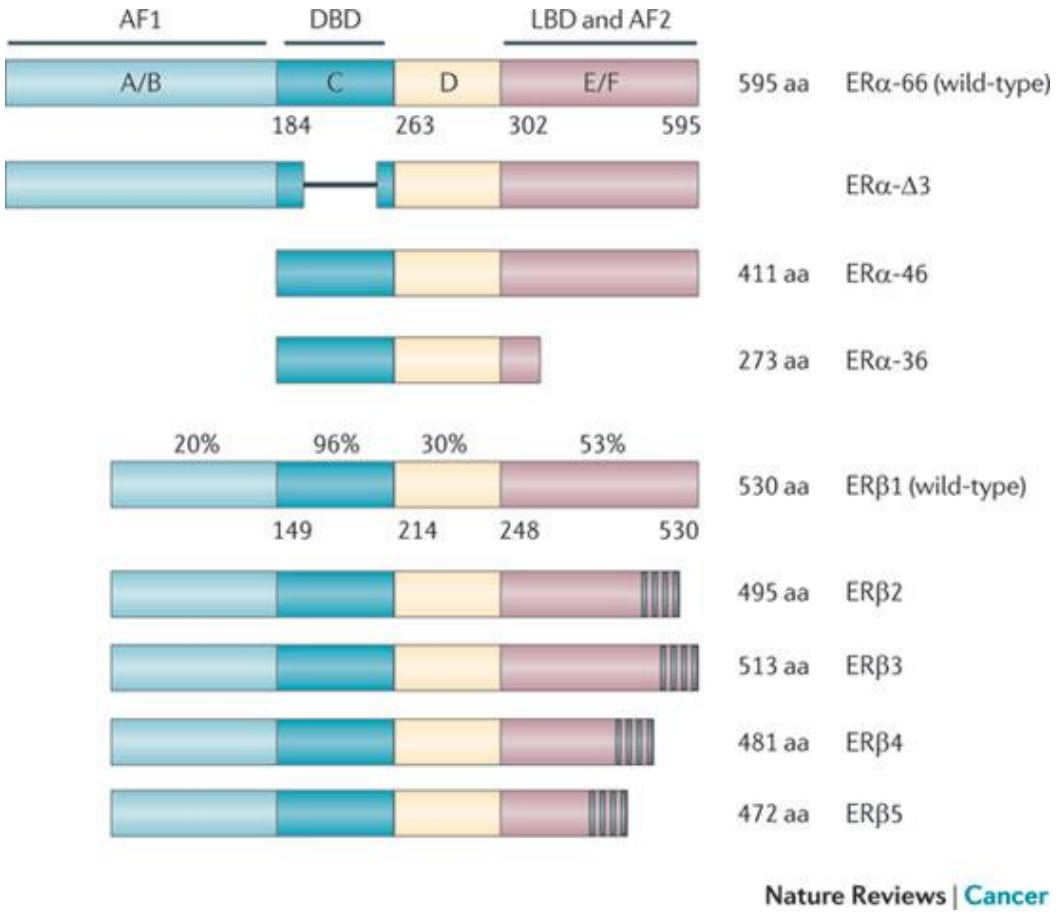


Figure 1.1 The structural domains are labelled A–F with the amino acid numbers indicated below. Relative positions of some of the known functional domains are represented by solid bars. The percentage amino acid homologies between wild-type estrogen receptor-α (ERα) and ERβ are also shown. The amino-terminal A/B regions contain a transactivation domain (AF-1) with ligand-independent function and a co-regulatory domain that is responsible for the recruitment of co-activators and co-repressors. The C region corresponds to the DNA-binding domain (DBD), which is required for binding to specific estrogen response elements (EREs) in the proximal promoter region or at distal regulatory elements of estrogen-responsive genes. The carboxy-terminal regions E and F contain the ligand-binding domain (LBD) and have a ligand-dependent transactivation function. This region is also responsible for the binding to co-regulatory and chaperone proteins, as well as for receptor dimerization and nuclear translocation. Finally, the D region contains several functional domains, including the hinge domain, part of the ligand-dependent activating domain and the nuclear localization signal. Human ERα and ERβ variant isoforms are presented below the wild-type forms. Most of these variants are expressed in malignant tissues and influence cancer biology. ERβ variants are formed from alternative splicing of the last coding exon (shown by the striped bars). From: Thomas et al, 2011 [151].

Also for ERβ different splice variants have been identified. ERβcx has a deletion which makes it unable to bind ligand. However, it can heterodimers with preferentially ERα and inhibit ERα induced gene transcription. Another ERβ splice variant is called ERβ2 and shows impaired E2 binding ability. ERβ2 may function as a dominant negative partner of both ERα and ERβ with reduced transcriptional activity. The expression of slice variants appears to be tissue specific [34]. Steroid receptor expression and proliferation are strictly regulated in the normal mammary gland, but not in malignant tumors. In normal mammary gland there is a minimum expression of ERα [35, 36]. ERα expression increases when normal mammary cells are proliferating such as the case of pregnancy and puberty period [37, 39]. However in BC increased ERα expression appears to occur early in the premalignant to malignant progression, and these tumor cells will continue expressing ERα [40]. In fact, it facilitates epithelial mammary cells to turn from a condition of hormone dependence to hormone independence. Recent transcriptome analyses confirmed observations made over a century ago, that estrogens stimulated the development of the disease in at least one out of five patients. If, from one side, the expression of ERα is a risk factor for the development of BC, from the other side, this expression is associated with responsiveness to hormonal treatment and with favorable prognosis [41]. Nevertheless, clinical evidence shows that more than 30% of hormone receptor-positive mammary tumors are unexpectedly non responsive to endocrine treatment. Reasons for therapy failure seem to lie not only in ER dysfunction, but also in mutations affecting the intracellular signal pathway of estrogens. In this regard characterizing ER networks will help to elucidate molecular mechanisms responsible for hormone resistance in breast tumors and will allow to detect new molecular markers for a more accurate BC prognosis. Although ERβ is widely expressed in both normal and malignant breast tissue, it is not thought to be as important as ERα in predicting response to

endocrine therapy [42]. Its function, if any, in BC progression, is not well understood. Additionally, ER-negative breast tumors are poorly differentiated and more aggressive [43]. The difference in ER expression between normal (low ER expression) and tumor cells (variable ER expression) raises the question as to why ERα is absent both in the normal breast epithelial cells and in the worse prognostic breast cancers while ERα is present in the breast epithelia cells in luminal like? Should the presence of ERα be our only marker or there are other elements to determine the prognosis of the disease. There are two possibilities as to why lack of ER is associated with a poor prognosis: i) an ER-positive tumor has the ability to lose ER expression and in doing so be transformed into a more aggressive ER-negative tumor or ii) ER-negative tumors originate from cells without expression of ERα. There are active investigations in support of both of these possibilities [44-48]. The tissue distribution of ERα and ERβ is in part different. Classical estrogen targets are the uterus, mammary gland, placenta, central nervous system, cardiovascular system and bone. These tissues have a high ERα content. Non-classical target include prostate, testis, ovary, adrenals, pancreas, skin and urinary tract [49]. The expression ERα is either low or not measurable in these tissues. Besides the classic estrogen tissues, ERβ is also highly expressed in many non-classical estrogen target tissues [50]. ERβ has a broader tissue distribution than ERα suggesting that the two receptors have distinct biological functions. This is evident hen studying the different phenotypes of ERα and ERβ knock-out mice (αERKO and βERKO, respectively). Both single and double knock-out mice can survive to adulthood, albeit with retarded growth. The most striking phenotypes in αERKO mice are complete infertility in both sexes. In contrast, male βERKO mice are fertile whereas the females are sub fertile; they have fewer litters with reduced number of pups [51]. The basal release of endometrium-derived NO is decreased in male αERKO [52] and the estrogen mediated production

of NO is abolished [53]. βERKO mice developed hypertension in both sexes as they age [54], which confirms their role in the cardiovascular system.

1.4 Estrogen receptor signaling

It was for many years believed that the only mechanism by which estrogen affected expression of estrogen-responsive genes was by direct binding of the activated ER to specific estrogen response elements (ERE) on DNA (Figure 1.2). However, evidence for signaling pathways that deviate from this classical model has emerged. Today, it is accepted that ER may regulate transcription

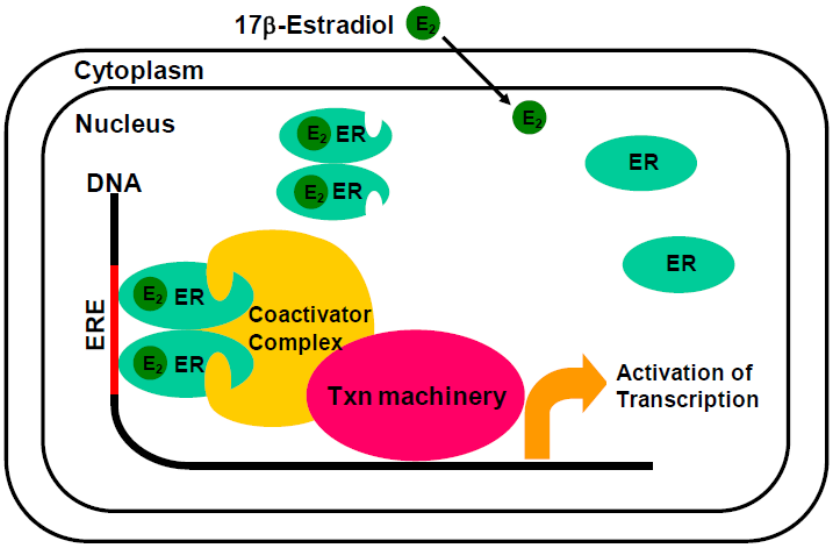


Figure 1.2 A simplified model of estrogen dependant gene transcription. From: Shi et al, 2007 [152].

from target genes by a number of distinct mechanisms, both in the presence and absence of estrogen (Figure 1.3). Activation of ER appears to be a multi-step process relying on a number of molecular events, including dimerization, the actual binding of ligand, phosphorylation, interaction with cofactors and DNA binding.

Classical ligand-dependent activation of ER. In the absence of ligand, ERs are preferentially located to the cell nucleus in a multiprotein complex containing heat shock proteins [55]. When estrogens, which can diffuse across the plasma and nuclear membranes of cells, bind to the ER a conformational change occurs that promotes receptor dimerization. The activated ERs bind as homodimers or heterodimers to EREs located in the regulatory regions of target gens. The ERE sequence is a 13 base par palindromic inverted repeat with the consensus sequence: 5'-GGTCAnnnTGACC-3'. The binding of ERs to the EREs facilitates the assembly of basal transcription factors into a stable pre-initiation complex and increases transcription rate for target mRNA synthesis [56]. The conformational change of activated ERs also leads to that an interaction surface for co-activators is provided. Ligand-dependent activation of transcription by ERs is mediated by the interactions of a number of different nuclear receptor co-activators.

Coregulatory proteins. Interactions of the DNA-binding domain of ER with promoters of target genes can be further modulated by the presence of specific nuclear co-regulator proteins that are recruited to gene promoters, where they interact with receptor-ligand complexes (Figure 1.4). When ER is in an inactive, unliganded conformation, it is bound to nuclear co-repressors (NCoR) such as NCoR1, and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT). NCoRs recruit histone deacetylases (HDAC) to promote deacetylation of histones, thus maintaining the chromatin in a condensed, transcriptionally inactive state [57]. E2-binding to ER induces a conformational change in the AF-2 domain of the receptor, facilitating direct interaction with co-activator proteins. Histone acetyltransferases (HAT) are then recruited, leading to acetylation of histones, unwinding of chromatin and activation of transcription [58]. The p160 family of proteins are steroid receptor co-activators (SRC) that have the ability to stimulate steroid receptor transcriptional activity.

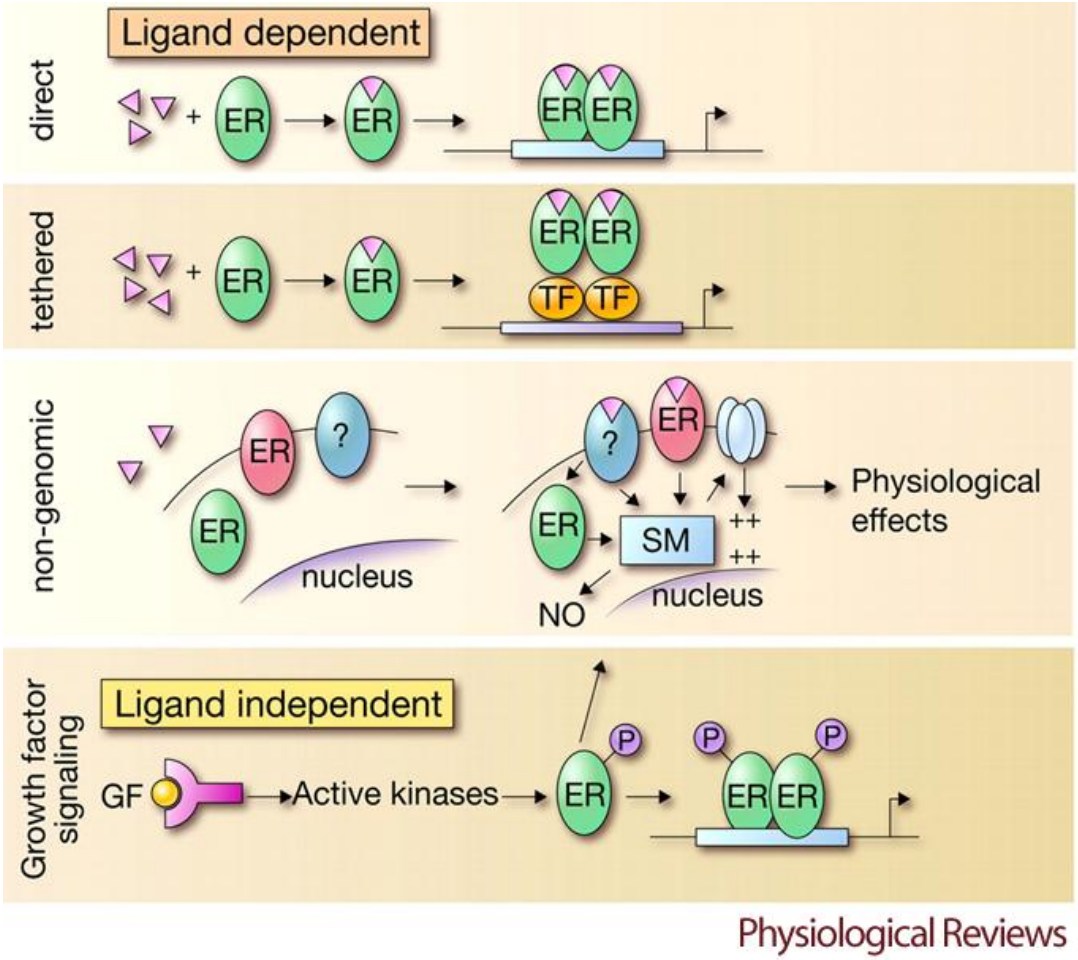


Figure 1.3 Model representing the mechanistically distinct molecular pathways used in the regulatory actions of ERs. The classical (direct) pathway includes ligand activation and a direct DNA binding to estrogen response elements (ERE) before modulation of gene regulation. The tethered pathway includes protein-protein interaction with other transcription factors after ligand activation, and thereby gene regulation is affected by indirect DNA binding. A third mechanism, also called nongenomic with rapid effects, is not as well understood as the genomic mechanism but has been observed in many tissues. The ligand activates a receptor, possibly associated with the membrane; either it is a classical ER, an ER isoform or a distinct receptor or, alternatively, a signal activates a classical ER located in the cytoplasm. After this rather unclear event, signaling cascades are initiated via second messengers (SM) that affect ion channels or increase nitric oxide levels in the cytoplasm, and this ultimately leads to a rapid physiological response without involving gene regulation. The ligand-independent pathway includes activation through other signaling pathways, like growth factor signaling. In this case, activated kinases phosphorylate ERs and thereby activate them to dimerize, bind DNA, and regulate genes. From: Heldring et al, 2007 [153].

