Université de Montréal

Growth Factor Activation of ErbB2/ErbB3 Signaling Pathways Regulate the Activity of Estrogen Receptors (ER)

par

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Thèse présentée à la Faculté des Études Supérieures

en vue de l'obtention du grade de Philosophiæ Doctor (Ph.D.)

En Biochimie

Avril, 2010

Université de Montréal
Faculté des études supérieures

Cette thèse intitulée:

Growth Factor Activation of ErbB2/ErbB3 Signaling Pathways Regulate the Activity of Estrogen Receptors (ER)

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Résumé

La signalisation par l'estrogène a longtemps été considérée comme jouant un rôle critique dans le développement et la progression des cancers hormono-dépendants tel que le cancer du sein. Deux tiers des cancers du sein expriment le récepteur des estrogènes (ER) qui constitue un élément indiscutable dans cette pathologie. L'acquisition d'une résistance endocrinienne est cependant devenue un obstacle majeur au traitement des cancers hormono-dépendants devenus hormono-indépendants.

L'émergence de cancers hormono-indépendants est décrite comme étant causée par une variété de voies telles que l'activation de ER en absence d'estrogène, l'hypersensibilité du récepteur aux faibles concentrations plasmique d'estrogène ainsi que l'activation de ER par des modulateurs sélectifs. De plus, l'activité du ER est fortement influencée par l'environnement cellulaire tel que l'activation de voie de signalisation des facteurs de croissances, la disponibilité de protéines co-régulatrices et des séquences promotrices ciblées. Présentement, les études ont principalement considéré le rôle de ERα, cependant avec la découverte de ERβ, notre compréhension de la diversité des mécanismes potentiels impliquant des réponses ER-dépendantes s'est grandement améliorée. L'activation hormono-indépendante de ER est retrouvée dans les tumeurs mammaires estrogène-dépendantes. L'activation des voies des kinases entraîne le développement d'un phénotype tumoral résistant aux traitements actuels. Nos connaissances concernant les voies impliquées dans l'activation de la signalisation du ER ainsi que le rôle des évènements de phosphorylation affectant l'activité du ER sont restreintes. ERα est considéré comme le sous-type dominant et corrèle avec la plupart des facteurs de pronostic dans le cancer du sein. Par contre, le rôle de ERβ reste imprécis. Les résultats présentés dans cette thèse ont pour objectif de mieux comprendre l'implication de ERβ dans la prolifération cellulaire par l'étude du comportement de ERβ et ERα suite à l'activation des voies de signalisation par les facteurs de croissance.

Nous démontrons que l'activation des récepteurs de surfaces de la famille ErbB, spécifiquement ErbB2/ErbB3, inhibe l'activité transcriptionnelle de ERβ, malgré la présence du coactivateur CBP, tout en activant ERα. De plus, l'inhibition de ERβ est attribuée à un résidu sérine situé dans la région charnière, absente dans chez ERα. Des études supplémentaires de ErbB2/ErbB3 ont révélé qu'ils activent la voie PI3K/Akt ciblant à son tour la sérine de la région charnière de ERβ. En effet, cette phosphorylation de ERβ par PI3K/Akt induit une augmentation de l'ubiquitination du récepteur qui promeut sa dégradation par le système ubiquitine-protéasome. Cette dégradation est spécifique pour ERβ. De façon intéressante, la dégradation par le protéasome requiert la présence du coactivateur CBP normalement requis pour l'activité transcriptionnelle des récepteurs nucléaires. Malgré que l'activation de la voie PI3K/Akt corrèle avec une diminution de l'expression des gènes sous le contrôle de ERβ, une augmentation de la prolifération des cellules cancéreuses est observée. L'inhibition de la dégradation de ERβ réduit cette prolifération excessive causée par le traitement avec Hrgβ1, un ligand de ErbB3.

Un nombre croissant d'évidences indique que les voies de signalisations des facteurs de croissance peuvent sélectivement réguler l'activité transcriptionnelle des sous-types de ER. De plus le ratio de ERα/ERβ dans les cancers du sein devient un outil de diagnostique populaire afin de déterminer la sévérité d'une tumeur. En conclusion, la caractérisation moléculaire du couplage entre la signalisation des facteurs de croissance et la fonction des ERs permettra le développement de nouveaux traitements permettant de limiter l'apparition de cellules tumorales résistantes aux thérapies endocriniennes actuelles.

Mots-clés : Cancers hormone-dépendant; Récepteurs aux Estrogènes, ER; Facteurs de croissances; Récepteur de tyrosine kinase, RTK; ErbB; CBP/p300; Phosphorylation; Ubiquitination; Mdm2; PI3K/Akt

Abstract

It has long been appreciated that estrogenic signaling plays a critical role in the development of hormone-dependent cancers such as breast cancer. Two-thirds of breast cancers express estrogen receptor (ER) which has been demonstrated to play an irrefutable role in tumour development and progression. However the acquisition of endocrine resistance has become a major obstacle in the treatment of hormone-dependent cancers that have acquired a hormone-independent state.

Hormone-independent cancers emerge from an array of pathways involving ER activation in the absence of estrogen, hypersensitivity of ER to low serum levels of estrogen and activation by estrogen antagonists. The activity of ER is critically influenced by the cellular environment such as growth factor signaling pathways, availability of coregulatory proteins and the promoter sequence of target genes. The mechanisms studied have mostly considered the role of ERα, however with the discovery of the second subtype, ERβ, the understanding on the diversity of potential mechanisms involving ER-dependent responses have improved. Hormonal-independent activation of ER can occur in estrogen-dependent breast tumours, with concomitant rise in kinase signaling pathways, resulting in the acquisition of a therapeutic resistant phenotype in treated women. Our knowledge is relatively limited on which pathways trigger ER signaling and how these phosphorylation-coupled events affect ER activity. ERα is considered the dominant subtype and correlates with most of the prognostic factors in breast cancers. Conversely the role of ERβ remains unclear. The results presented in this thesis were carried out with the objective of gaining a better understanding of ERβ's role in cellular proliferation by examining the behavior of ERβ and ERα during the activation of growth factor signaling pathways by cell-surface receptor-tyrosine kinases.

We demonstrate here that the activation of cell surface receptors of the ErbB family, specifically ErbB2/ErbB3, inhibits the transcriptional activity of ERβ despite the presence of the coactivator CBP, yet activated ERα. Furthermore the inhibition of ERβ was attributed to a specific serine residue located within the hinge region, not present in ERα. Additional studies of ErbB2/ErbB3-initiated signaling revealed that it triggered the activation of the PI3K/Akt pathway which targeted the serine residue within the hinge region of ERβ. In fact, phosphorylation of ERβ by the PI3K/Akt pathway led to an increase in receptor ubiquitination which promoted its degradation by the ubiquitin-proteasome system which was subtype specific. Interestingly, proteasomal degradation required the presence of the coactivator CBP, which is normally involved in assisting nuclear receptor transcriptional activity. Although the activation of the PI3K/Akt pathway correlated with a decrease in the expression of ERβ target genes it led to an increase in the proliferation of breast cancer cells. Inhibiting the degradation of ERβ reduced the enhanced proliferation of breast cancer cells brought about by the treatment of ErbB3's ligand, Hrgβ1.

Increasing evidence indicates that growth factor signaling pathways can selectively regulate the transcriptional activity of ER subtypes, and the ratio of ERα/ERβ expression in breast tumours is becoming a popular prognostic factor to evaluate the severity of the tumour. Therefore the molecular characterization of the coupling between growth factor signaling and ER function should provide improved therapeutical approaches to overcome or delay the onset of resistance to endocrine therapy in hormone-dependent cancers.

Keywords: Hormone-dependent cancer; Estrogen Receptor, ER; Growth factors; receptor tyrosine kinase, RTK; ErbB; CBP/p300; phosphorylation; Ubiquitination; Mdm2; PI3K/Akt

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Abbreviation List

ACTH	adrenocorticotrophin hormone
ADP	adenosine diphosphate
AMP	adenosine monophosphate
AF	Activation Function
AIB1	Amplified in breast cancer
AP-1	Activator Protein-1
AR	Androgen Receptor
$P19^{Arf}$	P19 Alternate reading frame
ATF	Activating transcription factor
ATP	Adenosine Triphosphate
BARD	BRCA1 associated RING domain
BRCA1	Breast Cancer suppressor protein
cAMP	cyclic AMP
CARM1	coactivator-associated arginine methyltransferase
CBP	CREB Binding Protein
Cdk	Cyclin-dependent protein
CREB	cAMP-responsive element-binding protein
DBD	DNA Binding Domain
DFS	Disease free survival
DHEA	dehydroepiandrosterone
DHT	dihydroxytestosterone
DNA	Deoxyribonucleic Acid
DRIP	Vitamin D Receptor Interacting Protein
E_2	Estradiol
E1A	viral protein E1A
E6AP	E6-associated protein

EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ER	Endoplasmic Reticulum
ER	Estrogen Receptor
ERE	Estrogen-Response Element
ERK	extra-cellular regulated kinase
FGF	Fibroblast growth factor
FSH	Follicle-stimulating hormone
GATA-1	TF binding gata DNA sequence
GH	growth hormone
GPCR	G Protein-Coupled Receptor
GPER	G protein-coupled estrogen receptor
GR	Glucocorticoid Receptor
GRIP1	GR Interacting protein-1
HAT	Histone acetyl transferase
hCG	human chorionic gonadotropin
HDAC	histone deacetylase
HIF	Hypoxia-inducible factor
Hrgβ1	Heregulin β1
Hsp	heat shock protein
IGF	Insulin Growth Factor
IKKα	IκB Kinase-α
JNK	Jun N-terminal Kinase
KDa	KiloDaltons
KO	Knockout
LBD	Ligand Binding domain
LH	Luteinizing hormone
MAPK	mitogen-activated protein kinase
Mdm2	Murine Double Minute 2
MEK	mitogen-activated/extracellular signal regulated kinase, MAP kinase kinase

MR	Mineralocorticoid Receptor
NCoR	Nuclear Corepressor
NF-κB	Nuclear Factor-Kappa B
NHR	Nuclear hormone receptor
OHT	Hydroxy-tamoxifen
Pak1	p21-activated kinase-1
P/CAF	p300/CBP Associated Factor
P/CIP	p300/CBP interacting protein
PDGF	Platelet –derived growth factor
PI3K	Phosphatidyl Inositol 3 Kinase
PIP_2	Phosphatidyl Inositol bisphosphate
Pit1	Pituitary-specific positive transcription factor 1
PKA	Protein Kinase A
PKC	Protein Kinase C
PML	Promyelocytic leukaemia
PR	Progesterone Receptor
PRMT	Protein arginine methyltransferase
SRC	Steroid Receptor Coactivator
RIP 140	receptor interacting protein 140
RING	Really interesting gene
Rpn	Proteasome non-ATPase subunit
Rpt	Regulatory particle ATPase
RTK	Receptor Tyrosine Kinase
RNA	Ribonucleic Acid
SERM	selective estrogen receptor modulator
SF	Steroidogenic factor
SMAD	designation for homologues Sma and Mad
SMRT	silencing mediator of retinoid and thyroid hormone
SREBP	sterol regulatory element-binding protein
STAT	signal activator of transcription

SUG1	Proteasome 26S subunit ATPase 5
SUMO	small ubiquitin-like modifier
Tal-1	T-cell acute lymphocytic leukemia 1
TFIIB	Transcription factor II B
TGF-β	transforming growth factor-β
TIF2	transcriptional intermediary factor 2
TM	transmembrane
TNF-α	Tumour Necrosis Factor-α
TRAP220	Thyroid hormone receptor-associated protein
TRIP	TGF-β receptor interacting protein
Wt	wild-type

To the ones who travelled with me on this journey,

"If we knew what we were looking for, it would not be called research, would it?"-
Albert Einstein

"A desire presupposes the possibility of action to achieve it; action presupposes a goal which is worth achieving".
Ayn Rand

Remerciements/Acknowledgements

This has been so far the most challenging, amazing, dramatic, introspective, insightful and incredibly fulfilling adventure I have had the chance of engaging. Nevertheless this journey would have never been as valuable if it wasn't for a number of influential, inspirational and certainly instructive individuals who have made every moment extremely worthwhile. Therefore this is my occasion to engrave and relate about the ones who made a difference.