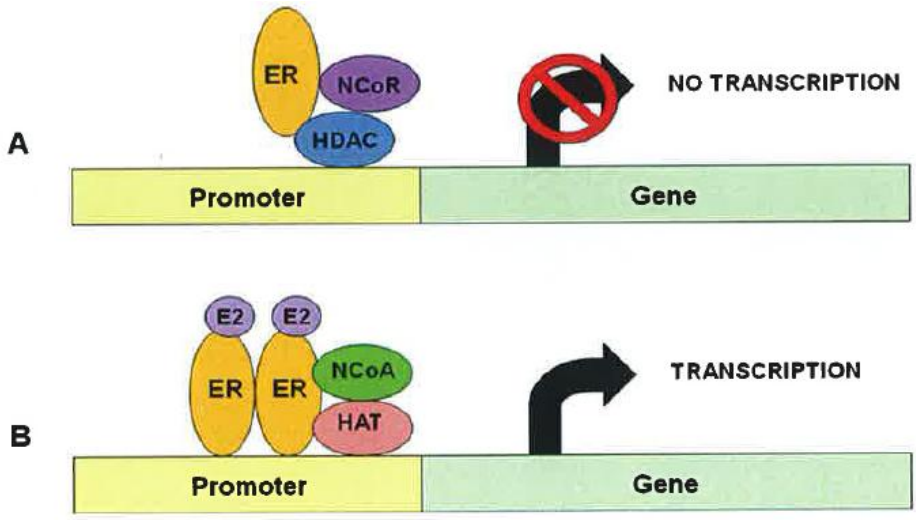


Figure 1.4 The role of co-activators and co-repressors in transcriptional repression and activation of ER-regulated genes. **A**: in the absence of ligand, ER is present in cells as a monomer, bound to nuclear co-repressors (NCoR) and histone deacetylases (HDAC), which maintain chromatin in an inactive state (REPRESSION). **B**: upon ligand-E2 binding ER dimerises and binds to the promoter, releasing bound NCoRs, and favoring association with co-activator proteins (NCoA). The formation of this complex at promoter regions of ER target genes recruits proteins with histone acetyltransferase (HAT) activity, such as CBP/p300, leading to chromatin unwinding and transcriptional activation (ACTIVATION). From: Glass et al, 2000 [58].

This family includes SRC-1 (also known as NCoA1, SRC-2 and SRC-3). The SRC proteins contain multiple structural and functional domains; the receptor interaction domain (RID) in the central region of the protein contains several conserved motifs (LLXXL; where L= leucine, X= any aminoacid) that allow these co-activators to interact with ligand-bound nuclear receptors at promoters of target genes [59, 60]. Two transcriptional activation domains (AD) are located in the C-terminal region of SRCs. AD1 contains multiple LXXLL motifs that allow SRCs to bind to the co-integrators CREB-binding protein (CBP) and p300, facilitating transcription through histone acetyltransferase activity [61]. AD2 binds to a protein called co-activator associated arginine methyltrasferase 1 (CARM1), which acts as a secondary ER co-activator, to AIB1 [62].

Ligand-independent activation of ER. The ERs can also be activated without any estrogen present. Within the AF-1 site of ER there are well-conserved serine residues, which are target for phosphorylation. Binding of growth factors, such as IGF-1 and epidermal growth factor, to its cognate receptor results in the intracellular activation of mitogen-activated protein kinase (MAPK) signal transduction cascade that influences the transcriptional activity of the ERα by phosphorylation of serine residues [63, 64]. Trembley et al. showed a similar ligand-independent activation of the ERβ [65]. Phosphorylation events have been demonstrated to be the foremost mechanism in the ligand-independent activation of ER. Estrogen also induces phosphorylation of serine residues, but this appears to be independent of MAPK [66]. It is also described that a combined stimulation with growth factors and estrogen gives potentiated effect [67]. In bone cells, mechanical strain has a similar effect on increasing ERE activity as more prolonged exposure to estrogen [68]. It is suggested that strain has its effects on increased ERE activity by phosphorylation of the ER using kinase-dependent signaling pathways [69]. Strain-induced ER phosphorylation does not require the presence of estrogen, but is dependent on extra-cellular regulated kinase (ERK), a member of the MAPK family [70]. ER may also be activated by cAMP induced signaling [71]. Activation via cAMP signaling pathway requires the AF-2 site, in contrast to the MAPK which requires the AF-1 site, and appears to be dependent on protein kinase A that is activated by cAMP. This represents a pathway distinct from activation via peptide growth factors.

Non-ERE-dependent activations of ER. In addition to binding to the ERE, the activated ERs can interact with other DNA-bound transcription factors to regulate the transcription of certain sets of genes. In this mechanism, ERs do not themselves bind DNA; instead it is tethered by protein-protein interactions to a transcription factor complex that contrasts the DNA. AP-1 sites and SP-1 sites are well characterized motifs that could mediate estrogen signaling via other bound transcription factors, such as

FOS/JUN [72]. The discovery of this mechanism explains how estrogen regulates genes in which no consensus ERE has been found.

Non-genomic signaling. There is evidence that estrogen has non-genomic effect too, since very rapid effects of estrogen have been observed [73]. These effects are too rapid to be accounted for by transcriptional activation or repression of target genes, which occurs with a time lag of several hours. These effects occur within seconds to minutes after estrogen treatment and cannot be blocked by transcription or translational inhibitors. Studies have suggested that these effects may be the result of estrogen activation of MAPK and ERK signaling [74] or release of intracellular calcium [75]. The MAPK pathway is rapidly activated by estrogen in various cell types, for example endothelial cells [76]. Some of the protective effects of estrogen in the cardiovascular system are mediated by a non-genomic mechanism involving rapid activation of eNOS by estrogen through the MAPK pathway [77]. The activated eNOS releases NO which promotes vasodilatation. eNOS is also regulated on the genomic level by estrogen by activating an ERE-sequence in its promoter region [73].

1.5 Breast cancer treatment: endocrine therapy

Given the critical importance of ER signaling in BC initiation and progression, efforts have been made to block this pathway therapeutically. In addition to chemotherapeutic regimens, eligible patients receive endocrine therapy. Patients will be categorized into ER-positive group versus ER-negative group based on the result of immunohistochemistry (IHC) test, which is a widely used test for assessing therapeutic biomarkers (such as ER) and has become a major part of practical diagnosis for various malignancies [78]. The ER-positive patients are eligible for endocrine therapy [79].

Current endocrine therapies are based on synthetic compounds that either act as estrogen antagonists (antiestrogens) or block the function of aromatases (the enzymes that catalyze the last step of estrogen biosynthesis) have been thought. Antiestrogens are designed to antagonize hormone induced proliferation and ERα target gene expression in mammary tumor cells (Figure 1.6) [80]. Within the antiestrogens it is possible to distinguish two major classes of drugs, depending on their functional effects. The "Selective Estrogen Receptor Modulators" (SERMs) are separate antiestrogens able to act as both receptor agonists and antagonists (mixed agonists-antagonists), depending on the cellular and promoter context as well as the ER isoform targeted (tissue-specific properties). The "Selective Estrogen Receptor Downregulators/Disruptors" (SERDs) are classical pure antiestrogens (pure antagonists), capable to completely block the activity of E2, to increase ER turnover and to disrupt its nuclear localization with a concomitant reduction in the number of detectable ER molecules in the cells both *in vitro* and *in vivo*. In general, this class of ER ligands plays an important role as a second line therapy against advanced BC in patients who develop resistance to SERM treatment [81].

The molecular targets of these anti-hormone therapies are the ERs. All ERα ligands bind exclusively to the carboxy-terminal (C-t) ligand-binding domain (LBD). The LBD of the ER recognizes a variety of compounds diverse in their size, shape, and chemical properties. The ERα activity is mediated by at least two separate activation functions (AFs), AF-1 in the amino-terminal (N-t), and AF-2 in the LBD. The activity of AF-1 is regulated by growth factors acting through MAP-kinase signaling pathway [82], while AF-2 activity is responsive to agonist ligands [83]. The binding of agonists triggers AF-2 activity, whereas the binding of antagonist does not [84]. Several studies suggest that ligands regulate AF-2 activity by directly affecting the structure of the LBD. Any ligand-induced conformational change involving the repositioning of helix 12, the most C-t helix of the

LBD, is essential for AF-2 activity (Figure 1.5). Indeed, from X-ray crystallography studies the ligand-dependent orientation of helix 12 has emerged as the principal determinant that distinguishes the function of estrogens from antiestrogens. In the ER complexes with estrogens, the position of helix 12 creates a co-activator binding site, whereas the position of helix 12 in ER-antiestrogen complexes blocks co-activator binding. [85, 86]. Mutational and structural studies highlighted that the helices 3, 5, 6, 11 and 12 form a hydrophobic pocket which envelops the steroid ligand and represents the static region of the AF-2 and a recognition surface, created in presence of agonist, for the co-activators linking the ER to the RNA pol II transcriptional machinery. [87- 89]. The first antiestrogen introduced in the clinical practice is tamoxifen (also referred to in the literature as Nolvadex®), that is also known as the SERM prototype. [90, 91].

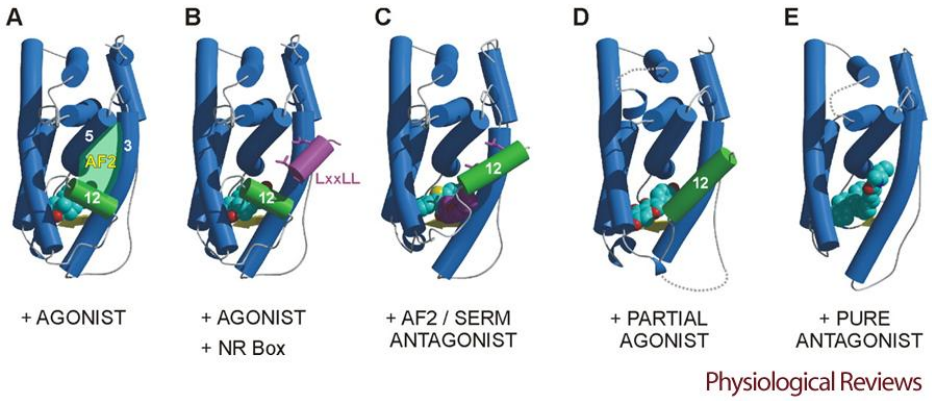


Figure 1.5 Major conformations induced by ER agonists and antagonists. Schematic representation of ERs bound to E2 **(A)**, E2/LXXLL NR-box **(B)**, raloxifene **(C)**, genistein **(D)**, and ICI **(E)**. The location of AF-2 between H3-H5 and H12 is indicated in A. The position of H12 is indicated by a green cylinder. The LXXLL peptide is shown as a purple cylinder with leucines in stick form in B. From: Heldring et al, 2007 [153].

In 1967 Dr Arthur L. Walpole discovered the compound ICI 46,474 in the laboratories of what is now the AstraZeneca pharma company [92], formerly Imperial Chemical Industries (ICI) and performed the initial studies in rodents. This drug, which later was named tamoxifen, tested on immature

rat, showed both estrogen agonist and antagonist effect, while on mouse only showed a full estrogen agonist effect. It was found that this compound, while functioning as a contraceptive in rats, did not exhibit equivalent activity in humans; however, it was initially proposed to act as a post-coital contraceptive agent and showed promise as an inducer of ovulation for women with challenged fertility. It was discovered latterly that, in humans, tamoxifen was modestly active in treating breast cancer, similarly to the high doses of estrogens or androgens that were already in clinical use [93]. Laboratory research into tamoxifen activity was temporary shelved until later in the 1970s, when it was discovered that is metabolic activation to 4-hidroxytamoxifen increased its binding affinity for ER by approximately 100-fold [94]. Tamoxifen is a no-steroidal antiestrogen that antagonizes the action of estrogen and is effective in both the treatment [95] and prevention [96] of ER-positive breast cancer [97, 98]. Although concerns were raised regarding the potential antiestrogenic effects on normal tissue, paradoxically tamoxifen acts as an estrogen on bone, blood lipids and the endometrium [99]. In the adjuvant and prevention settings this may increase the risk for endometrial cancer and thrombotic events in women taking tamoxifen, although the risk has been perceived to be small in relation to the substantial benefit from reduction in BC related events. Indeed, BC incidence was observed to be reduced by 48% in an at-risk population [96]. Nevertheless, breast epithelial cells and established carcinomas adapt to chronic antiestrogens exposure and develop resistance to tamoxifen, which may also result from the drugs partial agonistic activity stimulating tumor regrowth. [100, 101]. Tamoxifen has been a significant clinical success story for the treatment of hormone-responsive breast cancer, with accompanying observations that five years of adjuvant therapy in pre-menopausal ER positive patients was the optimal duration to improve disease free survival (DFS) and overall survival (OS) [102].

Raloxifene (also referred to in the literature as LY 156,758, keoxifene, LY 139, 481-HCL, Evista®), a second-generation SERM, was not developed as an antiestrogen for breast cancer, and a few data exist on the activity of raloxifene in patients with advanced cancer disease [103]. The goal was to introduce a new hormone replacement therapy to prevent osteoporosis but this drug proved to decrease the incidence of endometrial cancer and BC in the general population as a beneficial side effect [104]. Raloxifene is a no-steroidal antiestrogen produced by altering the triphenylethylene ring structure of tamoxifen to get a benzothiophene "fixed ring" structure. It has been evaluated in more than 11,000 postmenopausal women and has been found to maintain bone density with a decrease in BC incidence, in particular ER-positive tumors, in postmenopausal women by 76%, and no increase in endometrial thickness. Raloxifene exhibits binding affinity for the ER similar to that of tamoxifen but a lower estrogenic activity. It also has a potent antiestrogenic activity. Indeed, raloxifene exhibits estrogen-like effects in bone cells, preserving the bone mineral density, but not in uterine cells. It can rather block the uterotrophic action of both estrogen and tamoxifen. Furthermore raloxifene causes a decrease in circulating cholesterol, showing a beneficial side-effect in cardiovascular tissue [103]. Overall, raloxifene displays the profile of a SERM that could be applied as a potential preventive for osteoporosis in postmenopausal women but with the additional benefits of preventing BC and coronary heart disease and resulting into a better toxicity profile in terms of gynecological problems compared to tamoxifen [105]. At present, raloxifene is mainly used in a prophylactic capacity, to prevent the onset of BC in particularly high-risk patients [106]. As molecular effects of SERM compounds, ER leads to loss of heat shock proteins (HSPs), dimerization and phosphorylation of receptors, but with a specific conformational shape leading to co-activator action at AF-1 only and not at AF-2 site. The most striking feature of the structures of the tamoxifen- and raloxifene-liganded ERα LBD's is that helix 12 directly affects the structure

and function of the AF-2 surface in two ways. First, since helix 12 residues form an integral part of the AF-2 surface, AF-2 surface is incomplete when helix 12 is in the SERM-bound conformation. In particular, the critical aminoacids, such as Leu-539, Glu-542, and Met-543 are incorrectly oriented for co-activator recognition. The alkylaminoethoxy bulky side chain of the tamoxifen and the benzothiophenes "fixed ring" of the raloxifene project out of the hydrophobic pocket between helices 3 and 5 [107, 108] and, therefore, prevent helix 12 from repositioning and sealing, as a "lid", the ligand in the hydrophobic pocket. Second, residues from the static region of the AF-2 surface are bound to helix 12 and are prevented from interacting with co-activators. Indeed the aminoacids, that are critical for the co-activator recruitment are now masked [108]. The relative balance in a given cell type of co-activator and co-repressor proteins may also determine the given response to a particular ligand. Likewise, in the endometrium tamoxifen, but not raloxifene, may have estrogenic-like effects due to recruitment of co-activators to a subset of genes and this aspect may vary in different tissues depending on the background level of expression of co-activators (Figure 1.4) [109].

Fulvestrant (also known as ICI 182,780, Faslodex®), a SERD prototype, is a steroidal molecule devoid of estrogen-like activity in any body tissues. It was designed in order to treat patients with hormone sensitive breast tumors, that, after tamoxifen first line therapy, developed SERM resistance and showed on the gynecological tract side effects due to the SERM agonist activity on ER [110]. The absence in SERDs of agonist activity on the ER leads to the overcoming of the drug resistance development which limits the effectiveness of long-term tamoxifen therapy.

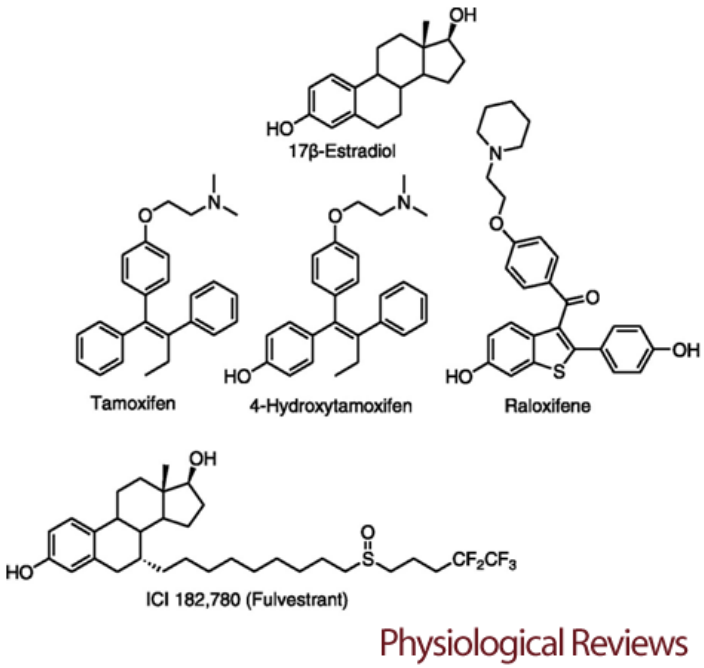


Figure 1.6 Molecular structures of the 17β-estradiol (E2) , tamoxifen, 4-hydroxytamoxifen (Tam), raloxifene (Ral), ICI 182,780 (fulvestrant). From: Heldring et al, 2007 [153].

Indeed, clinical studies demonstrated that proliferation of tamoxifen-resistant BC cell lines is again inhibited by fulvestrant [111, 112]. As far as the molecular activity concerns, fulvestrant competitively inhibits the binding of estrogen to the ER and binds to ER with an affinity that is about 100 times that of tamoxifen [110, 113]. As previously highlighted for SERMs, also for fulvestrant the alignment of the C-terminal transactivation helix (H12) over the LBD of ER is prevented, because of its long bulky 7a side chain. In addition the positioning of the terminal amide portion of the side chain precludes H12 from adopting its alternate orientation along the co-activator binding shallow groove of AF-2. As a result fulvestrant abolishes association between H12 and LBD, affecting the AF-2 functionality. Furthermore, the ER abnormal conformation results in loss of receptor dimerization and accelerated ubiquitylation and shuttling of the ER to the proteasome for degradation [111, 112, 114]. Indeed, a down regulation of the ER protein expression, that is, a reduction in the number of detectable ER molecules in

the cells both *in vitro* and *in vivo,* is observed. The ER rapid degradation plays a key role in the molecular basis of full antagonism of fulvestrant, because it also causes lack of the AF-1 agonist activity which, instead, is effective in SERMs. Fulvestrant shows a full antagonist activity on breast and endometrium without affecting bone density and serum lipids, and currently has been approved for the treatment of BC which has progressed on prior antiestrogen therapy in postmenopausal women. In the wake of emerging resistance to tamoxifen it was recognized that removal of E2 from the environmental of the tumor might prove to be a more effective method of blocking ligand-dependent ER-mediated signaling.

In this context, a spotlight has appeared on aromatase inhibitors as a novel endocrine therapy option in the past decade [57]. Instead of blocking binding of E2 to ER, these compounds act by preventing E2 biosynthesis catalyzed by the aromatase enzyme in extra gonadal tissue.

2. AIMS OF THE STUDY

Since clinical evidence shows that more than 30% receptor positive mammary tumors are unexpectedly non-responsive to endocrine treatments, and the reasons for such failure have been suggested to depend on the functions of ER and/or the intracellular signaling pathway controlled by estrogens, in this regard, dissection of the ER signaling networks in hormone-responsive BC cells, a useful approach to identify the molecular mechanisms of cell responsiveness to estrogen, may provide new insights on resistance of breast tumors to endocrine therapies. Interaction proteomics led so far to the identification of a large number of E2−ERα interactors in BC cell nuclei, including transcriptional coregulators and components of the nuclear actin pathway [115-118].

The main purpose of this study was to apply this technology to map the nuclear interactomes of ERα bound to the antiestrogenic compounds commonly used for BC treatment (i.e., ICI, Ral, and Tam), aiming at providing new mechanistic information to help explain the pharmacological activities of these drugs in BC cells in vitro and in vivo.

To this purpose, the following specific aims were defined:

1) isolate and identify the ERα interacting proteins when the receptor is bound to several antiestrogen compounds of common clinical use or to the endogenous ligand;

2) compare the compositions of the different ERα interactomes (receptosome) in order to identify the specific molecular partners of each antiestrogen compound and, in particular, those common to the two SERMs;

3) investigate the molecular processes and biological functions globally more representative for each ligand.

3. RESULTS AND DISCUSSION

3.1 Evaluation of ligand effects on the intracellular localization of wild-type and TAP-tagged ERα

The cellular model used in this study was derived from hormone-responsive human BC MCF-7 cells, naturally expressing ERα and widely used to investigate signal transductions by ERs in BC and to test the pharmacological effects of ER ligands. MCF-7 cells were stably transfected with an expression vector encoding ERα fused at the C-terminus with a TAP tag [119, 120] that can act as a "bait" for isolation of native ER containing multiprotein complexes by tandem affinity purification (TAP). These cells have been used successfully for mapping and functional characterization of E2-induced ERα nuclear interactome by TAP [117, 118, 121] As antiestrogens have been described to influence in different ways the cellular level and/or localization of ERα, the behavior of the exogenous fusion protein with respect to the endogenous receptor was assessed upon cell treatment with either E2, ICI, Ral, or Tam. In all cases, the nuclear levels of TAP-ERα were assessed by Western blotting and compared to those relative to endogenous ERα in the same samples or, for E2, in wt MCF-7 cells (Figure 3.1). Results, summarized in the histogram, show that the exogenous receptor behaves similarly to the endogenous one in all cases, as demonstrated by Ambrosino et al. [117]. Interestingly, comparing the results obtained for edogenous receptor in E2- treated wt and TAP-ERα cells, the former exhibit higher ERα levels, possibly due to inhibition of endogenous receptor expression by the exogenous protein [117]. The pure antiestrogen ICI disrupts nuclear-cytoplasmic shuttling of both ERα and TAP-ERα, possibly by inducing proteasome-dependent ER degradation [122, 123], as 1 h treatment of the cells with this compound causes a modest increase of receptor concentration in the nuclear extracts, when compared to that elicited

by E2 (Figure 3.1). On the other hand, the two SERMs (Ral and Tam) induce substantial nuclear accumulation of both receptor forms, with Tam being less effective than E2, but more than Ral, as described for endogenous ERα in this cell type [124]. Kinetic evaluation of nuclear translocation of ER was performed after 1, 6, and 12 h of treatment with each of the compounds studied by WB analysis of ERα and TAP-ERα both in the nucleus and in the cytoplasm, with β-actin and α-tubulin, respectively, used as controls. Results, reported in Figure 3.2, show that while E2 and SERMs induce a substantial accumulation of both ERs in the nuclear compartment for up to 12 h of treatment, ICI effects are not only less pronounced but also dynamic, since nuclear ER concentration decreases between 1 and 6 h and then rises again at 12 h.

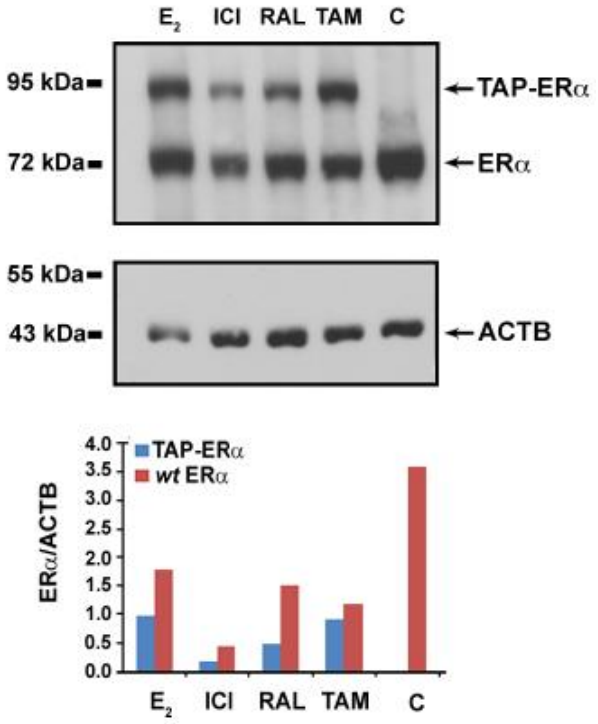


Figure 3.1 Western blot analysis of ERα and TAP-ERα in MCF-7 nuclear extracts, normalized to nuclear β-actin (ACTB) concentration in the same samples, from cells treated with E2, ICI, Ral, and Tam (10^{-8} M; 1 h). (C) Wild-type MCF-7 cells, not expressing TAP-ERα, stimulated with E2, used as negative control. Relative quantitation of TAP-ERα and ERα compared to ACTB is shown in the histogram.