First and foremost I would like to thank my supervisor, André, who took a chance and gave me one of the best opportunities one could be given; to do what you want the way you want to do it. But before you get there, you need to learn and no one knows more than your supervisor! The transition from a junior to a senior has taken a few years and André was there from the basics to the obscure. He was there when I needed him, there when I didn't think I needed him anymore (but still did!) and will always be there. Thank you for the opportunity, thank you for your commitment and most of all, thank you for your guidance, which I have deemed indispensable throughout this expedition.

Next I would like to thank the "ladies". Since I have started in the lab, few men have trespassed yet a number of women have left their mark. To the ones who started off with me, Cath, Véro and Roby, thank you for your support and your encouragement, I am privileged to have been able to count on you as my friends then and my friends now. To the ones who are seeing me off, I hope to be surrounded one day by such remarkable women as I have been these last few years. Amélie you have been my voice of reason, my chocolate pusher and a dear friend. Amélie, Nath, Karine and Véro the endless discussions on receptors, relationships, westerns blots, food, ChIP assays, life, antibodies and the outings have made lab life far more stimulating than people can probably imagine. Thank you!

To my dearest friends who were there since before it all began and are still there now that my adventure comes to a close. Marie and Isa, you have been more than friends; you have been my family since I arrived to Montreal and I cannot thank you enough for being part of it

all. Thank you for helping me through the challenges, rejoicing through the good times and for the life lessons. It is my privilege to have you both as my sisters.

To mum and dad, two of the most charismatic, determined and influential individuals I know. Thank you for it all. Thank you for your advice, your sacrifices, your unrelenting strength, your dedication, your support and most of all, thank you for making it happen. Mum, your everlasting energy, resourcefulness and guidance are my inspiration. Papa, your tenacity, determination and achievements are my motivation. Steven, although I don't get to have you in my life as much as I would want to, you're a great source of insight and your endeavors never cease to amaze me. A mi yayo, que aunque me hubiera gustado que estuvieras aqui, te doy las gracias por todo lo que me has ensenado y con mucho trabajo algun dia espero llegar a ser tan sabio como tu! A mi yaya que con ese amor incondicional, esa dulzura y ese caracter, gracias por todo lo que has hecho! Aux Sanchez et Van Der Knaap, merci de toujours être présent et pour tout le support que vous m'avez apporté, et Dom, tu as été une partenaire autant dans les sports qu'au jour le jour, merci pour tout!.

Thank you Dr. Cheri Deal, Dr. Michelle Brochu and Dr. Guy Van Vliet and everyone on the first floor of the research center at Ste-Justine, for your advice and for being part of my journey,

Finally to David, with whom I have the wonderful opportunity of sharing everything with. Thank you for your love, your insight, your perception, your discussions, your strength and your persistence to forever to push me to be better than I can be. What a privilege to have met you and what a gift to be with you. Thank you for everything and thank you for letting me be part of your adventure!

Merci à tous!

CHAPTER 1: INTRODUCTION

1 Hormone-Dependent Cancers

Steroid-hormone dependent cancers which include those of the breast, ovaries, endometrium and prostate are a leading cause of morbidity and mortality amongst diagnosed patients. Breast cancer is probably the most common form of cancer in women comprising 25% of the total incidence of diagnosed cancer and accounts for approximately 18% of mortality (Parkin and Fernandez, 2006)(Canadian Cancer Society/National Cancer Institute of Canada; *Canadian Cancer Statistics, Toronto, Canada 2008*) (Parkin et al., 2005). Public health data show that the global burden of breast cancer in women (measured by incidence, mortality and economic costs) is significant and increasing (Mackay J et al., 2006). The rate of breast cancer incidence is greatest in North America with 99.4 per 100,000 persons, closely followed by Western Europe (84.6 per 100,000) and Asia exhibiting the least number of incidences (roughly 25 per 100,000) (Botha et al., 2003).

Approximately 95% of breast cancers, whether in pre- or postmenopausal women, are initially hormone-dependent (Henderson et al., 1988; Soule and McGrath, 1980; Osborne et al., 1985; Lippman et al., 1986), however after a period of time (which may last several years) the tumour can potentially become hormone-insensitive by mechanisms which still need to be clarified. The majority of breast cancers develop during the postmenopausal period, when the ovaries are no longer functional. Despite the low levels of circulating estrogens, the concentration of estrogen metabolites (estradiol, estrone and their sulphates) are several times greater in the affected tissue than those found in the plasma, implying that a specific tumoural biosynthesis and accumulation of these hormones occur (Pasqualini et al., 1996; Chetrite et al., 2000).

The pathways involved in reaching a state of hormone independence is complex and physiologically important, especially when considering that over 30% of all human breast tumours which express both estrogen receptors (ERs) and progesterone receptors (PRs) fail to regress following anti-estrogen treatments (Clark and McGuire, 1988; Gurpide, 1991).

The perception that the interaction between estrogen and ER, by itself, can entirely mediate estrogen-target gene transcription is a great oversimplification. In fact, recent progress in understanding ER function has revealed a complex signaling cross-talk between ERs and other signal transduction pathways, mainly triggered by receptor tyrosine kinases (RTKs) and the expression and activity of coregulatory proteins. At least 70% of breast cancers are determined to be ER-positive breast cancers (Normanno et al., 2005). Therefore knowing that ERs remain present in ligand-resistant tumours it is primordial to fully understand the cellular mechanisms which are elicited during the development of ER ligand-independent regulation.

2 Steroid Hormones

Lipophilic hormones such as steroids (sex-steroids, corticosteroids, mineralocorticoids and ecdysteroids) as well as non-steroids (such as retinoic acid, thyroid hormone and vitamin D_3) are molecules implicated in signal transduction in vertebrates and invertebrates (Reichel and Jacob, 1993; Beato, 1989). Steroids are widely dispersed throughout the animal kingdom functioning as regulators of numerous biochemical and physiological processes with their synthesis beginning in the adrenal gland (Figure 1).

Minutes after the hypothalamic adrenocorticotrophic hormone (ACTH) stimulates the adrenal glands, we observe steroid hormone production (Figure 1). The first step is the conversion of cholesterol which is cleaved to pregnolenone, the steroid precursor to all steroidogenesis (Figure 2). Only certain cells in humans are able to convert cholesterol to pregnolenone: Leydig cells, placental trophoblast cells, ovarian theca cells, corpus luteum cells, the adrenal cortex cells and some neuronal cells in the brain. This is a tightly regulated step and it allows for a rapid production of steroids in response to stimuli. Next,

The Adrenal Gland

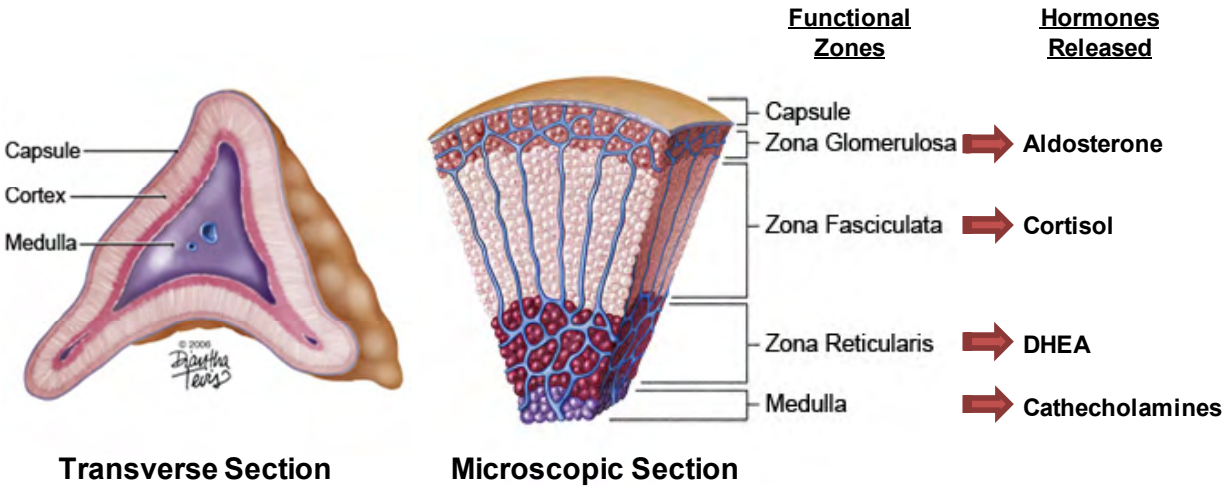

Figure 1 The adrenal gland

The adrenal gland is made up of the cortex and the medulla. The three functional zones that comprise the adrenal cortex each make distinctive steroids and this zone-specific synthesis parallels their distinct expression of steroidogenic enzymes. The zona glomerulosa (outer zone) produces mineralocorticoids (aldosterone) which monitors salt and water balance. The zona fasciculate (middle layer) makes cortisol regulating carbohydrate metabolism and vascular response to cathecholamines. The innermost layer, the zona reticularis, makes the androgen precursors such as DHEA and its sulfate (DHEA[S]). Neuroendocrine cells which make up the medulla synthesize and secrete catecholamines. *Image from illustrator Diantha Tevis-2006.*

pregnolenone is converted into the various intermediates and active steroid hormones. Few organs are capable of making steroids from cholesterol however many can transform circulating steroids, such as adrenal dehydroepiandrosterone DHEA (Figure 2), which is converted to testosterone in peripheral tissues, a route generating the major source of testosterone in women (Labrie et al., 2003). Hormones can potentially become more powerful or activated in target tissues, such as testosterone, which is converted to its active form, dihydrotestosterone (DHT) in the prostate gland. A general principle in steroidogenesis is that reactions are unidirectional as most are irreversible, and the hydroxysteroid dehydrogenase reactions (HSD) (Figure 2), while reversible, predominantly proceed in one direction. The enzymes necessary for these steps are 3β-HSD2, 17βHSD and the family of P450 enzymes, which include CYP19A1 (aromatase).

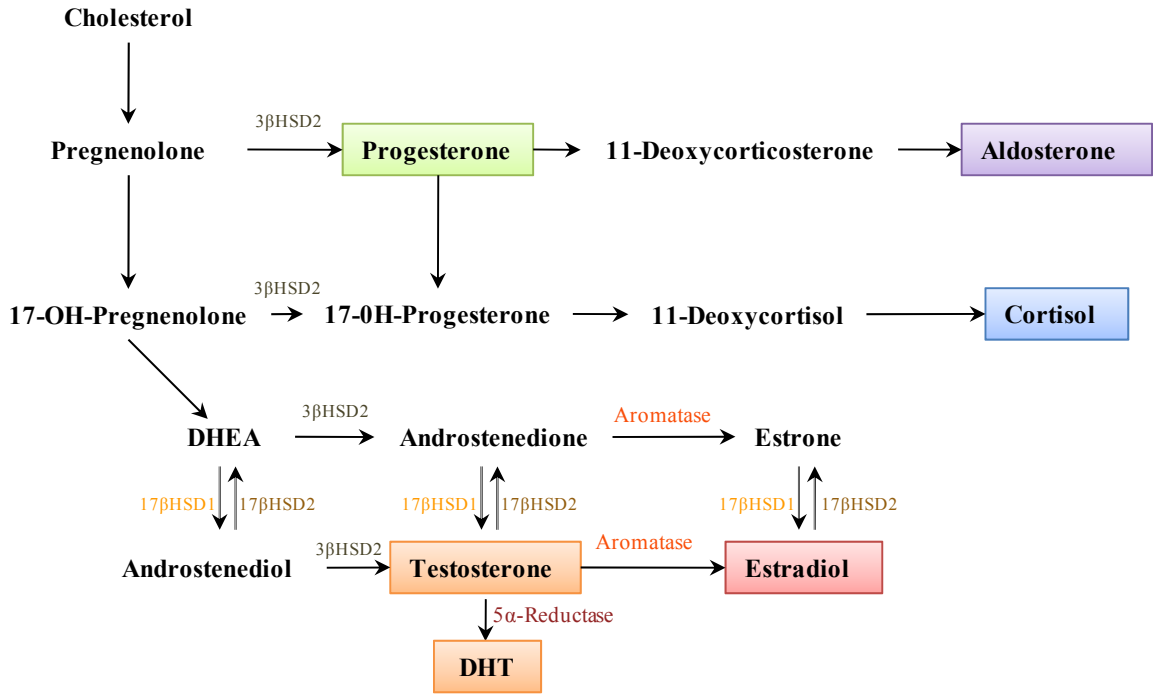

Figure 2 Human steroid biosynthesis. Adapted from (Ghayee and Auchus, 2007).