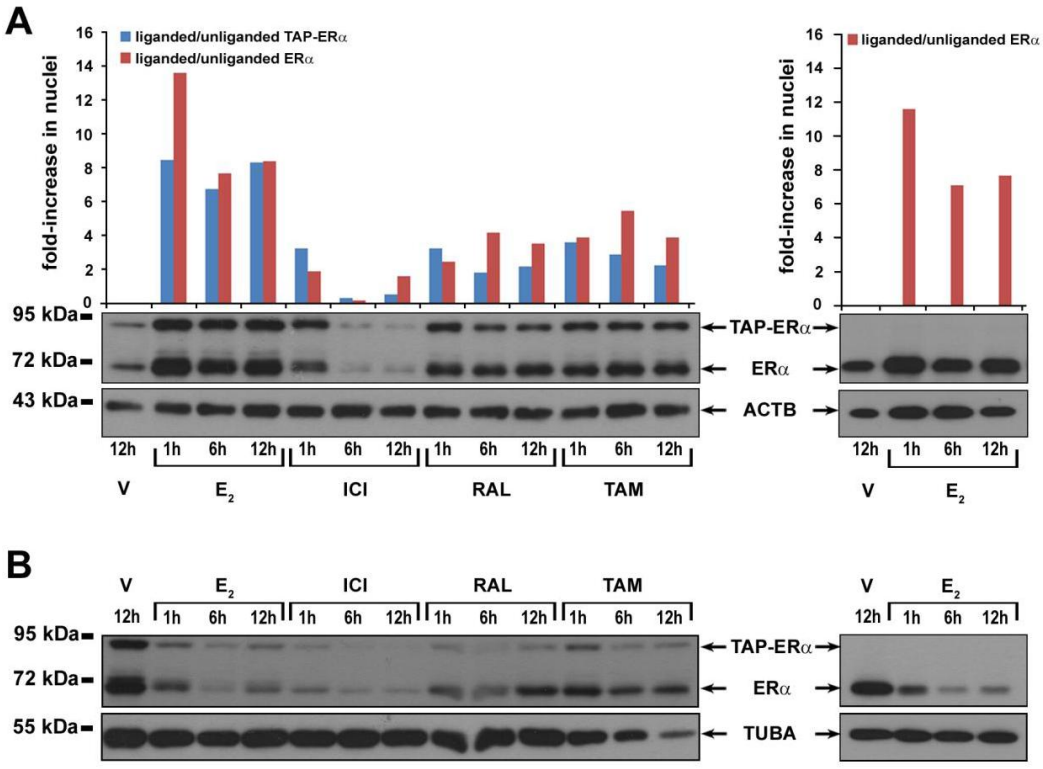


Figure 3.2 Time-course analysis of ERα and TAP-ERα nuclear traslocation induced by E2, ICI, Ral, Tam (10^{-8}M) or vehicle alone (V) by Western Blotting. (**A**) Nuclear extracts (TAP-ERα cells to the left and *wt* cells to the right). The histograms show TAP-ERα and ERα levels, relative to β-actin (ACTB), in treated *vs* untreated cells. (**B**) Cytoplasmic extracts (TAP-ERα cells to the left and *wt* cells to the right). TUBA: α-Tubulin.

3.2 Effects of estrogen and antiestrogens on the transcriptome of TAP-ERα expressing cells

ERα exerts its biological effects through several mechanisms, that all converge on regulation of target gene expression. As the different ligands affect recruitment of co-regulators on ERα, their influence on the ER dependent transcriptome in MCF-7 cells was investigated by gene expression profiling with oligonucleotide microarrays. To this end, TAP-ERα expressing MCF-7 cells were stimulated for 12 h with either E2, ICI, Ral, or Tam, and total RNA was extracted, fluorescently labeled, and analyzed on high-density oligonucleotide microarrays. This time of stimulation was chosen in order to better evaluate early, primary responses to the ligands, with respect to late,

secondary events in TAP-ERα cells, as shown by Cicatiello et al. for E2 in wt MCF-7 [125] and another hormone-responsive BC cell line [126]. Untreated TAP-ERα cells were used as a control. The results obtained are summarized in the heatmaps reported in Figure 3.3, where data are reported relative to mRNAs that showed a ≥2 fold-change, with respect to the control, in response to stimulation with each of the four compounds tested. In each case, changes in expression of the same mRNA under all four conditions are also reported, side by-side, to highlight similarities and differences in gene response to different ER ligands. Results show that gene activation clearly prevails over inhibition in response to E2 stimulation, as 2/3 of the transcripts show significantly higher levels in treated vs untreated cells, while, on the contrary, gene down-regulation events appear predominant following anti-estrogen treatment, independently from the nature of the drug used. As expected, under these conditions most of the genes regulated by estrogen do not respond similarly to antiestrogens. When comparing the overall responses of the MCF-7 cell transcriptome to the three antiestrogens tested, the effects appear strikingly different. First of all, the total number of genes responding to ICI is very low, when compared to the responses elicited by two SERMs, in agreement with the low nuclear ER concentration in the presence of this ligand and the known effects of ICI in hormone-responsive BC cells [127], with ICI regulated genes generally responding similarly also to Tam and Ral. Furthermore, Tam-responsive genes are more numerous than ICI-regulated ones and show similar regulation by Ral, but not by E2. This is in accordance with the observation that these SERMs promote ERα translocation to the nucleus and induce very similar conformational changes of the receptor, that are different from those promoted by binding of the cognate hormone. The effects of Ral on the cell transcriptome, however, are much more evident and, in most cases, unique to this drug. Taken together, the results shown in Figures 3.1, 3.3, and 3.4 reproduce correctly the known biological effects of these drugs in hormone-responsive BC cells, that are here confirmed to be

strictly related to the chemical structure of each compound and its ER binding properties, to indicate that the experimental model described here is suitable to identify functional protein partners of antiestrogen-bound ERα by interaction proteomics**.**

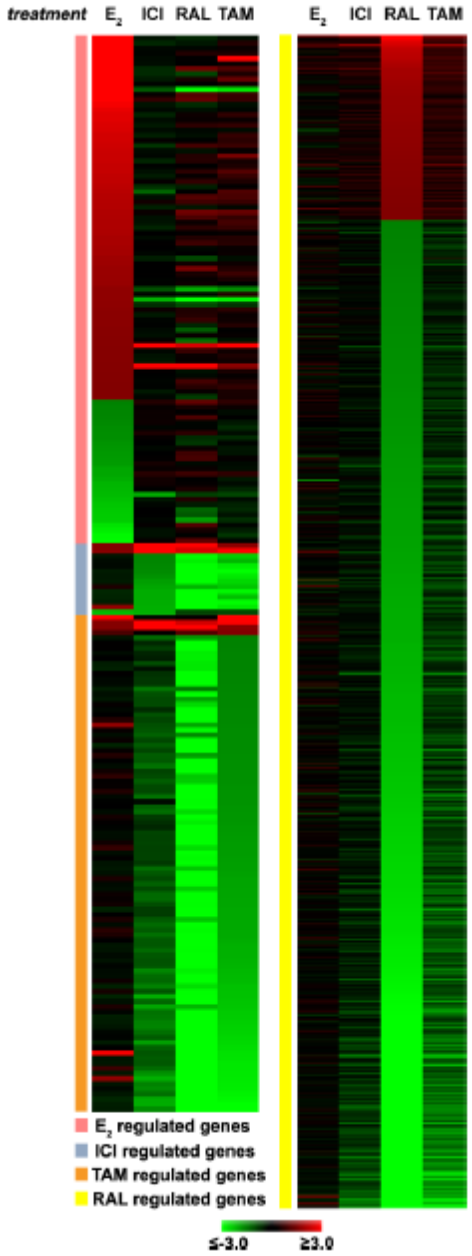


Figure 3.3 Interaction genomics of agonist- and antagonist-bound ERα in MCF-7 cells. Heatmaps summarizing the results of transcriptome analyses of TAP-ERα-expressing MCF-7 cells treated (10^{-8} M; 12 h) with E2 (top-left), ICI (midleft), Tam (bottom-left), or Ral (right). For each ligand, gene expression fold-changes were calculated with respect to the untreated control.

3.3 Identification of proteins recruited by ERα in the nucleus of BC cells in response to E2, ICI, Ral, or Tam

In order to identify ERα partner proteins specifically recruited by the receptor upon binding of an agonist (E2) or of different antagonist (ICI, Ral, Tam) ligands, partially purified ERα protein complexes isolated from native MCF-7 cell nuclear extracts were subjected to MS analysis (nanoLC-MS/MS). As control, wt cells, lacking the TAP-tagged receptor, were subject to the same purification−identification protocol and all proteins identified in these samples were considered not specific, and, for this reason, when present, they were discarded from the lists of specific ERα interactors and not considered further, as described by Tarallo et al. [118]. In addition, when focusing on SERMs specific ERα complexes, the proteins identified in ICI-treated samples were subtracted from the number of the Ral and Tam interactors and listed as a separate set. Two biological replicates were performed, and when the resulting MS data were analyzed separately, a very good reproducibility was observed (>60% identified proteins in common between the replicas), suggesting reliability of the purification procedure. To identify ligand-specific ER-associated nuclear proteins, the MS results from the two biological replicates were combined and analyzed together, to obtain more robust data sets. Results of this analysis, detailed in the Materials and Methods section, are listed in Tables 3.2-3.5 for E2, ICI, Ral, Tam, respectively, and are summarized in Figure 3.4. Receptor activation by E2 resulted in interaction with a set of nuclear proteins (210) significantly larger than those observed with ICI (46), Ral (23), or Tam (49). This result, that confirms our previous observations [117, 118], is likely to be due to the optimal receptor conformation promoted by the agonist, as well as to formation of stable ER homodimers, that provide an efficient docking site for interacting proteins. Furthermore, this could be explained, at least in part, by the relatively higher concentration of ERα in these samples, which may have

facilitated isolation and/or MS identification of interacting proteins. It is worth mentioning that the number of molecular partners of ERα in the sample treated with E2 for 1 h is comparable to what was previously reported for a 2 h stimulation with E2 of the same cells, but the two sets share only about 50% of proteins [118], in agreement with the highly dynamic ER−protein interactions occurring on BC cell chromatin during the earlier phases of hormonal stimulation [128].

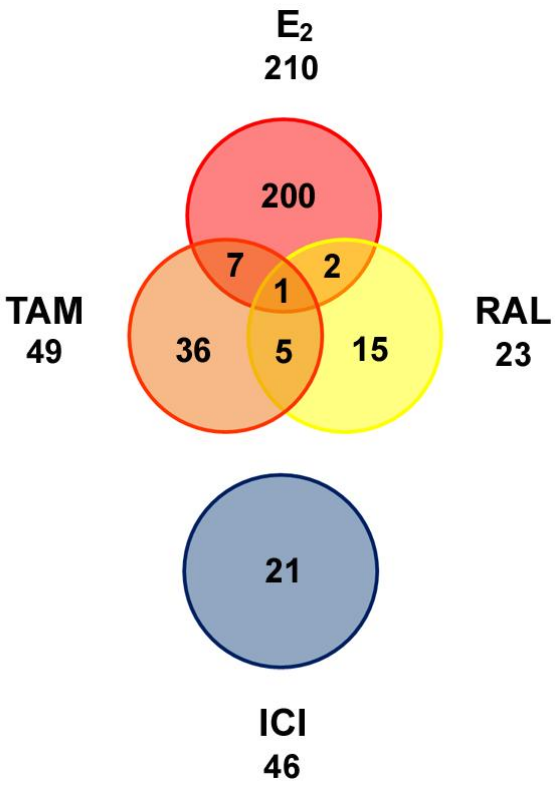


Figure 3.4 Interaction proteomics of agonist- and antagonist-bound ERα in MCF-7 cells. Venn diagram showing overlaps of interactomes identified following cell treatment with E2, Tam, or Ral (10^{-8} M; 1 h). Numbers reported below each symbol indicate the total number of specific interactors identified in purified samples by MS. The number within the isolated circle at the bottom of the panel indicates interactors specific for ICI treated cells.

The vast majority of the interactors identified are ligand specific and, as mentioned above, their number in SERM treated cells is significantly lower than that in E2-stimulated cells, with the compositions of the Ral and

Tam interactomes being rather different from each other and clearly distinct from those of ICI samples. Indeed, comparative analysis of the lists of ERα interactors identified with the four compounds tested shows that the majority of them are ligand-specific: 200 for E2, 15 for Ral, 36 for Tam, and 21 for ICI (Figure 3.4). Interestingly, five proteins not present in the E2 treated samples are specific to both SERMs (Table 3.1), suggesting that they might represent specific SERM effectors (see also below). It is worth mentioning that while the patterns of interactors detected with the two SERMs and E2 are very different from each other, the SERD appears to promote recruitment of a relatively larger number of proteins in common with E2 (19/ 46), a result that could relate to the fact that ICI, unlike SERMs, has a steroid structure like that of E2 and could therefore induce a conformational change on the receptor that, to some extent, is structurally closer to that elicited by binding of the natural hormone. The number of interactors identified does not seem to correlate only with concentration of ERα in nuclear extracts or purified samples, since, for example, the amount of receptor in Tam samples is only slightly lower than that in E2 samples but much higher than that in Ral or ICI samples, while the difference in number of binding proteins identified in E2 vs Tam samples is significant and that in Tam vs Ral or ICI samples is very small (compare results in Figure 3.4 with those in Figures 3.1 and 3.6A). On the basis of this observation, the known differences in biological activity in the BC cells of the compounds tested, and the results reported in Figure 3.3, we suggest that the lists of ERα interactors identified in this study, the majority of which were not shown before to be partners of the antiestrogen-bound receptor, represent a snapshot of the early and specific functional complexes formed by this protein in BC cell nuclei upon binding to antiestrogens, exploitable now to identify the molecular mechanisms that determine the variegated pharmacological effects of these drugs in BC cells. Functional analysis of the biological processes that involve the ERα interacting proteins identified in this study by gene ontology highlights

significant differences between agonist (E2)-, SERD-, and SERM-specific interactomes (Figure 3.5), that reflect also known effects of these ligands in BC cells.