The testis and ovaries have little capacity to synthesize aldosterone and cortisol. Alternatively, steroid metabolism in these organs is focused on making androgens and estrogens, while the corpus luteum of the ovary produces progesterone. The testis efficiently completes the biosynthesis of testosterone and can export this potent androgen whereas the ovaries synthesize primarily androstenedione to convert it into estrogens as well as variable amounts of testosterone (Figure 2). Steroidogenesis in the ovary is compartmentalized in a cell-specific manner, where the theca cells primarily produce androstenedione and the granulose cells complete the synthesis of estradiol (E_2). In adrenals and gonads, ACTH or Luteinizing hormone (LH) mobilize cholesterol into the steroidogenic pathways in bursts or pulse. Follicle-stimulating hormone (FSH) is critical in promoting granulose cell development and estradiol synthesis during ovulation. In the placenta, steroid production is less pulsatile and dependent on chorionic gonadotropin (hCG) early in gestation and later is mostly unregulated.

2.1 Estrogen; From Synthesis to Function

Steroid hormones are among the most powerful and enduring signaling molecules in the body. When transported via the circulation, steroids travel great distances from the site of synthesis in an endocrine organ to a distant target organ. Alternatively, steroids can act as local autocrine or paracrine signals that impact only the microenvironment, including in the brain. The half-life of steroids is several-fold greater than that of other blood-borne signaling molecules, such as insulin, which disappear within minutes to hours. Estradiol is the final end product of 6 enzymatic conversions from its precursor cholesterol (Figure 2), and it is the most potent steroid, being active at concentrations in the femtomolar range. The critical p450 enzyme aromatase is the rate-limiting step in estradiol synthesis from androgen precursors and is a nodal point of regulation (as discussed in chapter 8).

2.1.1 Synthesizing Estrogen

Estradiol production in the ovary is dependent on the action of 17βHSD. In ovarian granulose cells of developing follicles in cycling humans and rodents, 17βHSD1 converts estrone to estradiol. Upon ovulation, follicles luteinize and transform into corpora lutea which continue to secrete estradiol at high concentrations although, during luteinization, 17βHSD1 expression declines rapidly in the ovary. The expression of 17βHSD1 is mostly abundant in the granulose cells and the syncytiotrophoblasts of the placenta (Sawetawan et al., 1994; Fournet-Dulguerov et al., 1987) and expressed at lower levels in the breast (Miettinen et al., 1999). In the ovary, 17βHSD1 is primarily induced by FSH acting through the cAMP-dependent protein Kinase A (PKA) signaling pathway (Kaminski et al., 1997).

In addition to the source of active steroid hormones derived from the circulation, there are numerous tissues such as epithelial cells of human breast and endometrium that express 17βHSD, 3βHSD and aromatase, having therefore the ability to synthesize active steroid hormones from circulating steroid precursors. The expression of these enzymes in target tissues is very important especially in humans where the adrenal glands will secrete high levels of DHEA. These steroids serve as substrates in peripheral tissues for their eventual conversion to testosterone by one of the isoforms of 17βHSD or to estrone or E_2 by aromatase. In these tissues 17βHSD1 catalyzes the conversion of estrone to the more potent form E_2 (Figure 2) (Penning, 1997).

The peripheral expression of aromatase is critical, especially in men and postmenopausal women. A major site of peripheral expression of aromatase is in the adipose tissue of men and women (Kamat et al., 2002; Simpson et al., 2002). The conversion of androgens to estrogens in adipose tissue increases with age in postmenopausal women and in elderly men (Kamat et al., 2002; Simpson et al., 2002). The primary site of expression within adipose tissue is in the stromal mesenchymal cells (Simpson et al., 2002). Aromatase is also expressed in osteoblasts and chondrocytes in

males and females (Sasano et al., 1997). Aromatase can also be observed in the brain being primarily expressed in the hypothalamus of male and females as well as in other areas such as the retina (Kamat et al., 2002; Simpson et al., 2002).

2.1.2 Importance of Estrogen

Estrogen has widespread physiological actions, targeting both genomic and non-genomic mechanisms. Estrogen is a key regulator of growth, differentiation and biological function in a wide array of target tissues, including the male and female reproductive tracts, mammary gland, skeletal, cardiovascular and central nervous systems. In breast tissue, estrogens stimulate the growth and differentiation of the ductal epithelium, induce mitotic activity of ductal cylindric cells and stimulate growth of connective tissues (Porter, 1974). Estrogens can also exert histamine-like effects on the microcirculation of the breast (Soderqvist et al., 1993). Estrogens are also thought to have neuroprotective actions such as synaptic and dendritic remodeling (Naftolin et al., 1990) as well as glial activation in brain tissue from adult rats (Garcia-Segura et al., 1999). In neurons of the hippocampus, an area involved in memory, estrogens increase the density of *N*-methyl-D-aspartate receptors and increase neuronal sensitivity to input mediated by these receptors (Woolley et al., 1997). Estrogens can reduce the generation of β-amyloid peptides, which build up in the brains of patients with Alzheimer's disease and observed in cultured human neuroblastoma cells (Green and Simpkins, 2000).

Estrogens are also known to cause short-term vasodilation by increasing the formation and release of nitric oxide (NO) and prostacyclin in endothelial cells (Kim et al., 1999). A protective role of estrogens against atherosclerosis was suggested by the findings that estrogen treatment reduced the progression of coronary-artery atherosclerosis in oophorectimized monkeys (Clarkson et al., 1996). Furthermore, estrogens can directly inhibit the functions of osteoclasts which express regulators of bone resorption, reducing the risk of fracture in women with osteoporosis (Lufkin et al., 1992; Lindsay et al., 1980).

It has been recently proposed that estrogen plays a role in insulin resistance leading to glucose intolerance and type II diabetes when pancreatic β-cells cannot meet the requirement for insulin (Godsland, 2005). A study showed that treatment of E_2 was able to protect β-cells from oxidative injury in mice resulted in protection from proinflammatory cytokine-mediated β-cell death (Le et al., 2006; Contreras et al., 2002). E_2 can also exert anti-inflammatory actions in different tissues and animal models (Vegeto et al., 2003; Zancan et al., 1999), through the inhibition of inducible nitric oxide synthase (iNOS) synthesis.

One of the most important and notable effects of estrogens is a very potent mitogenic action in hormone sensitive tissues such as the breast (Evans, 1988; Kumar et al., 1987; Weisz et al., 1990) and the uterus (Quarmby and Korach, 1984; Martin et al., 1973). Prolonged exposure of target tissues or cells to estrogens has long been considered an important etiological factor for the induction of estrogen-associated cancers in experimental animals (Nandi et al., 1995) as well as in humans (Nandi et al., 1995; Ziel and Finkle, 1975; Mack et al., 1976; Pike et al., 1993; McDonald et al., 1977; Grady and Ernster, 1997; Feigelson and Henderson, 1996; Jick et al., 1979).

2.2 Steroid hormones- ligands to steroid receptors

Steroid hormones have long been known as essential metabolic regulators, but the cloning of their respective hormone receptors was an indispensable prerequisite to understand the molecular basis of hormone action transposed into a transcriptional process. In vertebrates, the nuclear receptor superfamily contains intracellular receptor proteins targets (a group of structurally related receptors) with specific affinity to estrogens, androgens, progestins, glucocorticoids, mineralcorticoids and thyroid hormones. (Evans, 1988; Mangelsdorf et al., 1995; Beato et al., 1995; Glass, 1994).

Jensen and coll. in the early 60's (Jensen and Jacobson, 1962) laid the groundwork demonstrating the presence of a binding protein that would mediate the biological effects of E_2 in the uterus. This has paved the way twenty four years later to the cloning of the estrogen receptor, presently known as ERα (Greene et al., 1986; Green et al., 1986). With the cloning of the other steroid receptors such as glucocorticoid receptor (GR), progesterone receptor (PR) and androgen receptor (AR), a considerable progress has been accomplished over the last two decades in understanding the mechanisms of steroid hormone action. In addition, the identification of a growing number of interacting factors recruited to steroid receptors in order to facilitate transcriptional processes in response to hormone stimuli has helped in developing a comprehensive model of cofactor assembly and exchange to mediate target gene expression. However, given the complexity of these various regulatory aspects involved in steroid receptor functions that have emerged from these studies, it became evident that strategies developed to efficiently counteract deregulated functions associated to receptors had to consider the model's intricate network.

3 Steroid Receptors

The neodarwinian theory of evolution describes that new functions emerge as the phenotypic outcome due to the natural selection of random mutations. Complex organs and functions are believed to be generated from a gradual selective process of elaboration and optimization (Dawkins R, 1986). Vertebrate steroid hormones and the intracellular protein receptor that mediate their effects elegantly illustrate this theory.

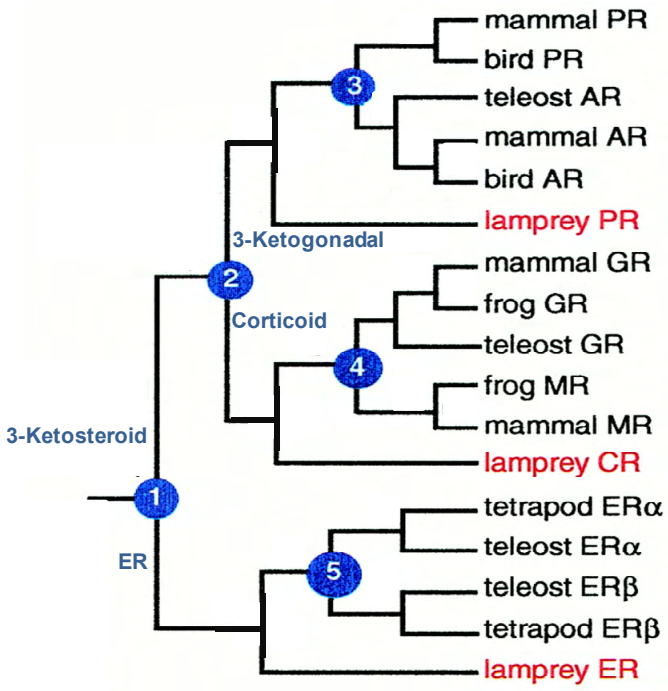

Figure 3 Phylogeny of steroid receptors. The blue circles represent gene duplication events. Adapted from (Thornton, 2001).

3.1 Evolution of Steroid Receptors

The receptor phylogeny suggests that two serial duplication of an ancestral steroid receptor occurred during the interval when vertebrates were evolving from invertebrates. In the ancestral vertebrate, the first duplication event created an estrogen receptor (ER) and a 3-ketosteroid receptor, whereas the second duplication came from the latter gene to produce a corticoid receptor and a receptor for 3-ketogonadal steroids (androgens, progestins or both) yielding three steroid receptors (Figure 3). At some point in time within the gnathostome lineage (jawed vertebrates comprised of fishes, amphibia, reptilia, aves and mammalia), each of these three receptors duplicated a second time to yield six steroid receptors currently expressed in jawed vertebrates: the ER to create ERα and ERβ, the

corticoid receptor giving the GR and mineralocorticoid receptor (MR) and the 3-ketogonadal receptor to create the AR and the PR (Figure 3). Although the timing of these events has remained unclear due to divergent hypotheses, gene mapping data supports serial duplications as the mechanism by which steroid receptors have diversified.

The fundamental role of steroids in regulating vertebrate development, reproduction, growth and homeostasis (DeGroot LJ, 1995; Gilbert SF, 1994; Evans, 1988) suggests that these steroid receptor duplications provided vertebrates a selective advantage over other organisms, which would be important in vertebrate competition with the diverse multicellular organisms present in early Cambrian, 540 to 480 million years ago, as well as for vertebrate survival during global extinctions that occurred about 440 and 370 million years ago (Raup, 1994). Nuclear receptors are an excellent example of how gene duplications and divergence can generate a protein family that responds to diverse signals to regulate a wide variety of physiological processes.

3.2 The Ancestral Steroid Receptor was an Estrogen Receptor

An ancestral protein is likely to have resembled in sequence and function to a descendent gene which evolved more slowly after the duplication event, compared to one with a more rapid evolutionary rate. Relative rate tests based on amino acid distances and reconstruction of branch lengths suggest that the ancestral steroid receptor was a functional estrogen receptor, the sequence of which was conserved among descendent ERs.

The reconstructed ancestral receptor is 71% identical to the human ERα however radically different to the PR, AR, GR and MR. This result indicates that the ancestral steroid receptor activated genes with estrogen-response palindromes (AGGTCA-figure 5) and bound estrogens. In the synthesis of estradiol and estrogens, progesterone and testosterone are synthesized as intermediates. These steroids, and the enzymes that produce them, would therefore have been present during the period when only one receptor for

estrogen existed. After gene duplications of the ancestral estrogen receptor gene and followed by considerable sequence divergence, receptors emerged to give these intermediate compounds novel signaling functions.

Redundant receptors created by gene duplication could then diverge in sequence from their ancestors and evolve affinity for these steroids, creating signaling functions for what were once intermediates. The expansion of the steroid receptor family by gene duplication and ligand application allowed a greater specificity in hormone control over physiological functions. Estrogen regulation appears to be the most ancient of all modes of steroid/receptor control which is supported by the apparent role of estrogen in branchiostome and echinoderm reproduction (Fang et al., 1994; Hines et al., 1992). Comparisons of the evolution of steroid receptors indicate that land animals show a slow sequence divergence. The fish estrogen and glucocorticoid receptors have about 70 and 60% sequence identity respectively, to their human orthologs. This puts these steroid

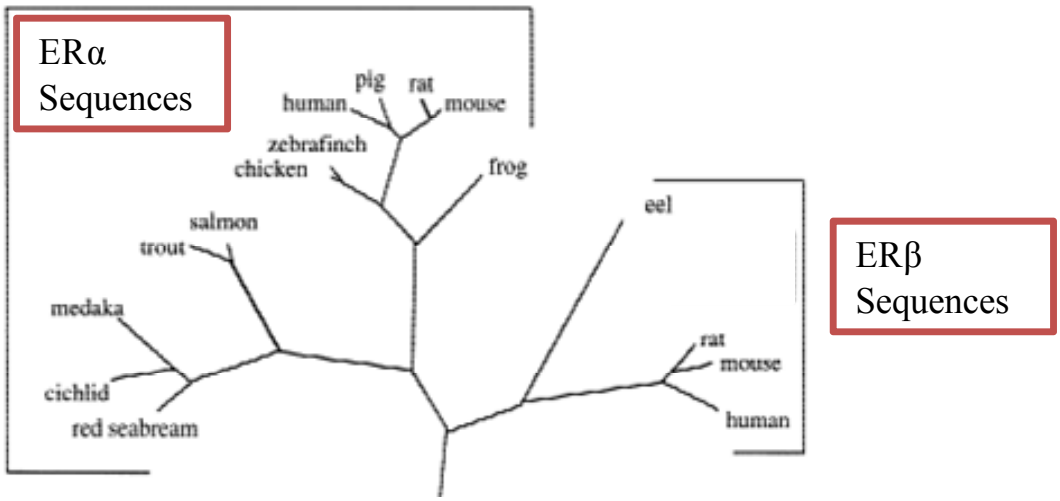

Figure 4 Phylogenetic tree of the evolutionary relationships of ERα and ERβ based on amino acid sequences. Adapted from (Kelley and Thackray, 1999).

receptors in the class of slowly changing proteins (Doolittle et al., 1996). The discovery of a second form of ER (Kuiper et al., 1996; Mosselman et al., 1996) termed ERβ, generated questions about the biological importance of this newly discovered gene. Previous sequence alignments have shown that ERβ sequences share common elements that are distinct from ERα sequences (Pettersson et al., 1997) supporting the idea that the ERβ sequences belong to a separate monophyletic clade (a group composed of a single ancestor and all its descendents) with respect to ERα and have evolved in parallel (Naylor and Brown, 1998) (Figure 4). The fact that the ERβ gene is widespread among chordates (animals which are either vertebrates or one of several closely related invertebrates) and comprises a separate genetic lineage dating back at least 450 million years argues that this gene performs distinct biological functions that have been maintained by natural selection for this long period of time.

4 Estrogen Receptors

In the 1950s, Jensen and Jacobson (Jensen and Jacobson, 1962; Jensen EV and Jacobson HI, 1960) used tritium labeled E_2 to demonstrate that it was specifically retained by estrogen target tissues which led them to hypothesize that a receptor must exist for this molecule.

4.1 ERα and ERβ: Discovery of two subtypes

In the next decade an ER was identified by Toft and Gorski (Toft and Gorski, 1966) and isolated from several mammalian species, including rat and human (Toft and Gorski, 1966; Jensen et al., 1968). However, it was not until the mid 1980s that the first ER, now called ERα, was cloned by two groups of investigators (Green et al., 1986; Greene et al., 1986; Walter et al., 1985). In the mid 1990s, a second ER, called ERβ, was identified from a library scan of rat prostate cDNA library (Kuiper et al., 1996) and subsequently cloned from several species including the mouse, human, and fish (Kuiper et al., 1996; Mosselman

et al., 1996; Tremblay et al., 1997) which meant that the biological properties associated to ER signaling in terms of subtype selectivity, ligand specificity, and tissue distribution had to be reviewed (Giguère et al., 1998; Gustafsson, 1999).

At first, a human ERβ with 477 amino acids was reported (Mosselman et al., 1996). A few months later, Enmark *et al.* (Enmark et al., 1997) identified an ERβ mRNA species of 485 amino acids, and it was hypothesized to reflect full-length ERβ. The following year, Ogawa *et al.* (Ogawa et al., 1998b) reported the cloning of an additional ERβ species consisting of 530 amino acids, which is now considered to represent full-length ERβ. A few months later, Moore *et al.* (Moore et al., 1998) also identified the same 530-amino acid sequence as the full-length ERβ in addition to various isoforms. Similarly to ERα, ERβ expression has also been associated with cancers of the breast (Dotzlaw et al., 1997; Dotzlaw et al., 1999; Speirs et al., 1999a; Speirs et al., 1999b), colon (Foley et al., 2000; Campbell-Thompson et al., 2001), and ovarian tissues (Pujol et al., 1998; Rutherford et al., 2000).

4.2 ERα and ERβ; Pertinent or redundant?

Although *in vitro* studies have demonstrated redundancy in the roles of these two receptors, tissue localization has revealed distinct expression patterns for each receptor suggesting that each ER subtype might perform specific functions. ERα is expressed in a variety of tissues classically associated with estrogenic activity including the uterus, ovaries (theca cells), cervix, vagina, breast, bone and several additional target organs such as in the prostate (stroma) and brain but to a lesser degree in bladder, liver and thymus. ERβ is predominantly expressed in normal colon, prostate (epithelium), ovary (granulosa cells), bone marrow and brain, with smaller amounts reported in uterus, bladder, lung and testis (Kuiper et al., 1997; Shughrue et al., 1998; Veeneman, 2005) and in the spleen, hypothalamus, and thymus (Couse et al., 1997). ER tissue expression is also tied to

developmental stage, specifically in both uterus and pituitary where ERβ is expressed during development but is later replaced by ERα (Shupnik, 2002; Nishihara et al., 2000).

The development of KO models has helped us to unmask unidentified estrogen signaling systems as well as those that are independent of either ERα or ERβ. Studies with ERα and ERβ knockout (KO) mice have revealed a role for ERs signaling in bone formation, male and female sexual maturation, fertility, cardiovascular and angiogenesis effects, and behavior (Bocchinfuso and Korach, 1997; Couse et al., 1995; Eddy et al., 1996; Hess et al., 1997; Korach et al., 1996; Krege et al., 1998; Lindberg et al., 2003; Ogawa et al., 1996; Ogawa et al., 1997; Ogawa et al., 1998a; Ogawa et al., 1998d; Rubanyi, 2000; Schomberg et al., 1999; Windahl et al., 2001; Windahl et al., 2002). Both sexes of the αERKO are infertile. In the female αERKO mice, estrogen insensitivity leads to hypoplasia in the reproductive tract with enlarged cystic and haemorrhagic follicles in the ovaries. Also, lack of pubertal mammary gland development and excess adipose tissue was observed in females (Couse and Korach, 1999). In αERKO males, testicular degeneration and epididymal dysfunction are the major phenotypes. Conversely, male βERKO mice are fertile and show no obvious phenotype. However female βERKO mice exhibit inefficient ovarian function and subfertility due to a block in the last step of follicular development. This can be overcome when animals are treated with FSH and LH. A recent report described an abnormal vascular function resulting in hypertension of βERKO mice (Zhu et al., 2002). The generation of mice that do not express either receptor isoform (DERKO) has provided additional information on the role of these two receptors in regulating physiological and behavioural processes. The adult ovarian phenotype is masculinised, coinciding with a reduction in oocyte number. In addition the ovaries do present structures that resemble testicular seminiferous tubules (Couse and Korach, 2001). Absence of both receptors leads to a significant drop in sexual and aggressive behaviour (Ogawa et al., 2000; Simpson and Davis, 2000; Zhu et al., 2002).

The difference in tissue distribution between ERα and ERβ can only partly explain the tissue specific effects of estrogens. Tissue specificity may actually be attributable to the type of dimers formed by these two receptors and their interaction with accessory proteins. Several groups have reported that ERα and ERβ can form functional heterodimers (Cowley et al., 1997; Ogawa et al., 1998b; Pettersson et al., 1997; Tremblay et al., 1999b).

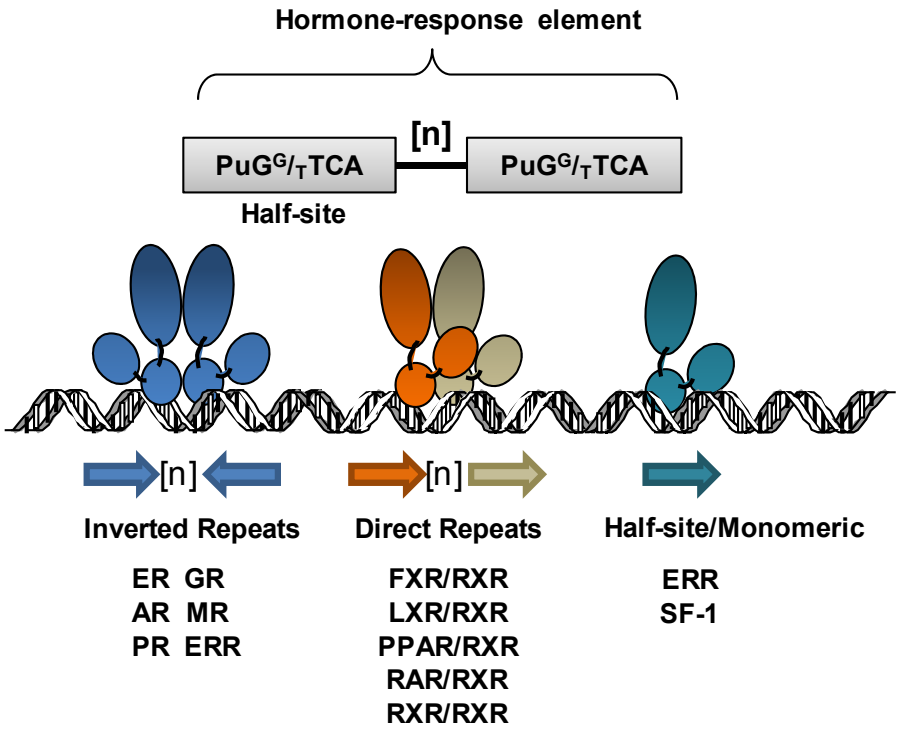

Figure 5 Hormone Response Element- Orientation of Hormone response element and their cognate receptors.