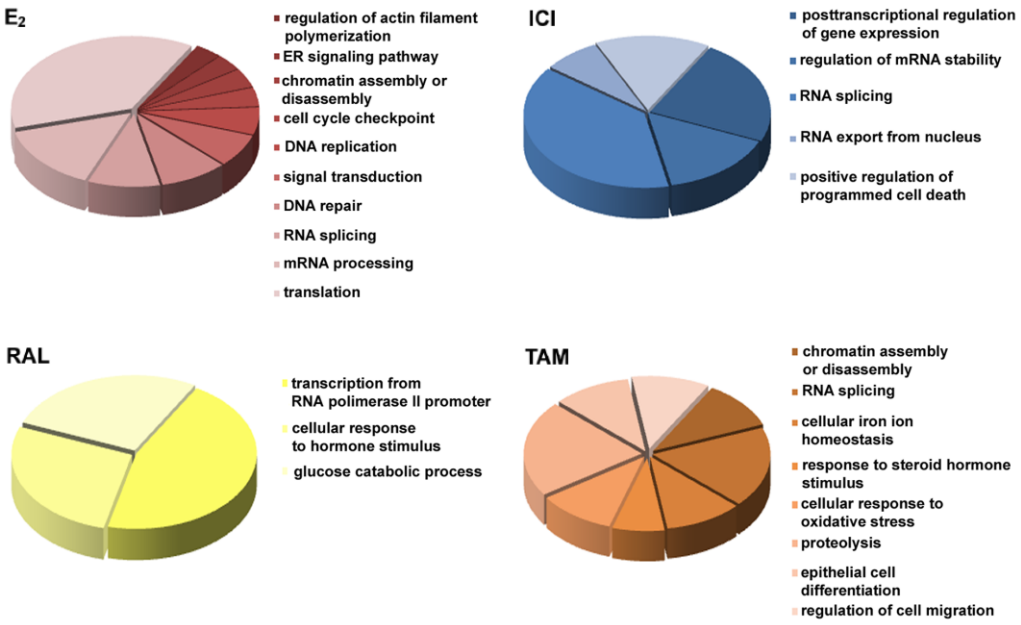


Figure 3.5 Gene ontology analysis of the biological processes involving the proteins interacting with E2- (**red pie chart**), ICI- (**blue pie chart**), Ral- (**yellow pie chart**), or Tam-bound (**orange pie chart**) ERα. For each treatment a pie chart highlights the most significant cellular processes involving the proteins recruited to the receptor by each of the ligands studied.

In particular, estrogen promotes recruitment by ER of proteins involved in DNA replication and cell cycle progression, chromatin remodeling, gene transcription, and RNA splicing and actin cytoskeleton organization, while components of the ICI-dependent interactome participate in the control of mRNA stability and translation and in regulation of apoptosis, all processes associated with the cytostatic actions of this drug. On the other hand, the proteins specifically bound to SERM-ER are specifically involved not only in regulation of gene expression and signal transduction but also in proteolysis, epithelial cell differentiation, cell migration, and response to oxidative stress. While most of these functions remain to be

elucidated in the context of hormone-responsive BC cells, this result confirms the existence of common pathways controlled by estrogen and SERMs in this cell type, clearly distinct from those specifically affected by ICI. Similar differences between the four lists of interactors were observed also when GO term enrichment analysis was performed using as background (reference) a list of MCF-7 proteins detected experimentally, obtained by combining published results.

SwissProt ID	Protein name	Gene name	Peptides matched		Sequence coverage (%)		MOWSE Score	
			RAL	TAM	RAL	TAM	RAL	TAM
B2RTY4	Myosin-IXa	MYO9A	6	6	2	2	30	27
P08559	Pyruvate dehydrogenase E1 component subunit alpha, somatic form	PDHA1	5	1	11	3	38	48
P12036	Neurofilament heavy polypeptide	NEFH	4	2	3	1	93	72
Q5VUG0	Scm-like with four MBT domains protein 2	SFMBT2	2	1	1	1	30	30
Q8TEK3	Histone-lysine N-methyltransferase, H3 lysine-79 specific	DOT1L	5	2	3	0	28	27

Table 3.1 Proteins identified specifically in nuclear extracts from SERM-treated cells.

To validate the results obtained by mass spectrometry, WB analysis was carried out using a selection of antibodies directed against some of the most interesting proteins exhibiting ligand specific association with ERα. Among these, we selected the ICI-ERα specific interactor KIAA1967 protein, also known as Deleted in breast cancer gene 1, whose expression in MCF-7 cell nuclei was unaffected by ligand treatment (Figure 3.6A).

In agreement with the MS results, KIAA1967 was prevalently detected by WB in purified TAP−ERα complexes from SERD treated cells (Figure 3.6B), despite the low concentration of ERα in these samples. A slight amount of this protein in E2 and Tam samples was close to that detected in the control sample, despite the very high concentration of receptor

under these conditions, confirming a preferential interaction of KIAA1967 with SERD-bound ERα.

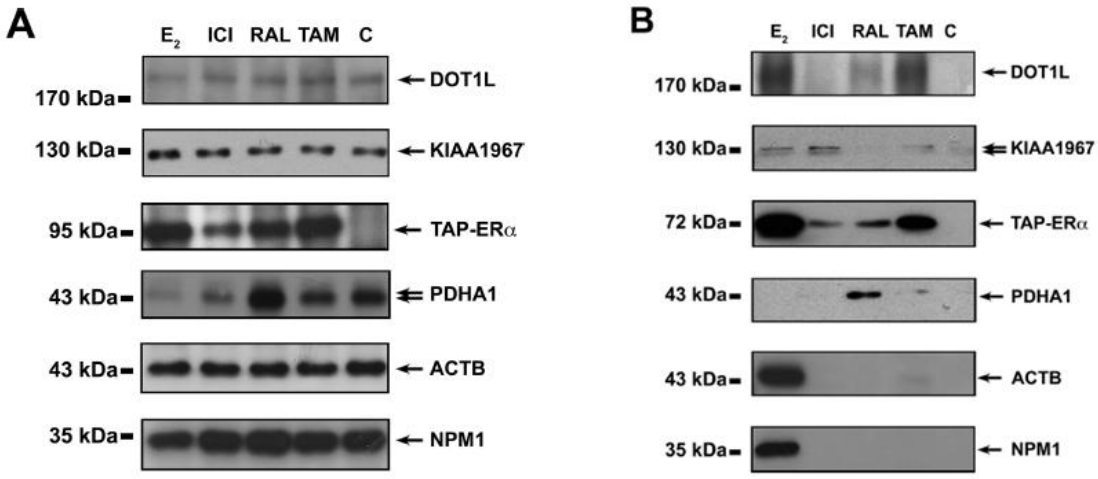


Figure 3.6 Western blot analysis of selected ER nuclear interactors identified by TAP. (**A**) Whole nuclear extracts from wild-type MCF-7 (C) or TAP-ERα cells stimulated with E2, ICI, Ral, or Tam (10^{-8} M; 1 h). (**B**) Validation of MS data. Confirmation of TAP-ERα interaction with DOT1L (DOT1-like, histone H3 methyltransferase), KIAA1967 (Deleted in breast cancer gene 1/KIAA1967), PDHA1 (pyruvate dehydrogenase (lipoamide) alpha 1), ACTB (nuclear β-actin), or NPM1 (Nucleophosmin/Nucleolar phosphoprotein B23/Numatrin), measured in partially purified samples (bound or eluate TAP fractions). Double arrows indicate the presence of two bands detected by the antibodies against human PDHA1 and KIAA1967 proteins.

Recruitment of KIAA1967 by ICI liganded ERα may have important antitumor effects, as this protein has been shown to be able to interact directly with SIRT1 and to inhibit the activity of this enzyme both in vitro and in vivo [129]. SIRT1 is involved in cancer cell growth and survival, due also to its antiapoptotic activity [130, 131] and ability to silence tumor suppressor genes [132]. Interestingly, KIAA1967 has been found over-expressed in cancer cells [133- 136], suggesting that recruitment of KIAA1967 by ICI-liganded ERα may target key cancer genes, resulting in their silencing by SIRT1.

Pyruvate dehydrogenase E1 component subunit α (PDHA1) appears, instead, to be a preferred interactor of ERα−Ral and, to a lesser extent, −Tam complexes, as confirmed by WB (Figure 3.6). Surprisingly, a significant

increase of PDHA1 can be observed in the crude nuclear extracts upon treatment of the cells with Ral and, to a lesser extent, with Tam, but not with E2 ICI (Figure 3.6A). This appears to be the result of drug-induced nuclear translocation of the protein, as the total cellular concentration of PDHA1 did not change significantly following treatment (Figure 3.7) and WB analysis of the cytoplasm fractions showed reduction of the cytoplasmic concentration of PDHA1 in correspondence with its increase in the nuclear compartment (data not shown). Pyruvate dehydrogenases, that exert a pivotal role in cellular metabolism, were recently assigned an additional function in the nucleus as coactivators in STAT5-dependent gene transcription in response to interleukin IL-3. This was reported specifically for the pyruvate dehydrogenase E2 component (PDHE2), which interacts both in the nucleus and in the cytoplasm with the E1 component (PDHE1) [137], suggesting that PDHA1 may contribute to the function of PDHE1 as coregulator of SERM−ERα complexes for target gene regulation.

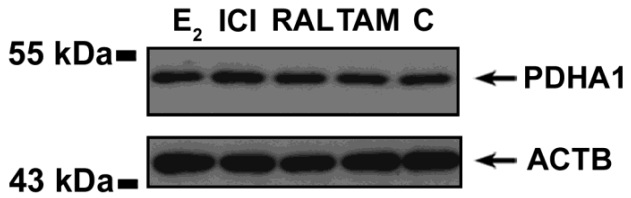


Figure 3.7 Western Blot analysis of PDHA1 in whole cell extracts from cells from E2-, ICI-, Ral- or Tam-stimulated (10^{-8}M; 1hr) TAP-ERα cells. C: *wt* MCF-7 cells stimulated with E2, used as negative control. ACTB: β-actin.

Nuclear levels of β-actin (ACTB) and Nucleophosmin (Nucleolar phosphoprotein B23/Numatrin NPM1), two well characterized functional partners of estrogen-activated ERα in BC cell nuclei [117], were, instead, not affected by treatments (Figure 3.6A), but these proteins were clearly detected in E2 stimulated samples (Figure 3.6B), in agreement with the MS results, and to a much lower extent, in Tam-treated samples, in agreement with

previously published results [117, 118, 138] and with the MS output data, that in Tam samples detected peptides from this protein but assigned a low MOWSE score. In view of the role that β-actin plays in regulation of gene expression, by recruitment of chromatin remodeling complexes and a positive effect on RNA polymerase II activity, and the known role of Nucleophosmin in ribosome biogenesis, a reduced recruitment of these proteins to antiestrogen-bound ERα will result in reduction of receptor effects on the above-mentioned processes, which might explain the differences in gene regulation shown in Figure 3.3 and the antiestrogenic effects of SERMs and SERDs in BC cells both in vitro and in vivo. Interestingly, an additional SERM-spcecific interactor, Myosin- IXa, is itself a component of the actin-based motors involved in intracellular movements and, in particular, in collective epithelial cell migration that facilitate formation and maintenance of continuous cell layers. In MCF-7 cells, estrogens promote acquisition of mesenchymal-like features associated with metastasis development and stimulate movement of a subset of estrogen-treated cells as cell clusters (collective motility). Antiestrogens, such as Tam, prevent both phenomena. [139] Myosin-IXa has been suggested to locally regulate Rho proteins and assembly of thin actin bundles associated with nascent cell−cell adhesion, which is required to sustain the collective migration of epithelial cells. Recruitment of this protein by Ral- and Tam-bound ERα, identified here in the nucleus, could also occur in the extranuclear compartment, where it may result in reduction of the collective cell migration. Alternatively, binding of this protein to SERM-ER could result in accumulation of this protein in the nucleus, diverting it from its activities outside this compartment. Another interesting SERM-specific ERα interactor discovered here is the DOT1-like, histone H3 methyltransferase DOT1L protein, a histone code “writer” lacking the SET domain. DOT1L, that is responsible for regulating gene expression through histone-methylation (H3K79) [140], can bind to several MLL-fusion partners found in acute leukemia and, through this binding, is thought to

promote oncogenesis [141, 142]. In order to further investigate DOT1L-ERα interaction by an independent experimental approach, coimmunoprecipitations were performed. wt MCF-7 cells were stimulated with E2, ICI, Ral, or Tam (10^{-8} M, 1 h) or the vehicle alone (V).

Whole nuclear protein extracts were immunoprecipated with specific antibodies against either DOT1L or ERα, and the immunoprecipitates were analyzed by Western Blotting with both Abs.

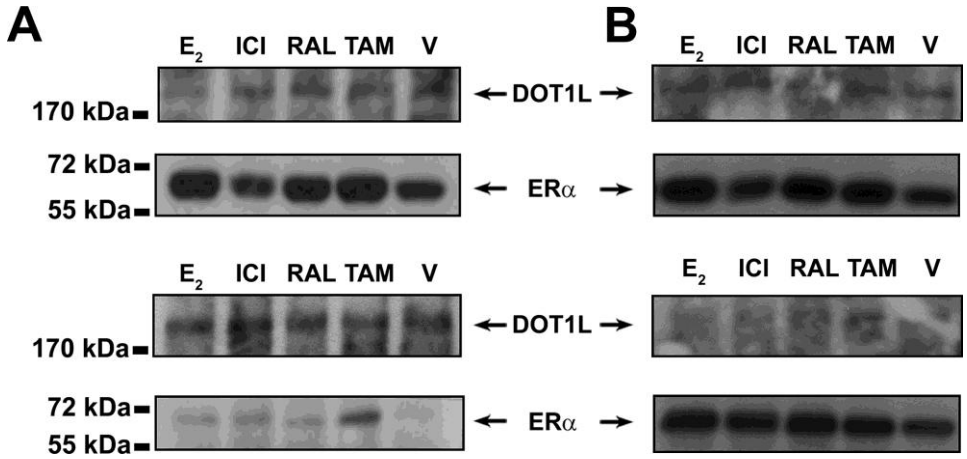


Figure 3.8 DOT1L-ERα coimmunoprecipitation. (**A**) Immunoprecipition with anti-DOT1L Abs. Upper panel: whole nuclear extracts from wild-type MCF-7 cells stimulated with E2, ICI, Ral, Tam (10^{-8} M; 1 h), or vehicle alone (V); lower panel: immunoprecipitates from the same samples. (**B**) Immunoprecipitation with anti- ERα Abs. Upper panel: whole nuclear extracts from wild-type MCF-7 cells stimulated with E2, ICI, Ral, Tam (10^{-8} M; 1 h), or vehicle alone (V); lower panel: immunoprecipitates from the same samples.