Moreover, the ERα/ERβ heterodimer appeared to form preferentially over each homodimer when both receptors are expressed, and was shown to bind to the consensus estrogen response element (ERE) sequence (Figure 5) with an affinity comparable to that of the ERα homodimer and greater than the ERβ homodimer (Tremblay et al., 1999b; Cowley

et al., 1997). Consequently, profiles in gene expression may diverge upon ER dimerization properties, and therefore the ratio of different receptor types in tissues may be an important determinant of a biological response.

4.3 Structure and isoforms of the ERs

ERα and ERβ are each encoded by unique genes localized on chromosome 6 and 14 in humans respectively (Enmark et al., 1997; Tremblay et al., 1997; Giguère et al., 1998). Both ER subtypes share the distinctive modular structure of functional domains characteristic of the superfamily of nuclear hormone receptors (Kumar et al., 1987; Evans, 1988). Nuclear receptors have been clustered into 6 subclasses based on sequence comparison and phylogenetic analysis (Laudet, 1997) and a unified nomenclature was proposed thereafter (Laudet et al., 1999). Members include receptors for estrogen (ER), glucocorticoids (GR), progesterone (PR) and androgens (AR), as well as the orphan estrogen-related receptors (ERRs), which are contained within the NR3 subclass, reflecting their apparent abilities to bind to response elements organized as inverted repeats (Figure 5) (Beato et al., 1995). Receptors that share the heterodimerization partner retinoid X receptor (RXR) bind response elements organized as direct repeats, such as for retinoic acids (RAR), prostaglandins and fatty acids (PPAR), thyroid hormones (TR), vitamin D (VDR) and oxysterols (LXR), and are mostly found in the NR1 subclass (Truss and Beato, 1993; Glass, 1994; Mangelsdorf et al., 1995). Members of NR2 subclass are able to bind as homodimers on direct repeat elements including RXR, HNF4 and COUP-TF. Subclasses NR4-6 are comprised of orphan nuclear receptors for which no specific ligand has yet been identified (Laudet, 1997).

4.3.1 Structure of ERs

There are six functional domains that characterize ERα and ERβ termed A through F (Figure 6). These consist of a highly conserved (Umesono and Evans, 1989) (97%) DNA-binding domain (C) containing two Zn^{2+}-finger motifs necessary for dimerization and specific binding to genomic response elements (Figure 5), a globular C-terminal region (EF) relatively well conserved (60%) (Warnmark et al., 2003) that contains the LBD and an activation function (AF-2) that mediates receptor trans-activation. The AF-2 domain is characterized by a highly conserved amphipathic α-helix (H12), essential for ligand dependent activation of transcription and interaction coregulatory proteins (McKenna et al., 1999; Heery et al., 1997). The low rate of change and the conservation of critical residues within the DBD and the LBD imply that there has been strong selective pressure to maintain these functions in both ERα and ERβ. In addition, the amino acids that make

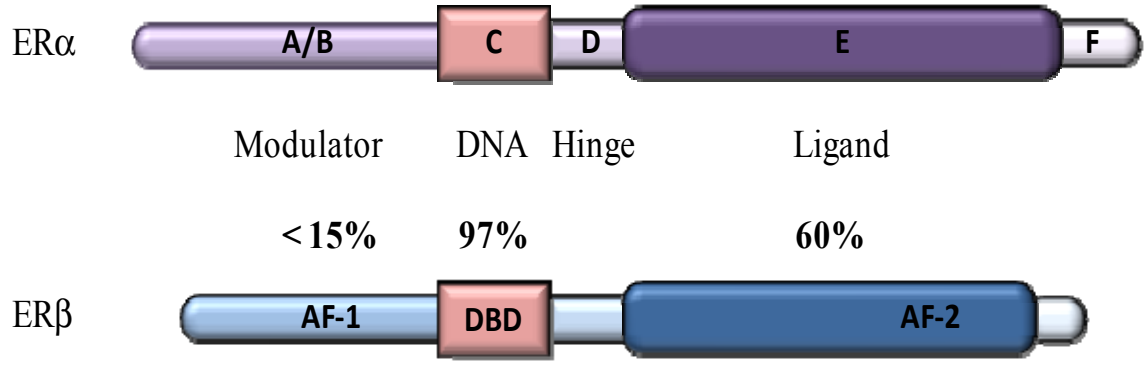

Figure 6 ERα and ERβ functional domains and sequence homology. Adapted from Sanchez *et al.* TEM.

direct contacts with the DNA in the crystal structure of the DBD are completely conserved (Schwabe et al., 1993). Also the amino acids of the ligand binding cavity, identified in the crystal structure of the LBD, involved in the direct and indirect hydrophobic interactions with the ligand (Brzozowski et al., 1997), are conserved with only a few changes within the clade.

The LBD region is preceded by a flexible hinge region (D) that was previously describe to possess signals for nuclear localization and the binding of chaperones such as heat shock proteins (hsp), which provide the receptors proper folding and a means to interact with protein trafficking systems. However, over the past few years, studies have demonstrated the hinge region to play a much more extensive role in the regulation of both receptor isoforms, through different post-translational modifications (Sanchez et al., 2007; Giordano et al., 2010; Berry et al., 2008; Herynk et al., 2009).

The N-terminal (AB) domain (Figure 6) is the region that differs dramatically between both ER subtypes with a feeble 15% homology. Although the N-terminal appears to be relatively unconstrained compared with the DBD and the LBD, it plays an important role in the transactivation of gene expression. Experiments have shown that transcriptional activation functions (AF) in the N-terminal domain (AF-1) and the LBD are both required for full receptor activity (Kumar et al., 1987; Tzukerman et al., 1994). From the structure-function analysis presently available, the apparent differences between AF-1 and the AF-2 in conformation suggest that the two activation functions have evolved different approaches to achieve transcriptional activation (Warnmark et al., 2003). The N-terminal region of ERs contains serine residues which have been implicated in cross-talk with various cell signaling pathways (Tremblay et al., 1997; Weigel, 1996; McInerney and Katzenellenbogen, 1996). The serine phosphorylation sites in the N-terminal domain of ERα and ERβ are not conserved, suggesting that ERα and ERβ may be regulated differently by cell signaling pathways. Indeed the AF-1 ligand-independent domain which controls the

recruitment of coregulators can be both similar and unique from those employed by the AF-2 (McKenna et al., 1999; Webb et al., 1998). Finally the F terminal domain comprised of the last 30-45 amino acids (depending on the subtype) has approximately 18% homology (Gustafsson, 1999) and appears to regulate the conformation of ERs in order to control the transcriptional response to its ligand (Yang et al., 2008).

4.3.2 Isoforms/variants of ERα and ERβ

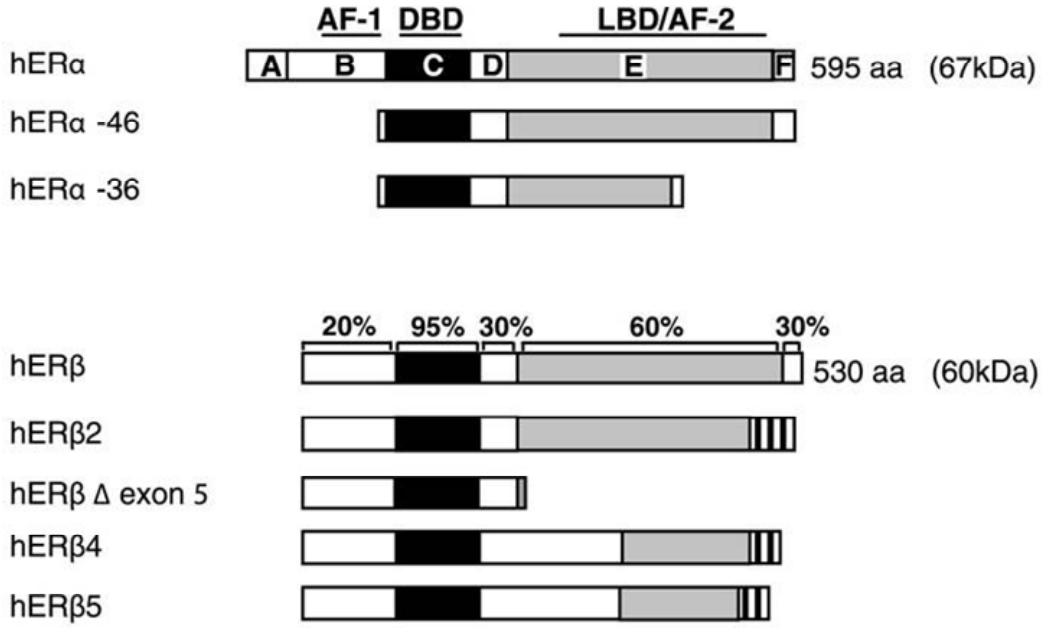

Figure 7 Isoforms of ERs- Schematic representation of the different splicing produtcs from the ERα and ERβ *Heldring et al Physiol Rev 87:905-931, 2007.*

From the eight total exons that code for ERα, detection of up to five different isoforms/variants have been discovered in humans from alternative splicing, intronic exons and exonic duplications (Hirata et al., 2003). The full-length ERα is defined as being 595 aa (hERα-66 (66KDa)), however shorter transcripts have been observed to be expressed in

different cell lines, such as hERα-36 (36 KDa) and hERα-46 (46KDa) (Flouriot et al., 2000; Wang et al., 2005). hERα-36 lacks both AF domains but contains sites that could be myristoylated suggesting that it would have the potential of anchoring itself in the plasma membrane. hERα-46 (46KDa) lacks the AF-1 but still manages to show antagonizing activity on the proliferative effects of the full length hERα-67 in MCF-7 cells (Penot et al., 2005) (Figure 7).

As for ERβ, five different variants (ERβ1-5) have been cloned (Figure 7) and examined. ERβ1 is considered the full-length receptor and is the only variant to contain fully functioning helices 11 and 12 (Wurtz et al., 1996) and therefore capable of interacting with ligands and recruiting coregulatory complexes (Henttu et al., 1997). Few studies have looked at the preference in dimerization partners between ERα and ERβ, however in gel-shift assays, ERβ4 and ERβ5 heterodimerised with ERα (Poola et al., 2005) which affected the response to estrogen signaling, similar to the heterodimerization of ERβ2 (ERβcx) with ERα. ERβcx is the only variant to possibly exhibit clinical relevance. Although this protein does not respond to any particular ligand, it has a dominant negative effect on ERα transcriptional activity (Ogawa et al., 1998c; Zhao et al., 2007).

4.4 Activation of ERs

Full transcriptional activity of a nuclear receptor is accomplished not only by the synergism between its AFs but also relies on a number of events. The transcriptional potential of each AF is dependent on external determinants such as cell type, posttranslational modifications, promoter context and interaction with coregulatory complexes (Berry et al., 1990; Aronica and Katzenellenbogen, 1993; Hadzopoulou-Cladaras et al., 1997; Tzukerman et al., 1994; McInerney et al., 1998; Pham et al., 1992; Cenni and Picard, 1999).

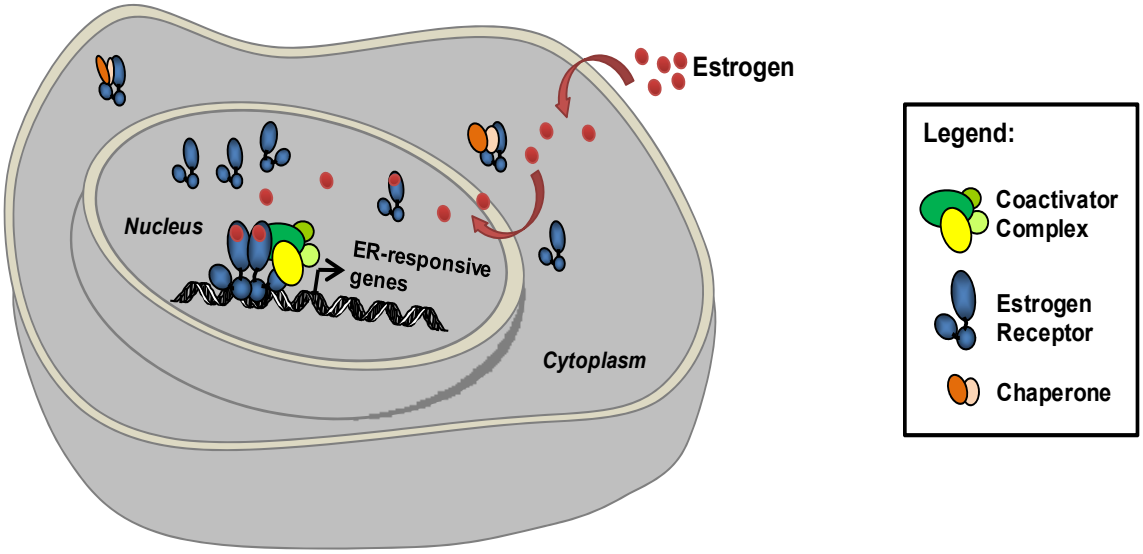

Figure 8 Ligand-dependent activation of ERs.

4.4.1 Ligand-Dependent Activation of ERs

ERs, in absence of estrogen, are attached to receptor-associated proteins which function as chaperones (Figure 8) stabilizing the receptor in an inactivated state by masking the DNA binding domain (Couse et al., 1999). Following the binding of E_2, an activating conformational change is generated within the ERs promoting dimerization and high affinity binding to specific DNA response elements found within the regulatory regions of target genes (refer to figure 5). ERα and ERβ have been shown to interact with identical DNA response elements and exhibit a similar binding affinity profile for naturally occurring estrogens such as E_2 when assayed *in vitro*. Both ERs recognize a distinct palindromic sequence, normally specific to the type of nuclear receptor. In the case of ERs it is an inverted repeat sequence separated by three nucleotides; AGGTCAnnnTGACCT (Parker et al., 1993) (Figure 5). However, only a small number of estrogen-responsive

genes contain the consensus sequence. Several of the genes identified having a functional ERE sequence not only consist of one or more changes from the consensus but are made up of multiple copies of imperfect EREs (Driscoll et al., 1998). Depending on the cell and promoter context, the different combinations of DNA-bound ERs exert either a positive or negative effect on the expression of downstream target genes (Wood et al., 1998).

4.4.2 Ligand-Independent Activation of ERs

The responsiveness of steroid receptors to cell signaling pathways in the absence of their hormone can be different. ERs are quite responsive to cell signaling pathways. In fact, endogenously expressing ER-positive cells maintained in phenol-red free, charcoal-stripped serum used to minimize steroids, frequently display a considerable transcriptional activity in the absence of estrogen (Smith et al., 1993). Although ERs belong to a family of ligand-activated receptors, they are also phosphoproteins and their activity can equally be regulated by phosphorylation of specific sites which can occur as part of both the ligand-induced activity (Ali et al., 1993; Arnold et al., 1994; Kato et al., 1995) and/or ligand-independent activity (Arnold et al., 1995b; Bunone et al., 1996; Tremblay et al., 1999a; Tremblay et al., 1998). Studies from other transcription factors such as CREB and PR, have shown that phosphorylation can play roles in nuclear translocation, DNA binding, interaction with other proteins and trans-activation (Hill and Treisman, 1995; Denner et al., 1990a). Polypeptide growth factors can activate ERs and increase the expression of ER target genes (Smith, 1998). Phosphorylation occurs predominantly at specific serine/threonine or tyrosine residues and is catalyzed by enzymes such as mitogen-activated protein kinases (MAPK) (Shao and Lazar, 1999). MAPKs are composed of several serine-threonine kinases that are activated in response to various cell-growth signals and transduce extracellular signals to intracellular targets by way of membrane receptors.

Activation of ERs by signaling pathways (section 5.3) in the absence of E_2 was first identified in the early 1990s (Ignar-Trowbridge et al., 1992). Ovariectomized mice were

treated with epidermal growth factor (EGF), promoting the translocation of ER towards the nucleus and stimulating its activity. Furthermore, EGF antibodies were administered to ovariectomized mice 3 days prior to hormone treatment resulting in a marked decrease of uterine DNA synthesis (Ignar-Trowbridge et al., 1995), leading to believe that EGF had a role in the proliferative effects of estrogen in reproductive tissues. Further assessment of the cell-surface receptor tyrosine kinases (RTKs) demonstrated their role in the recruitment of multiple signal transduction cascades that act to increase the activation of MAPK Erk1/2, PKB/Akt, Jnk, p38 and protein kinase C (PKCα and δ), key elements in the regulation of cell proliferation and survival signals (Bonni et al., 1999; Campbell et al., 2001; Gibson et al., 1999; Stambolic et al., 1999) (Amit et al., 2007). Chapter 5 will focus on the detailed description of the different signaling pathways regulating the activity of ERs.

5 Cell-Surface Receptors

It is now widely documented that the activation of growth factor (GF) signaling cascades through a supply of GF ligands via up-regulation and increased activation of their target growth factor membrane receptors and their recruited downstream signaling elements, can promote hormone-like responses.

5.1 Members of ErbB Clan

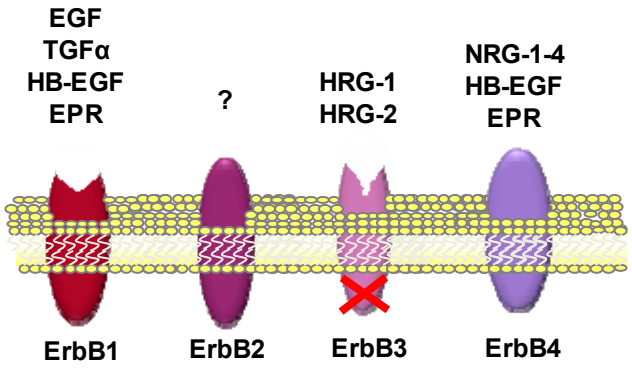

Figure 9 Four types of ErbB receptors and their ligands.

Growth factors and their receptors play a fundamental role in the communication between outside the cell surface and the inside compartments (Schlessinger and Lemmon, 2006; Scaltriti and Baselga, 2006). The human epidermal growth factor family (ErbB/HER) is comprised of four closely related receptors (Figure 9); epidermal growth factor receptor (EGFR, HER1, ErbB1), human EGFR-2 (HER2, ErbB2), ErbB3 and ErbB4. They are transmembrane oncoproteins containing an extracellular ligand binding domain and an intracellular receptor tyrosine kinase (RTK) domain sharing 40-45% homology to one another. This family of proteins has an important role in tumour processes including angiogenesis and metastasis and is associated with a poor prognosis in many human malignancies due to their overexpression or constitutive activity (Salomon et al., 1995; Hemming et al., 1992). Although all the aforementioned receptors share a strong homology within their TK domains, they are quite distinct in their extracellular N-terminal and cytoplasmic C-terminal domain (Klapper et al., 2000).

The significance of ErbBs in normal development was, as with ERs, obtained from knockout-generated mice. Null mutations in individual ErbB loci are lethal demonstrating that ErbB receptors play a pivotal role in regulating vertebrate embryogenesis and development. ErbB1 KOs are lethal at the embryonic and up to perinatal stages as mice develop abnormalities in the brain, lungs, gastrointestinal tract and the skin (Threadgill et al., 1995; Sibilia and Wagner, 1995; Sibilia et al., 1998; Miettinen et al., 1995). ErbB2 and ErbB4 KOs are lethal at the stage of midgestation due to malformations of the heart (Gassmann et al., 1995; Lee et al., 1995). ErbB3 KO mice are embryonically lethal due to malformations of the heart valves in addition to neural crest defect and lack of Schwann cell precursors (Riethmacher et al., 1997; Erickson et al., 1997).

5.2 Activation of ErbB

Several ligands bind to the ErbB receptors (Figure 9). Members of the EGF superfamily include epidermal growth factor (EGF) (Todaro et al., 1980), transforming growth factor-α (TGF- α) (Shoyab et al., 1988) and amphiregulin, which specifically bind to ErbB1 (EGFR). Heparin-binding EGF and epiregulin (EPR) bind to both EGFR and ErbB4 (Figure 10) (Toyoda et al., 1995). Neuregulins 1 and 2 (also known as heregulins (HRG) or neu differentiating factor) bind to both ErbB3 and ErbB4 (Falls, 2003). Binding of GF to ErbBs induces receptor dimerization and activation of intracellular protein tyrosine kinase with subsequent initiation of numerous downstream signaling events (Figure 10) (Press and Lenz, 2007). All ErbB ligands exist initially as membrane-anchored precursors that require proteolysis to liberate them as soluble mature ligands (Massague and Pandiella, 1993; Harris et al., 2003b).

In the absence of ligand, ErbB1, ErbB3 and ErbB4 are monomeric and can be partially or completely inhibited (Schlessinger, 2002; Ferguson et al., 2003; Bouyain et al., 2005). This inhibition is caused by the extracellular portion autoinhibiting the ligand surface due to its conformation status. Binding of a ligand leads to an alteration within the extracellular domain which creates a ligand-binding pocket and protrusion of a dimerization area. This change aids in receptor oligomerisation and the formation of homo- and heterodimers (Hynes and Lane, 2005; Citri et al., 2003; Leahy, 2004). Dimerization brings the intracellular kinase domains of the two receptors close together encouraging transphosphorylation of tyrosine kinase residues in the cytoplasmic tail of one receptor by the kinase domain of the adjacent receptor (Figure 10) (Jorissen et al., 2003; Schlessinger, 1988). Unlike its family counterparts, ErbB2 has not yet had a ligand identified to regulate its activity (Yarden and Sliwkowski, 2001) (Klapper et al., 1999; Citri et al., 2003).

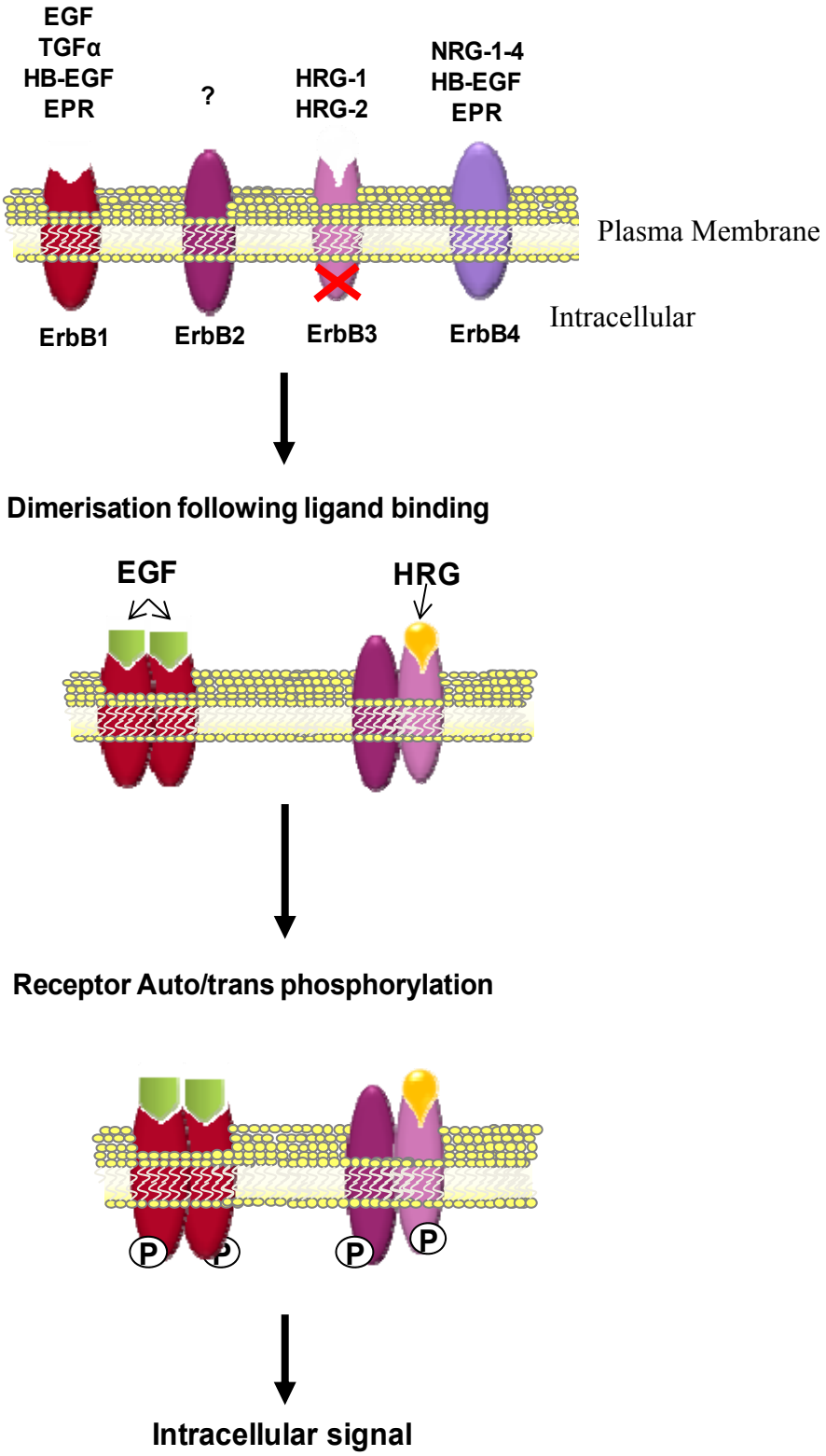

Figure 10 ErbB Receptor Activation.