The results shown in Figure 3.8 confirm preferential DOT1L interaction not only with SERM- but also with ICI-bound ERα. Interestingly, inhibition of DOT1L has been shown to be a valid therapeutic strategy in tumor treatment [143]. Recruitment of DOT1L by antiestrogen−ERα complexes (Figures 3.6B, 3.8 and Table 3.1) could thus play a role in controlling the enzymatic activity of DOT1L and, therefore, modulate its downstream targets.

Table 3.2
Proteins specifically identified in partially purified sample upon E2 treatment, including in **bold,** *italic* and ***bold italic*** those in common with Ral, Tam and both SERM ligands, respectively.

SwissProt ID	Protein name	Gene name	Peptides matched	Sequence coverage (%)	MOWSE Score
O00159	Myosin-Ic	MYO1C	12	11	254
O14974	Protein phosphatase 1 regulatory subunit 12A	PPP1R12A	3	2	53
O14979	Heterogeneous nuclear ribonucleoprotein D-like	HNRPDL	4	11	47
O15020	Spectrin beta chain, brain 2	SPTBN2	8	3	29
O15143	Actin-related protein 2/3 complex subunit 1B	ARPC1B	3	11	28
O15144	Actin-related protein 2/3 complex subunit 2	ARPC2	1	4	31
O15400	Syntaxin-7	STX7	2	5	43
O15523	ATP-dependent RNA helicase DDX3Y	DDX3Y	6	7	64
O43143	Putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15	DHX15	5	2	30
O43707	Alpha-actinin-4	ACTN4	16	19	482
O43795	Myosin-Ib	MYO1B	30	26	935
O43866	CD5 antigen-like	CD5L	1	2	52
O60832	H/ACA ribonucleoprotein complex subunit 4	DKC1	9	18	244
O60841	Eukaryotic translation initiation factor 5B	EIF5B	1	0	30
O75420	PERQ amino acid-rich with GYF domain-containing protein 1	GIGYF1	5	4	28
O76021	Ribosomal L1 domain-containing protein 1	RSL1D1	11	20	290
O94906	Pre-mRNA-processing factor 6	PRPF6	8	8	83
O95782	AP-2 complex subunit alpha-1	AP2A1	9	8	123
P00367	Glutamate dehydrogenase 1, mitochondrial	GLUD1	8	14	274
P04843	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1	RPN1	6	10	99
P05141	ADP/ATP translocase 2	SLC25A5	6	22	326
P05388	60S acidic ribosomal protein P0	RPLP0	9	40	631
P06576	ATP synthase subunit beta, mitochondrial	ATP5B	3	6	53

P06748	Nucleophosmin	NPM1	15	37	563
P07197	Neurofilament medium polypeptide	NEFM	4	3	31
P08238	Heat shock protein HSP 90-beta	HSP90AB 1	7	8	29
P08708	40S ribosomal protein S17	RPS17	3	23	38
P08754	Guanine nucleotide-binding protein G(k) subunit alpha	GNAI3	2	7	227
P0C0S5	Histone H2A.Z	H2AFZ	1	7	71
P11387	DNA topoisomerase 1	TOP1	33	35	1187
P11388	DNA topoisomerase 2-alpha	TOP2A	15	9	381
P14649	Myosin light chain 6B	MYL6B	3	19	115
P15153	Ras-related C3 botulinum toxin substrate 2	RAC2	2	9	133
P15880	40S ribosomal protein S2	RPS2	3	10	44
P15924	Desmoplakin	DSP	13	4	29
P17066	Heat shock 70 kDa protein 6	HSPA6	7	13	245
P17480	Nucleolar transcription factor 1	UBTF	16	18	226
P17844	Probable ATP-dependent RNA helicase DDX5	DDX5	27	38	1065
P18077	60S ribosomal protein L35a	RPL35A	6	38	60
P18124	60S ribosomal protein L7	RPL7	4	19	215
P19338	Nucleolin	NCL	21	28	1216
P22087	rRNA 2'-O-methyltransferase fibrillarin	FBL	5	21	149
P23246	Splicing factor, proline- and glutamine-rich	SFPQ	18	27	317
P23396	40S ribosomal protein S3	RPS3	3	11	55
P26599	Polypyrimidine tract-binding protein 1	PTBP1	2	3	44
P30050	60S ribosomal protein L12	RPL12	5	27	304
P35249	Replication factor C subunit 4	RFC4	3	9	48
P35268	60S ribosomal protein L22	RPL22	6	46	435
P35579	Myosin-9	MYH9	28	15	511
P35580	Myosin-10	MYH10	7	4	66
P35659	Protein DEK	DEK	8	20	107
P36578	60S ribosomal protein L4	RPL4	13	28	385
P36955	Pigment epithelium-derived factor	SERPINF1	1	1	32
P38159	Heterogeneous nuclear ribonucleoprotein G	RBMX	5	15	128
P39656	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit	DDOST	1	1	55
P40429	60S ribosomal protein	RPL13A	5	9	42

	L13a				
P42285	Superkiller viralicidic activity 2-like 2	SKIV2L2	6	5	28
P46087	Putative ribosomal RNA methyltransferase NOP2	NOP2	6	8	102
P46776	60S ribosomal protein L27a	RPL27A	4	22	243
P46777	60S ribosomal protein L5	RPL5	14	40	319
P46778	60S ribosomal protein L21	RPL21	3	21	37
P46779	60S ribosomal protein L28	RPL28	12	52	331
P47756	F-actin-capping protein subunit beta	CAPZB	5	18	123
P49207	60S ribosomal protein L34	RPL34	4	22	37
P49916	DNA ligase 3	LIG3	3	2	42
P50583	Bis(5'-nucleosyl)-tetraphosphatase [asymmetrical]	NUDT2	1	7	31
P50914	60S ribosomal protein L14	RPL14	4	20	121
P51116	Fragile X mental retardation syndrome-related protein 2	FXR2	3	4	31
P52272	Heterogeneous nuclear ribonucleoprotein M	HNRNPM	11	15	187
P52907	F-actin-capping protein subunit alpha-1	CAPZA1	3	15	155
P53680	AP-2 complex subunit sigma	AP2S1	1	4	40
P55084	Trifunctional enzyme subunit beta	HADHB	4	7	67
P60468	Protein transport protein Sec61 subunit beta	SEC61B	2	26	91
P60866	40S ribosomal protein S20	RPS20	1	5	50
P61247	40S ribosomal protein S3a	RPS3A	5	18	49
P61313	60S ribosomal protein L15	RPL15	4	19	139
P61353	60S ribosomal protein L27	RPL27	3	20	114
P61513	60S ribosomal protein L37a	RPL37A	2	18	144
P61803	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit DAD1	DAD1	1	8	44
P62140	Serine/threonine-protein phosphatase PP1-beta catalytic subunit	PPP1CB	6	20	87
P62244	40S ribosomal protein S15a	RPS15A	5	34	102
P62249	40S ribosomal protein S16	RPS16	4	19	60
P62266	40S ribosomal protein	RPS23	6	30	318

	S23				
P62269	40S ribosomal protein S18	RPS18	4	24	157
P62277	40S ribosomal protein S13	RPS13	5	30	51
P62280	40S ribosomal protein S11	RPS11	6	27	140
P62424	60S ribosomal protein L7a	RPL7A	3	9	39
P62701	40S ribosomal protein S4, X isoform	RPS4X	6	19	99
P62750	60S ribosomal protein L23a	RPL23A	4	13	143
P62753	40S ribosomal protein S6	RPS6	2	9	102
P62829	60S ribosomal protein L23	RPL23	4	17	90
P62841	40S ribosomal protein S15	RPS15	3	13	32
P62851	40S ribosomal protein S25	RPS25	1	8	61
P62873	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	GNB1	6	19	116
P62879	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2	GNB2	6	19	101
P62888	60S ribosomal protein L30	RPL30	2	18	123
P62899	60S ribosomal protein L31	RPL31	6	32	154
P62913	60S ribosomal protein L11	RPL11	5	23	413
P62917	60S ribosomal protein L8	RPL8	6	21	259
P63010	AP-2 complex subunit beta	AP2B1	5	6	59
P63173	60S ribosomal protein L38	RPL38	1	14	44
P68032	Actin, alpha cardiac muscle 1	ACTC1	26	34	4608
P78559	Microtubule-associated protein 1A	MAP1A	4	34	29
P83111	Serine beta-lactamase-like protein LACTB, mitochondrial	LACTB	6	11	83
P83731	60S ribosomal protein L24	RPL24	2	10	30
P84103	Serine/arginine-rich splicing factor 3	SRSF3	1	5	39
Q00325	Phosphate carrier protein, mitochondrial	SLC25A3	4	9	64
Q00577	Transcriptional activator protein Pur-alpha	PURA	2	4	89
Q01780	Exosome component 10	EXOSC10	8	10	35
Q01831	DNA repair protein complementing XP-C cells	XPC	2	2	46

Q02543	60S ribosomal protein L18a	RPL18A	3	14	74
Q02878	60S ribosomal protein L6	RPL6	6	20	178
Q02880	DNA topoisomerase 2-beta	TOP2B	50	30	1432
Q03113	Guanine nucleotide-binding protein subunit alpha-12	GNA12	2	4	227
Q07020	60S ribosomal protein L18	RPL18	3	18	122
Q07666	KH domain-containing, RNA-binding, signal transduction-associated protein 1	KHDRBS1	2	5	72
Q07955	Serine/arginine-rich splicing factor 1	SRSF1	2	6	43
Q08945	FACT complex subunit SSRP1	SSRP1	4	5	44
Q09028	Histone-binding protein RBBP4	RBBP4	3	8	52
Q12905	Interleukin enhancer-binding factor 2	ILF2	4	11	353
Q13243	Serine/arginine-rich splicing factor 5	SRSF5	3	11	44
Q13402	Myosin-VIIa	MYO7A	8	3	47
Q14247	Src substrate cortactin	CTTN	1	2	30
Q14344	Guanine nucleotide-binding protein subunit alpha-13	GNA13	2	6	227
Q14444	Caprin-1	CAPRIN1	2	1	46
Q14498	RNA-binding protein 39	RBM39	2	3	27
Q14789	Golgin subfamily B member 1	GOLGB1	9	3	32
Q14839	Chromodomain-helicase-DNA-binding protein 4	CHD4	9	6	38
Q14980	Nuclear mitotic apparatus protein 1	NUMA1	11	6	119
Q15233	Non-POU domain-containing octamer-binding protein	NONO	25	45	561
Q16643	Drebrin	DBN1	2	5	104
Q16698	2,4-dienoyl-CoA reductase, mitochondrial	DECR1	4	14	102
Q49A26	Putative oxidoreductase GLYR1	GLYR1	3	5	100
Q53GS9	U4/U6.U5 tri-snRNP-associated protein 2	USP39	3	6	34
Q5H9F3	BCL-6 corepressor-like protein 1	BCORL1	4	1	38
Q5JNZ5	Putative 40S ribosomal protein S26-like 1	RPS26P11	3	20	72
Q5JTH9	RRP12-like protein	RRP12	8	5	51
Q5JWF2	Guanine nucleotide-binding protein G(s) subunit alpha isoforms XLas	GNAS	3	3	288
Q5SSJ5	Heterochromatin protein 1-binding protein 3	HP1BP3	7	12	192

Q5T1R4	Transcription factor HIVEP3	HIVEP3	6	2	35
Q5T280	Uncharacterized protein C9orf114	C9orf114	3	7	37
Q5TIE3	von Willebrand factor A domain-containing protein 5B1	VWA5B1	4	3	34
Q5VXU9	Uncharacterized protein C9orf84	C9orf84	5	3	27
Q68E01	Integrator complex subunit 3	INTS3	3	5	36
Q6PJG2	Uncharacterized protein C14orf43	C14orf43	4	3	55
Q6UN15	Pre-mRNA 3'-end-processing factor FIP1	FIP1L1	2	3	40
Q6UXE8	Butyrophilin-like protein 3	BTNL3	2	4	27
Q6ZWH5	Serine/threonine-protein kinase Nek10	NEK10	6	6	34
Q71UM5	40S ribosomal protein S27-like	RPS27L	2	10	56
Q7RTP6	Protein MICAL-3	MICAL3	9	5	39
Q7Z406	Myosin-14	MYH14	10	5	265
Q86UE4	Protein LYRIC	MTDH	5	10	67
Q86V81	THO complex subunit 4	THOC4	2	7	49
Q8IWU2	Serine/threonine-protein kinase LMTK2	LMTK2	2	1	31
Q8IY81	Putative rRNA methyltransferase 3	FTSJ3	16	17	351
Q8IZL8	Proline-, glutamic acid- and leucine-rich protein 1	PELP1	6	5	103
Q8N3K9	Cardiomyopathy-associated protein 5	CMYA5	13	3	27
Q8N3X1	Formin-binding protein 4	FNBP4	4	3	28
Q8N9T8	Protein KRI1 homolog	KRI1	2	3	77
Q8TBZ0	Coiled-coil domain-containing protein 110	CCDC110	3		42
Q8TCU5	Glutamate [NMDA] receptor subunit 3A	GRIN3A	3	2	33
Q8TDD1	ATP-dependent RNA helicase DDX54	DDX54	10	11	93
Q8TDI0	Chromodomain-helicase-DNA-binding protein 5	CHD5	9	4	38
Q8TDN6	Ribosome biogenesis protein BRX1 homolog	BRIX1	4	10	60
Q8WZ42	Titin	TTN	52	1	34
Q92901	60S ribosomal protein L3-like	RPL3L	5	8	243
Q969L2	Protein MAL2	MAL2	1	63	43
Q969Q0	60S ribosomal protein L36a-like	RPL36AL	2	14	39
Q96CW1	AP-2 complex subunit mu	AP2M1	6	13	40
Q96GQ7	Probable ATP-dependent RNA helicase DDX27	DDX27	12	14	305
Q96HS1	Serine/threonine-protein phosphatase PGAM5,	PGAM5	6	18	182