ErbB2 is the preferred heterodimerising partner of ligand-bound ErbB3 but can also bind ErbB1 and ErbB4 (Karunagaran et al., 1996), (Graus-Porta et al., 1997). Dimers containing ErbB2 are known to enhance and prolong the signaling of several ErbB ligands, and this may be due to the reduced dissociation of the receptor complex (Karunagaran et al., 1996) in addition to the reduction in the rate of internalization of the complex leading to recycling rather than degradation (Holbro et al., 2003). ErbB3 harbors a substitution in crucial residues of the C-terminal intracellular domain rendering its kinase activity dead (Guy et al., 1994) therefore ErbB3 homodimers are inactive. This forces the receptor to heterodimerize with other ErbBs to become phosphorylated and trigger an intracellular signal (Kim et al., 1998). In addition, ErbB3 contains seven copies of the Tyr-X-X-Met motif in its c-terminal motif recognized by phosphatidylinositol 3-Kinase (PI3K) which leads to the activation of the Akt pathway (Prigent and Gullick, 1994; Songyang et al., 1993). ErbB3's preferred dimerizing partner is ErbB2, in fact, the ErbB2-ErbB3 heterodimer is the most prevalent receptor complex and one of the most potent signaling pathways that regulate cell growth and transformation (Pinkas-Kramarski et al., 1996; Karunagaran et al., 1996).

5.3 ErbB Intracellular signaling

Autophosphorylation of the C-terminal tyrosine residues serving as docking sites for cytoplasmic signaling proteins contain Src-homology (SH-2) and phosphotyrosine-binding (PTB) domains (Olayioye et al., 2000; Yarden and Sliwkowski, 2001). Each ErbB receptor exhibits a phosphotyrosine profile that allows for binding of enzymes such as Src, phospholipase Cγ, and the p85 regulatory subunit of PI-3K, or adapter molecules such as Shc and Grb2 linking ErbB activity to many downstream effectors (Figure 11) (Olayioye et al., 2000; Hynes and Lane, 2005). Although the Ras-Raf-MAPK and PI3K pathways are the major signaling pathways by the ErbB family, each dimeric receptor complex can activate different combinations of these signaling cascades, resulting in a wide range of signaling events (Figure 11).

5.3.1 Ras/Raf/MAPK pathway

The Ras/Raf/MAPK pathway is the major downstream signaling pathway activated by the ErbB family (Salomon et al., 1995; Hemming et al., 1992). Initiation of the signaling cascade starts with the direct binding of Grb2/Sos complex adaptor to specific docking sites on the intracellular portion of EGFR, otherwise an indirect interaction through Shc adaptor proteins is observed as well (Batzer et al., 1994). Next, the Grb2 associated guanine nucleotide exchange factor Sos activates ras by the exchange of GDP to GTP, which in turn activates Raf kinase triggering the kinase cascade involving mitogen-extracellular kinase 1,2 (MEK1,2 /MAPKK) and Erk1/2 (MAPK) (Figure 11) (Liebmann, 2001). Erk1, 2 can subsequently phosphorylate several cytoplasmic and nuclear targets, such as ERα and ERβ (Hill and Treisman, 1995).

5.3.2 PI3K/Akt pathway

The PI3K/Akt pathway regulates cell growth, resistance to chemotherapy, tumour invasion and migration (Vivanco and Sawyers, 2002). Its activation occurs through the SH2 binding motifs of the p85 regulatory subunit of PI3K to the phosphotyrosine residues or indirectly through the binding of GTP-Ras to the p110 catalytic subunit of PI3K. Active PI3K converts membrane-bound lipid phosphatidylinositol 4, 5-bisphosphate (PIP2) to PIP3, which in turn phosphorylates and activated the serine/threonine Akt (Vivanco and Sawyers, 2002; Meier and Hemmings, 1999) (Figure 11). Similar to Erk, Akt can phosphorylate a variety of cytoplasmic and nuclear targets. Interestingly, while ErbB2 easily triggers the MAPK pathway through Grb2 and Shc adapters, it cannot, on its own, activate Akt, as it cannot recruit the p85 subunit of PI3K unless ErbB3 or ErbB4 is expressed (Prigent and Gullick, 1994; Olayioye et al., 2000).

Studies from breast cancer T47D cell line have demonstrated that depending on which ErbB ligand is present, such as EGF or Hrgβ1, the signaling cascade and biological response will differ accordingly. Hrgβ1 can activate the p38 MAPK pathway which in

conjunction with the PI3K/Akt pathway and MAPK can stimulate cell proliferation, however EGF treatment on EGFR, activates a robust MAPK response which does not lead to cell proliferation (Neve et al., 2002). Therefore, each ErbB can trigger a distinct set of signaling proteins generating unique responses that are cell-type and ligand-receptor specific. In particular, steroid hormone and growth factor cross-talk acts to modulate endocrine response in breast cancer (Nicholson et al., 1999). Abnormalities in GF signaling pathways may account for the endocrine-resistant phenotype and thus may represent a target for new therapies to overcome resistance and enhance clinical response rate as reviewed in chapter 8.

5.3.3 Membrane receptor signaling pathways can regulate ERs activity

The concept that overexpressed EGFR and ErbB2 plays a role in the development of anti-estrogen resistance is supported by data that demonstrates their hyperphosphorylation provoked their heterodimerization in resistant MCF-7 cells (Knowlden et al., 2003) leading to the recruitment of multiple signal transduction cascades that act to increase the activation numerous signaling pathways, such as MAPK, Akt and even Protein Kinase C (PKCα and δ), key elements in the regulation of cell proliferation and survival signals (Bonni et al., 1999; Campbell et al., 2001; Gibson et al., 1999; Stambolic et al., 1999; Coutts and Murphy, 1998; Kurokawa and Arteaga, 2003; Oh et al., 2001).

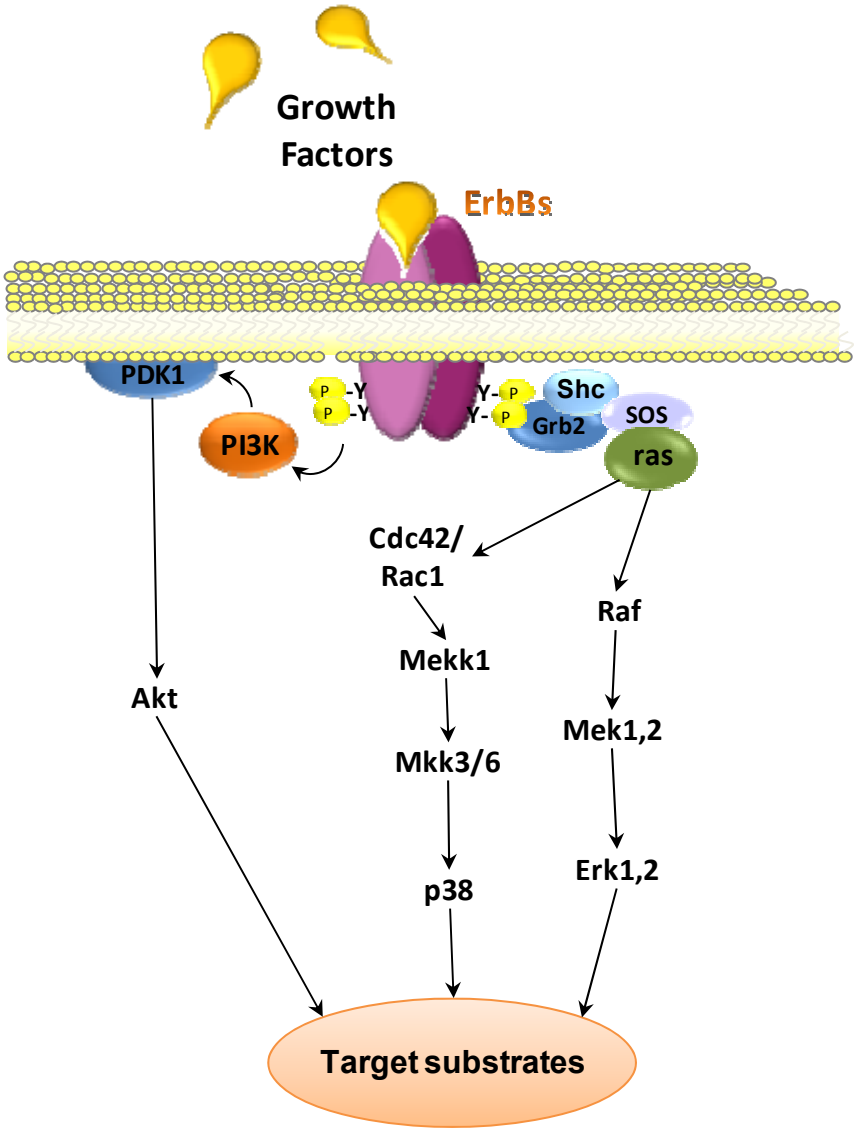

Figure 11 Intracellular pathways activated by ErbB receptors.

Such signaling would act as a counterbalance to the anti-proliferative and pro-apoptotic effects of anti-estrogens. MAPK increases are also reported to contribute to the growth of ER-positive breast cancer cells during adaptation to long-term estrogen deprivation (Martin et al., 2003; Jeng et al., 2000). Similarly, overexpression of PKCα in ER-positive breast cancer cells can promote hormone-independent growth through ERα (Tonetti et al., 2000). There is now substantial evidence of crosstalk between the ERs and growth factor receptor signaling pathways.

5.3.3.1 Impact on ERα

There are multiple pathways known to regulate hormone-independent activation of ERα. One of the best characterized pathways involves the activation of ERα by EGF. The serine 118 residue of the human ERα AF-1 was described as being a target of MAPK phosphorylation following treatment with EGF or IGF-1, enabling ERα to activate target gene transcription even in the absence of E_2 (Figure 12) (Kato, 2001; Kato et al., 1995). Point mutation converting this serine into an alanine residue proved to repress the activation (Ali et al., 1993). Ser-118 can be targeted by other kinases such as of cyclin-dependent kinases, cdk7 (Chen et al., 2000). In this case, the presence of Transcription Factor II H (TFIIH) which contains cdk7 (part of the RNA Polymerase II initiation complex) together with the ERα LBD mediate ERα phosphorylation. Cyclin A/Cdk2 can also target ERα phosphorylation which leads to an increase in its transcriptional activation (Rogatsky et al., 1999) but does not target ser-118. Instead cdk2 targets two other serine residues, ser 104 and 106 also present in the AF-1 domain (Figure 12). Extracellular signal-regulated kinase, ERK 7, activated by pathways independent of the documented MAPK (ERK1/2), targets ERα degradation in a hormone-dependent manner. ERK7 can enhance ser-118 phosphorylation nevertheless targeting the receptor for degradation (Henrich et al., 2003) (Figure 12). In fact cancer cell lines were found to express much less Erk 7 compared to levels found in normal tissue.