	mitochondrial				
Q96L21	60S ribosomal protein L10-like	RPL10L	1	5	35
Q96PK6	RNA-binding protein 14	RBM14	6	10	123
Q99729	Heterogeneous nuclear ribonucleoprotein A/B	HNRNPAB	1	2	62
Q9BQ04	RNA-binding protein 4B	RBM4B	4	9	70
Q9BV38	WD repeat-containing protein 18	WDR18	3	6	66
Q9BVP2	Guanine nucleotide-binding protein-like 3	GNL3	7	12	68
Q9BX40	Protein LSM14 homolog B	LSM14B	1	3	37
Q9BXX2	Ankyrin repeat domain-containing protein 30B	ANKRD30B	5	3	36
Q9BY44	Eukaryotic translation initiation factor 2A	EIF2A	4	8	35
Q9H0A0	N-acetyltransferase 10	NAT10	7	7	47
Q9H0D6	5'-3' exoribonuclease 2	XRN2	6	6	31
Q9H1H9	Kinesin-like protein KIF13A	KIF13A	8	4	46
Q9H6R4	Nucleolar protein 6	NOL6	5	4	31
Q9H6W3	Lysine-specific demethylase NO66	NO66	2	3	54
Q9HCM4	Band 4.1-like protein 5	EPB41L5	3	4	107
Q9NPC3	E3 ubiquitin-protein ligase CCNB1IP1	CCNB1IP1	2	3	34
Q9NR30	Nucleolar RNA helicase 2	DDX21	3	23	628
Q9NRC6	Spectrin beta chain, brain 4	SPTBN5	15	4	29
Q9NVI7	ATPase family AAA domain-containing protein 3A	ATAD3A	9	12	181
Q9NW13	RNA-binding protein 28	RBM28	8	10	54
Q9P2E9	Ribosome-binding protein 1	RRBP1	9	6	166
Q9UHB6	LIM domain and actin-binding protein 1	LIMA1	7	9	51
Q9ULV4	Coronin-1C	CORO1C	5	10	74
Q9UMS4	Pre-mRNA-processing factor 19	PRPF19	8	17	87
Q9UPU7	TBC1 domain family member 2B	TBC1D2B	5	5	31
Q9Y265	RuvB-like 1	RUVBL1	4	10	67
Q9Y3E5	Peptidyl-tRNA hydrolase 2, mitochondrial	PTRH2	1	7	39
Q9Y3I0	tRNA-splicing ligase RtcB homolog	C22orf28	3	3	39
Q9Y4P3	Transducin beta-like protein 2	TBL2	1	2	57
B9A064	**Immunoglobulin lambda-like polypeptide 5**	**IGLL5**	**4**	**27**	**449**
P09874	**Poly [ADP-ribose] polymerase 1**	**PARP1**	**11**	**14**	**386**
P09651	*Heterogeneous nuclear ribonucleoprotein A1*	*HNRNPA1*	*9*	*19*	*171*

P39023	*60S ribosomal protein L3*	*RPL3*	*12*	*21*	*520*
P60709	*Actin, cytoplasmic 1*	*ACTB*	*35*	*27*	*8110*
Q13151	*Heterogeneous nuclear ribonucleoprotein A0*	*HNRNPA0*	*6*	*27*	*151*
Q5QNW6	*Histone H2B type 2-F*	*HIST2H2BF*	*2*	*14*	*116*
Q6NXT2	*Histone H3.3C*	*H3F3C*	*3*	*22*	*50*
Q99459	*Cell division cycle 5-like protein*	*CDC5L*	*3*	*3*	*65*
P03372	***Estrogen receptor***	***ESR1***	***8***	***14***	***388***

Table 3.3
Proteins specifically identified in partially purified sample upon ICI treatment, including in **bold,** *italic* and ***bold italic*** those in common with E2, SERMs and both E2 and SERMs, respectively.

SwissProt ID	Protein name	Gene name	Peptides matched	Sequence coverage (%)	MOWSE Score
O60812	Heterogeneous nuclear ribonucleoprotein C-like 1	HNRNPCL1	1	3	52
O75643	U5 small nuclear ribonucleoprotein 200 kDa helicase	SNRNP200	5	3	28
P06753	Tropomyosin alpha-3 chain	TPM3	5	19	31
P06850	Corticoliberin	CRH	1	3	27
P08865	40S ribosomal protein SA	RPSA	3	3	47
P19021	Peptidyl-glycine alpha-amidating monooxygenase	PAM	1	10	40
P26196	Probable ATP-dependent RNA helicase DDX6	DDX6	11	27	332
Q13085	Acetyl-CoA carboxylase 1	ACACA	2	0	28
Q15717	ELAV-like protein 1	ELAVL1	2	7	70
Q1KMD3	Heterogeneous nuclear ribonucleoprotein U-like protein 2	HNRNPUL2	7	8	120
Q5VSP4	Putative lipocalin 1-like protein 1	LCN1P1	2	11	61
Q6Y7W6	PERQ amino acid-rich with GYF domain-containing protein 2	GIGYF2	6	5	94
Q6ZMI0	KLRAQ motif-containing protein 1	KLRAQ1	5	5	29
Q8IX12	Cell division cycle and apoptosis regulator protein 1	CCAR1	3	2	32
Q8IYT4	Katanin p60 ATPase-containing subunit A-like 2	KATNAL2	3	5	47
Q8N163	Protein KIAA1967	KIAA1967	5	7	70
Q8N7P1	Inactive phospholipase D5	PLD5	4	7	28
Q8NAT2	Tudor domain-containing protein 5	TDRD5	3	1	34
Q8ND56	Protein LSM14 homolog A	LSM14A	13	22	368
Q92900	Regulator of nonsense transcripts 1	UPF1	3	2	40
Q9NYF8	Bcl-2-associated transcription factor 1	BCLAF1	2	2	56

O43286	**Beta-1,4-galactosyltransferase 5**	**B4GALT5**	**1**	**1**	**29**
P11940	**Polyadenylate-binding protein 1**	**PABPC1**	**7**	**11**	**156**
P35637	**RNA-binding protein FUS**	**FUS**	**6**	**14**	**411**
P51991	**Heterogeneous nuclear ribonucleoprotein A3**	**HNRNPA3**	**3**	**8**	**48**
P62318	**Small nuclear ribonucleoprotein Sm D3**	**SNRPD3**	**2**	**14**	**120**
Q01844	**RNA-binding protein EWS**	**EWSR1**	**3**	**4**	**202**
Q08211	**ATP-dependent RNA helicase A**	**DHX9**	**9**	**20**	**137**
Q12906	**Interleukin enhancer-binding factor 3**	**ILF3**	**4**	**4**	**108**
Q6S8J3	**POTE ankyrin domain family member E**	**POTEE**	**7**	**17**	**69**
Q92804	**TATA-binding protein-associated factor 2N**	**TAF15**	**6**	**11**	**256**
Q92841	**Probable ATP-dependent RNA helicase DDX17**	**DDX17**	**4**	**6**	**160**
Q99728	**BRCA1-associated RING domain protein 1**	**BARD1**	**4**	**5**	**36**
Q9BUJ2	**Heterogeneous nuclear ribonucleoprotein U-like protein 1**	**HNRNPUL1**	**5**	**6**	**62**
Q9NTJ4	**Alpha-mannosidase 2C1**	**MAN2C1**	**1**	**0**	**29**
Q9Y2W1	**Thyroid hormone receptor-associated protein 3**	**THRAP3**	**6**	**6**	**106**
P02786	*Transferrin receptor protein 1*	*TFRC*	*7*	*10*	*124*
P59665	*Neutrophil defensin 1*	*DEFA1*	*1*	*9*	*50*
P61626	*Lysozyme C*	*LYZ*	*1*	*8*	*79*
Q6PJF5	*Inactive rhomboid protein 2*	*RHBDF2*	*5*	*4*	*44*
Q8N957	*Ankyrin repeat and fibronectin type-III domain-containing protein 1*	*ANKFN1*	*2*	*0*	*29*
Q9NZ71	*Regulator of telomere elongation helicase 1*	*RTEL1*	*3*	*2*	*30*
O60506	***Heterogeneous nuclear ribonucleoprotein Q***	***SYNCRIP***	***4***	***6***	***166***
Q7RTV0	***PHD finger-like domain-containing protein 5A***	***PHF5A***	***2***	***17***	***50***
Q9H1R3	***Myosin light chain kinase 2, skeletal/cardiac muscle***	***MYLK2***	***2***	***3***	***70***
Q9UIB8	***SLAM family member 5***	***CD84***	***1***	***2***	***51***

Table 3.4

Proteins specifically identified in partially purified sample upon Ral treatment, including in **bold,** *italic* and ***bold italic*** those respectively in common with E2, Tam and both E2 and Tam ligands.

SwissProt ID	Protein name	Gene name	Peptides matched	Sequence coverage (%)	MOWSE Score
O15399	Glutamate [NMDA] receptor subunit epsilon-4	GRIN2D	4	2	28
O75475	PC4 and SFRS1-interacting protein	PSIP1	3	4	35
O95996	Adenomatous polyposis coli protein 2	APC2	5	2	32
P10276	Retinoic acid receptor alpha	RARA	1	1	30
P11177	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	PDHB	1	3	30
P20849	Collagen alpha-1(IX) chain	COL9A1	3	3	28
P29803	Pyruvate dehydrogenase E1 component subunit alpha, testis-specific form, mitochondrial	PDHA2	1	2	40
P51610	Host cell factor 1	HCFC1	3	3	30
P54253	Ataxin-1	ATXN1	1	1	34
Q14204	Cytoplasmic dynein 1 heavy chain 1	DYNC1 H1	8	1	32
Q8NB25	Protein FAM184A	FAM184 A	4	3	32
Q92851	Caspase-10	CASP10	2	2	37
Q96RP8	Potassium voltage-gated channel subfamily A member 7	KCNA7	1	1	29
Q9H0C1	Zinc finger MYND domain-containing protein 12	ZMYND 12	1	2	29
Q9Y6U7	RING finger protein 215	RNF215	2	5	34
B9A064	**Immunoglobulin lambda-like polypeptide 5**	**IGLL5**	**5**	**30**	**497**
P09874	**Poly [ADP-ribose] polymerase 1**	**PARP1**	**1**	**0**	**44**
B2RTY4	*Myosin-IXa*	*MYO9A*	*6*	*2*	*30*
P08559	*Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial*	*PDHA1*	*5*	*11*	*38*
P12036	*Neurofilament heavy polypeptide*	*NEFH*	*4*	*3*	*93*
Q5VUG0	*Scm-like with four MBT domains protein 2*	*SFMBT2*	*2*	*1*	*30*
Q8TEK3	*Histone-lysine N-methyltransferase, H3 lysine-79 specific*	*DOT1L*	*5*	*3*	*28*
P03372	***Estrogen receptor***	***ESR1***	***4***	***8***	***63***

Table 3.5

Proteins specifically identified in partially purified sample upon Tam treatment, including in **bold,** *italic* and **bold italic** those respectively in common with E2, Ral and both E2 and Ral ligands.

SwissProt ID	Protein name	Gene name	Peptides matched	Sequence coverage (%)	MOWSE Score
A6NMY6	Putative annexin A2-like protein	ANXA2P2	1	3	55
P01833	Polymeric immunoglobulin receptor	PIGR	4	5	124
P02545	Prelamin-A/C	LMNA	2	3	31
P02787	Serotransferrin	TF	4	7	56
P02788	Lactotransferrin	LTF	13	21	321
P03973	Antileukoproteinase	SLPI	2	20	148
P04406	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	2	6	40
P05089	Arginase-1	ARG1	2	7	30
P06702	Protein S100-A9	S100A9	6	35	42
P07339	Cathepsin D	CTSD	4	9	35
P12273	Prolactin-inducible protein	PIP	3	23	112
P25311	Zinc-alpha-2-glycoprotein	AZGP1	2	7	29
P29508	Serpin B3	SERPINB3	6	11	156
P31025	Lipocalin-1	LCN1	4	21	176
P31151	Protein S100-A7	S100A7	2	22	100
P54652	Heat shock-related 70 kDa protein 2	HSPA2	7	13	352
P62805	Histone H4	HIST1H4A	1	7	39
P78363	Retinal-specific ATP-binding cassette transporter	ABCA4	2	0	32
P80188	Neutrophil gelatinase-associated lipocalin	LCN2	1	5	43
Q14103	Heterogeneous nuclear ribonucleoprotein D0	HNRNPD	1	2	40
Q14508	WAP four-disulfide core domain protein 2	WFDC2	1	6	29
Q16378	Proline-rich protein 4	PRR4	1	11	40
Q4VXU2	Polyadenylate-binding protein 1-like	PABPC1L	2	2	42
Q5D862	Filaggrin-2	FLG2	3	1	36
Q5VTT5	Myomesin-3	MYOM3	6	4	28
Q7Z7A1	Centriolin	CEP110	6	3	36
Q8TDL5	Long palate, lung and nasal epithelium carcinoma-associated protein 1	LPLUNC1	3	6	76
Q8WUQ7	Uncharacterized protein C19orf29	C19orf29	2	3	33

Q92529	SHC-transforming protein 3	SHC3	1	1	29
Q96DA0	Zymogen granule protein 16 homolog B	ZG16B	1	3	33
Q96JZ2	Hematopoietic SH2 domain-containing protein	HSH2D	1	2	32
Q9BV73	Centrosome-associated protein CEP250	CEP250	9	4	29
Q9HCF6	Transient receptor potential cation channel subfamily M member 3	TRPM3	2	1	30
Q9UDR5	Alpha-aminoadipic semialdehyde synthase, mitochondrial	AASS	3	3	30
Q9UGM3	Deleted in malignant brain tumors 1 protein	DMBT1	1	0	101
Q9Y592	Coiled-coil domain-containing protein 41	CCDC41	5	5	32
P60709	**Actin, cytoplasmic 1**	**ACTB**	**10**	**22**	**439**
P09651	**Heterogeneous nuclear ribonucleoprotein A1**	**HNRNP A1**	**3**	**8**	**74**
P39023	**60S ribosomal protein L3**	**RPL3**	**1**	**2**	**34**
Q13151	**Heterogeneous nuclear ribonucleoprotein A0**	**HNRNP A0**	**2**	**8**	**30**
Q5QNW6	**Histone H2B type 2-F**	**HIST2H 2BF**	**2**	**13**	**31**
Q6NXT2	**Histone H3.3C**	**H3F3C**	**2**	**15**	**43**
Q99459	**Cell division cycle 5-like protein**	**CDC5L**	**2**	**2**	**27**
B2RTY4	*Myosin-IXa*	*MYO9A*	*6*	*2*	*27*
P08559	*Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial*	*PDHA1*	*1*	*3*	*48*
P12036	*Neurofilament heavy polypeptide*	*NEFH*	*2*	*1*	*72*
Q5VUG0	*Scm-like with four MBT domains protein 2*	*SFMBT2*	*1*	*1*	*30*
Q8TEK3	*Histone-lysine N-methyltransferase, H3 lysine-79 specific*	*DOT1L*	*2*	*0*	*27*
P03372	***Estrogen receptor***	***ESR1***	***1***	***1***	***40***

4. CONCLUSIONS

This study provides for the first time a comparative analysis of the effects of antiestrogens on the nuclear ERα interactome of hormone-responsive human BC cells. The results clearly show that the protein complexes recruited by ERα upon estrogen (E2) and antiestrogen (ICI, Ral, Tam) stimulation share few components, as the majority of the receptor partners identified appear to be ligand-specific. This evidence points to the possibility, suggested by a number of indirect observations, that estrogenic and antiestrogenic compounds may induce different biological effects in BC cells via ERα by promoting recruitment to the receptor of specific molecular partners.

Comparison of the number of interactors shared between two receptor complexes to the total number of interactors identified suggests that ERα complexes recruited upon SERM stimulation share a relatively higher number of common interactors. This result is in agreement with the possibility of a direct relationship between the structure of the compound, the molecular composition of the interactome, and the biological effects elicited by the receptor.

The known functions of several proteins identified here open new venues to investigate the molecular mechanisms underlying SERM inhibition of BC cells proliferation and promotion of cell death and to understand the events that lead to loss of breast tumor sensitivity to antiestrogen-based therapies.

5. MATHERIALS AND METHODS

5.1 Cell cultures

The human hormone-responsive mammary carcinoma cell line MCF-7 (Clontech-Takara) was cultured in Dulbecco's modified Eagle's medium containing 1 mg/mL D-glucose (Sigma-Aldrich) and supplemented with 2 mM L-glutamine, 10% FBS (HyClone), 25 units/mL penicillin, 25 units/mL streptomycin, 250 ng/mL amphotericin B, and 100 μg/mL G418 (standard growth conditions). To study protein complexes assembly upon ligand treatments, cells were estrogen deprived (starved) by exchanging the medium to Dulbecco's modified Eagle's medium without phenol red (Sigma-Aldrich) supplemented with 2 mM Lglutamine and 5% stripped serum (dextran-coated charcoaltreated FBS) 5 days prior to performing the ligand treatments and to harvesting the cells, as described by Addeo [144]. MCF-7 cells were used to generate stable clones expressing TAP (control cells) or C-TAP-ERα (TAP-ERα expressing cells) as described by Ambrosino et al.[117].

5.2 Preparation of nuclear extracts

The cells were harvested by scraping, washed twice in cold 1× PBS, collected by centrifugation at 1000g, and resuspended in 3 volumes with respect to the cell pellet of hypotonic buffer (20 mM HEPES pH 7.4, 5 mM NaF, 10 mM sodium molybdate, 0.1 mM EDTA, 1 mM PMSF, and 1× protease inhibitor mixture (Sigma-Aldrich)). Upon incubation on ice for 15 min, 0.5% Triton X-100 was added, and a cytosolic fraction was discarded after centrifugation of the samples at 15,000g for 30 s at 4 °C. The nuclear pellet was first washed twice in hypotonic buffer to remove any residual cytosolic contaminations and then was resuspended in 1 volume of nuclear

lysis buffer (20 mM HEPES pH 7.4, 25% (v/v) glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 1 mM DTT, 1× protease inhibitor mixture (Sigma-Aldrich), and 1 mM PMSF), incubated for 30 min at 4 °C on a rotating platform. The nuclear extract was clarified by centrifugation at 15,000g, for 30 min at 4 °C and then was diluted by adding 2 volumes of nuclear lysis buffer w/ o NaCl. The nuclear extracts were assayed, and nonsignificant cross-contamination between the two cellular compartments could be detected by Western Blotting using an anti-α tubulin antibody [117].

5.3 Western blotting

Western blot analyses were performed using standard protocols as described by Nassa et al.[145]. In detail, protein samples were denatured, separated on a 7 or 10% polyacrylamide and 0.1% SDS (SDS-PAGE), and electrotransferred onto a nitrocellulose membrane (Whatman GmbH-Schleicher & Schuell). The membrane was blocked using 5% (w/v) fat-free milk powder in 1× TBS supplemented with 0.1% (v/v) Tween20 (TBS-T). The used primary antibodies were as follows: rabbit antihuman ERα (sc-543, HC-20, Santa Cruz Biotechnology), rabbit anti- TAP (CAB1001, Thermo Scientific-Pierce), rabbit anti-α- tubulin (T6199, Sigma Aldrich), mouse anti-β-actin (A1978, Sigma Aldrich), rabbit anti-α-tubulin (T6074, Sigma Aldrich), mouse anti-DBC1/3G4 (#5857, Cell Signaling), rabbit anti-Nucleophosmin (ab52644, Abcam), rabbit anti-DOT1L/ KMT4 (ab72454, Abcam), mouse anti-DBC1/3G4 (#5857, Cell Signaling), and mouse anti-Pyruvate Dehydrogenase E1- alpha subunit (ab110334, Abcam). All antibodies were first tested to evaluate specificity and sensitivity. After extensively washing with TBS-T, the immunoblotted proteins were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies

(GE Healthcare) and were detected by enhanced chemiluminescence (ECL Kit, GE Healthcare) and exposure to a medical Xray film (FujiFilm).

5.4 Isolation of ERα nuclear partners by Tandem Affinity Purification

Control and TAP-ERα expressing cells (approximately 6 × 108 cells in 500 cm^2 plates) were used for each tandem affinity purification (TAP) procedure. The cells were starved and stimulated with 1×10^{-8} M ligand (17β-estradiol/E2, 4- hydroxytamoxifen/Tam, Raloxifene/Ral, or Fulvestrant/ICI; all from Sigma-Aldrich) for 1 h. Cells were harvested, extensively washed with ice-cold 1× PBS, and lysed as described above. Nuclear extracts were incubated with 6 μL/mg protein IgG Sepharose beads (IgG-Sepharose 6 Fast Flow, GE Helthcare) at 4 °C for 4 h on a rotating platform. Before incubation, the beads were equilibrated in 10 volumes of TEV buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 1 mM DTT, 0.1% Triton X- 100, and 150 mM NaCl), and washed four times with 20 volumes of IPP150 buffer (20 mM HEPES, pH 7.5, 8% glycerol, 150 mM NaCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, and 0.1% Triton X-100) at 4 °C for 15 min. At the end of the incubation, the unbound proteins were collected by centrifugation and the beads were washed with 100 volumes of IPP150 and 30 volumes of TEV buffer in a Poly-Prep Chromatography column (0.8 cm × 4 cm, Bio-Rad) at 4 °C. Thereafter, 4 bead volumes of TEV buffer containing 1 unit of TEV protease/μL of beads (Invitrogen) were added and, following incubation for 2 h at 16 °C on a shaking platform (Thermomixer, Eppendorf), the eluted proteins were collected by sedimentation.

5.5 Nano LC-MS/MS analysis of TEV eluates

The partially purified protein samples from the different experimental points were concentrated by precipitation with acetone/TCA, dried, sonicated, and resuspended in Laemmli buffer followed by SDS-PAGE and visualization with Silver Staining, as described by Nassa et al. [145]. All lanes on the gels were excised and were sliced into six pieces, and the proteins were in-gel digested with trypsin solution (Sequencing grade Modified Trypsin, Promega) and incubated at 37 °C overnight as described by [146, 147]. The resulting peptides were acidified and dissolved by addition of 0.1% TFA (Sigma-Aldrich) and analyzed by LCMS/ MS using an Ultimate 3.000 nano-LC (Dionex, Sunnyvalle, CA, USA) and a QSTAR Elite hybrid quadrupole TOF-MS (Applied Biosystems/MDS Sciex, CA, USA) with nano-ESI ionization. The LC-MS/MS samples were first loaded on a ProteCol C18 trap column (10 mm × 150 μm, 3 μm, 120 Å) (SGE Incorporated, Austin, Texas, USA), followed by peptide separation on a PepMap100 C18 analytical column (15 cm × 75 μm, 5 μm, 100 Å) (LC Packings/Dionex) at 200 nL/min. The separation gradient consisted of 0−50% B in 50 min, 50% B for 3 min, 50−100% B in 2 min, and 100% B for 3 min (buffer A: 0.1% formic acid; buffer B: 0.08% formic acid in 80% acetonitrile). MS data were acquired using Analyst QS 2.0 software. The information-dependent acquisition method consisted of a 0.5 s TOF MS survey scan of m/z 400−1400. From every survey scan, the two most abundant ions with charge states +2 to +4 were selected for product ion scans. Once an ion was selected for MS/MS fragmentation, it was put on an exclusion list for 60 s. The LC-MS/MS data were searched against SwissProt release 22062011 (529056 sequences; 187423367 residues; Taxonomy Homo sapiens (human): 20236 sequences) for all samples using the in-house Mascot (version 2.2, Matrix Science) through the ProteinPilot 2.0.1 interface. The criteria for Mascot searches were the following: human-specific

taxonomy, trypsin digestion with one missed cleavage allowed, and oxidation of methionine as a variable modification and carbamidomethylation as a fixed modification. For the LC-MS/ MS spectra the maximum precursor ion mass tolerance was 50 ppm and the MS/MS fragment ion mass tolerance was 0.2 Da, and peptide charge states of +1, +2 or +3 were used. All reported protein identifications were statistically significant because, instead of a Standard Scoring, a MudPIT scoring was used which automatically filters low scoring peptide masses. To eliminate the redundancy of proteins that appear in the database under different names and accession numbers, the single protein member with the highest protein score (top rank) was selected from multiprotein families for the identification results.

5.6 Gene Ontology analyses

Statistically over-represented biological processes were identified among the sets of proteins identified by MS analyses in each of the four experimental conditions by means of GOFFA [148], a bioinformatics tool for the functional analysis of genomic and proteomic data, developed for ArrayTrack, that starting from a list of genes or proteins identifies Gene Ontology (GO) terms associated with each of them. GOFFA determines the statistical significance of a GO term using Fisher's Exact Test. For this study, the list of genes expressed in MCF-7 cells and identified by microarray-mediated gene expression profiling (see below), was used as a reference and for each dataset the GO terms over-represented respect to the reference with a p-value ≤ 0.05 were selected. In addition, GO analysis was performed also using as a reference a list of proteins identified experimentally in MCF-7 cells by means of the Gene Ontology Enrichment Analysis and Visualization Tool (GORILLA; http://cbl-gorilla.cs.technion.ac.il).

5.7 Protein complexes immunoprecipitation

For immunoprecipitation of endogenous ERα or DOT1L, to nuclear extracts from MCF-7 cells (800−2000 μg proteins) was added 2.0−2.5 μg/mg protein specific Abs (rabbit antihuman ERα: sc-543, HC-20, Santa Cruz Biotechnology and rabbit anti- DOT1L/KMT4: A300-954A, Bethyl), and the mixture was incubated for 1−3 h at 4 °C with stirring via rotation; then Protein A/G Plus-Agarose was added for 1 h. Immunoprecipitated proteins were collected by centrifugation, and after extensive washing, the beads were resuspended in Laemmli buffer and subject to SDS-PAGE and Western blotting as described by Ambrosino et al [117].

5.8 RNA purification

Total RNA was extracted from TAP-ERα expressing cells, using the standard RNA extraction with TRI Reagent (Sigma- Aldrich) method, as described by Grober at al. [149]. Cells were starved and total RNA was extracted after stimulation with 1×10^{-8} M ligand (E2, Tam, Ral, or ICI) or ethanol vehicle for 12 h. In each case RNA extracted from two independent biological replicates was used. Before use, the RNA concentration of each sample was assayed with a ND-1000 spectrophotometer (NanoDrop) and its quality assessed with the Agilent 2100 Bioanalyzer with an Agilent RNA 6000 Nano kit (Agilent Technologies).

5.9 RNA expression profiling

For mRNA expression profiling, 500 ng of total RNA was reverse transcribed, as described by Paris et al. [150]and used for synthesis of cDNA

and biotinylated cRNA according to the Illumina TotalPrep RNA Amplification Kit (Ambion, Cat. no. IL1791) protocol. For each sample, 750 ng of cRNA was hybridized for 18 h at 58 °C on Illumina Human HT-12v4 BeadChips (Illumina Inc.), as described by Grober et al. [149] and subsequently scanned with the Illumina iScan. Data analyses were performed with GenomeStudio software v2011.1 (Illumina Inc.), by comparing all values obtained at each time point against the 0 h values. Data were normalized with the quantile normalization algorithm, and genes were considered as detected if the detection p-value was lower than 0.01. Statistical significance was calculated with the Illumina DiffScore, a proprietary algorithm that uses the bead standard deviation to build an error model. Only genes with a DiffScore ≤ -30 and ≥ 30, corresponding to a p-value of 0.001, were considered as statistical significant [149, 150]. Raw microarray data have been deposited, in a format complying with the Minimum Information About a Microarray Gene Experiment (MIAME) guidelines of the Microarray Gene Expression Data Society (MGED), in the EBI ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) with Accession Number E-MTAB-1196.

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LIST OF AUTHOR'S PUBLICATIONS RELATED TO THE THESIS

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