In another study to demonstrate that ERα activity could be modulated by signals other than its specific ligand, the use of 8-bromo-cAMP (a protein kinase activator) was employed and *PR* expression was seen increased (Denner et al., 1990b), a gene target of ERs. Although ERα was activated by the cAMP pathway in uterine tissue, activation of ERα by cAMP was not observed in ovary and breast tissue in the absence of estrogen (Cho and Katzenellenbogen, 1993b) demonstrating that transcriptional activity of ERs can be cell-type specific. Similar results on *PR* gene were observed when stimulating with IGF-1. Treatment of IGF-1 promoted the increase of PR levels in rat uterine cells grown in primary cultures due to an increase in ERα phosphorylation (Aronica and Katzenellenbogen, 1993). Further studies demonstrated that this increase was due to the apparent indirect interaction between PKA and PKC in a ligand dependent manner, leading to phosphorylation of ERα and hence transcriptional activation (Cho and Katzenellenbogen, 1993a). A later study revealed that PKA could, once activated by cAMP, directly phosphorylate serines of ERα within AF-1 specifically targeting ser-104, 106 and 118, which demonstrated an increase in transcriptional activity that was further potentiated in the presence of its ligand, E_2 (Le Goff et al., 1994). TGFα which can also bind the EGFR, can increase ERα transcriptional activity through the use of MAPK signaling pathways as well as other second messenger signaling pathways, such as PKC and PKA (Ignar-Trowbridge et al., 1993).

Further downstream within the N-terminal AF-1 domain of ERα, ser-167 is also targeted for phosphorylation in a ligand-dependent manner by a highly specific serine/threonine protein kinase, Casein kinase II known to regulate the activity of several transcription factors (Edelman et al., 1987). Ser-167 appears to also be the target of the PI3K/Akt pathway (Figure 12). Studies have demonstrated that phosphorylation by Akt at this particular site activates ERα-mediated transcription in a PI3K dependent manner (Shah and Rowan, 2005). An additional kinase activated by the EGF pathway is the 90-kDa ribosomal S6 Kinase ($pp90^{rsk1}$), a Ser/Thr Kinase. During growth factor response, $pp90^{rsk1}$ targets the phosphorylation of ser-167, which is necessary in order to regulate the

transcriptional activity of the AF-1 in the ERα isoform (Joel et al., 1998). It appears that the phosphorylation of ser-167 plays a role in optimal DNA- binding not only *in vitro* but *in vivo* as well to endogenous promoters (Shah and Rowan, 2005; Likhite et al., 2006).

Recent studies have shown that p21-activated kinase (pak1) can target ERα ser-305 (Bostner et al., 2010) which can also promote transcriptional activation of cyclin D1 (Balasenthil et al., 2004).Furthermore, PKA which also targets ser-305 rendered cells less resistant to tamoxifen treatment (Kok et al., 2010). In addition targeting ser-305 prevents the acetylation of the nearby lysine residue at position 303 (Cui et al., 2004). In fact lys-303 is often mutated in breast cancer and seems to increase the sensitivity of ERα to E_2 (Fuqua et al., 2000). It has been shown that another MAPK, p38, can directly phosphorylate ERα on Thr-311 in ERα-expressing endometrial cancer cells, encouraging its nuclear localization and its interaction with transcriptional coactivating complexes (Lee and Bai, 2002).

Finally within the C-terminal domain, close to the LBD, lies the residue tyrosine-537, which when phosphorylated by the *src* family tyrosine kinases $p60^{c\text{-}src}$ and $p56^{lck}$ in MCF-7 and sf8 cells, serves to regulate hormone binding to the receptor, trigger homodimerization and enhance transcriptional activation on EREs (Arnold et al., 1995a) (Weis et al., 1996; Likhite et al., 2006). While it has not yet been described in tumours, the corresponding substitution in ERβ bestowed constitutive activity and increased the recruitment of coactivators (Tremblay et al., 1998). Multiple phosphorylated forms of ERα exist, and more importantly studies have demonstrated the correlation between the several phosphorylates sites within the receptor with human breast tumour biopsy samples (Murphy et al., 2009) revealing the significance of studying the regulation of ER phosphorylation.

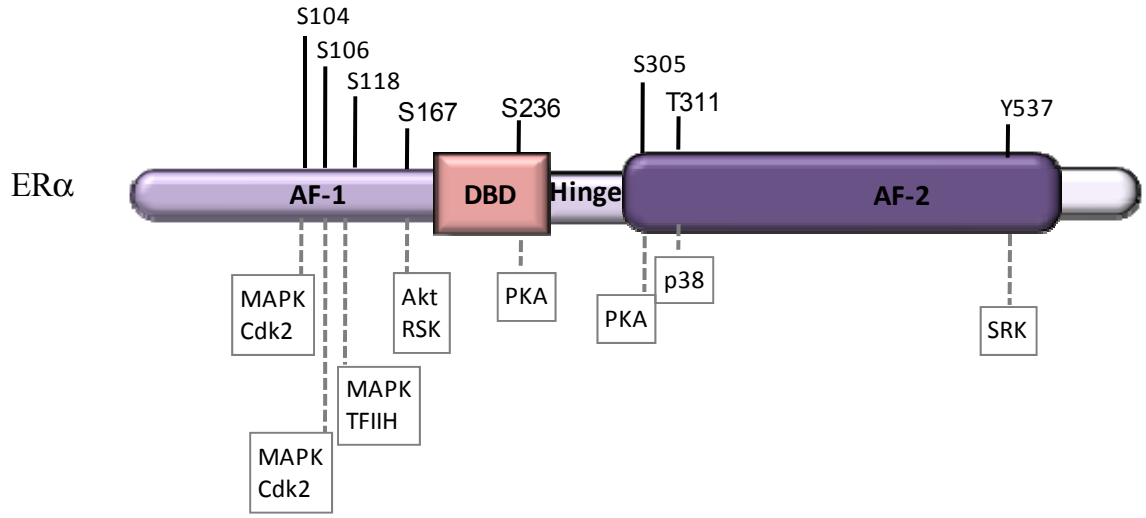

Figure 12 Schematic representation of experimentally-supported phosphorylation sites on ERα.

5.3.3.2 Impact on ERβ

The identification of ERβ has improved our understanding on the diversity of potential mechanisms by which ER-independent and estrogenic responses may be achieved. In the absence of estrogen, ERβ has been shown to regulate cycooxygenase-2 (COX-2) in fetoplacental endothelial cells (Su et al., 2009). The group did show that protein expression of COX-2 was not induced following estradiol treatment, however, specific knockdown of ERβ lead a decrease in the levels of COX-2. Although no signalling pathways were studied, the group was confident non-estrogenic signalling upon ERβ was at play. Earlier studies however did identify that by increasing the levels of cAMP, the transcriptional activity of ERβ was enhanced. Unlike ERα, this was not due to an increase in phosphorylation within the AF-1 region. It appears that the elevated level of cAMP, which activated a variety of protein kinases such as PKA and PKC, target the carboxy-terminal region, but specific targets have yet to be identified (Coleman et al., 2003). Also, during estrogen treatment, the expression of TGFβ-inducible *early gene* (*TIEG*) was regulated by ERβ in an AF-1-dependent manner due to the domain's ability to

recruit coactivators (Hawse et al., 2008). The MAPK pathway can target and enhance the activity of the murine ERβ through stimulating the recruitment of coactivating protein complexes to its N-terminus by phosphorylation of Ser-106 and Ser-124 (Figure 13) within the AF-1 domain (Tremblay et al., 1999a; Tremblay and Giguere, 2001). 15d-PGJ_2 (prostaglandin J_2) induces apoptosis in pancreatic cancer cells by attenuating ERβ-mediated *human telomerase reverse transcriptase* (*hTERT*) gene transcription through a reduction in ERβ phosphorylation (Kondoh et al., 2007). Since it has been reported that ERβ phosphorylation was mediated by the MAPK signaling pathway (Tremblay et al., 1999a), treatment of cells with MEK inhibitor PD98059 reduced ERβ phosphorylation affecting the binding of ERβ to the *hTERT* gene promoter and hTERT protein expression

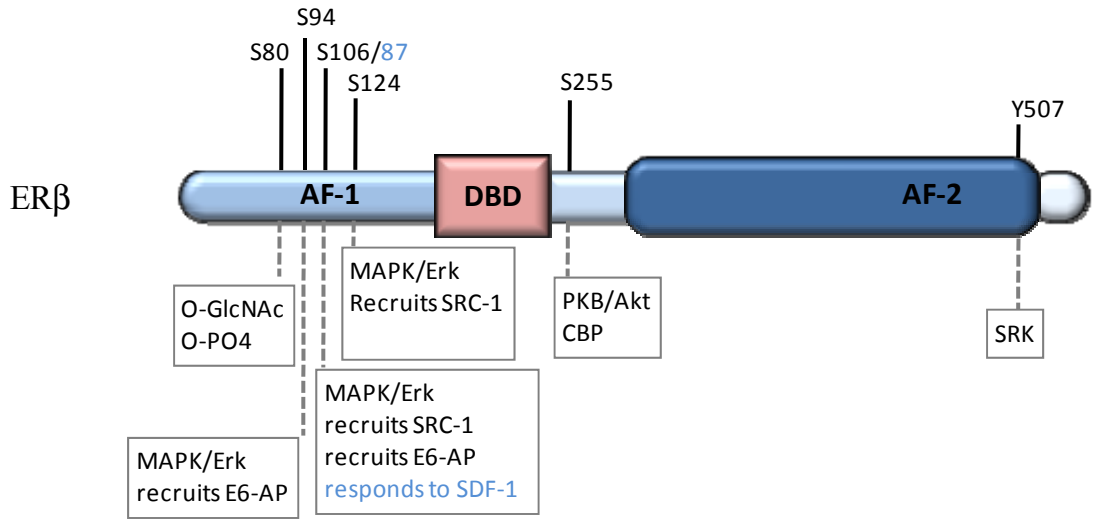

Figure 13 Schematic representation of experimentally-supported phosphorylation sites on ERβ.

suggesting that 15d-PGJ_2 suppressed the phosphorylation of ERK1/2 which in turn affected the activity of ERβ.

The p38 pathway can equally activate the transcriptional activity of ERβ in a ligand dependent manner although the mechanism, direct or indirect has not been established (Driggers et al., 2001). However, the outcome ER phosphorylation by protein kinases not only differ according to the identity of the activator but is also cell-type specific. It was recently shown that the activation of the p38 pathway by ErbB2/ErbB3 heterodimer was able to repress ERβ activity in pathological cell-lines by affecting the recruitment of coactivators to the N-terminal region (St Laurent et al., 2005a). Similarly, ErbB2/ErbB3 was able to repress ERβ through the Akt pathway by phosphorylating the receptor within the hinge region on Ser-255 (Figure 13) and leading to a decrease in its transcriptional activity (Sanchez et al., 2007). From the studies highlighted, it can be appreciated that ERβ, much like ERα, are regulated by several mechanisms either increasing or decreasing their activity depending on the activator, the sites targeted and the cell-type in which they are expressed.

5.4 GPCR can regulate ERs

Studies regarding the potential influence of GPCR signaling on ER-responsive genes are very few. A negative regulator of Rho-family of GTP-binding proteins, RhoGDIα, is able to regulate the activity of ERα transactivation. In fact RhoGDIα differentially modulated the expression of *PR* and *pS2* genes, which contain very different cis elements and trans-acting factors. While the *pS2* gene contains an imperfect ERE that interacts with ERα (Berry et al., 1989), the *PR* gene contains several activator protein-1 (AP-1) response elements and Sp1 sites through which ERα acts indirectly (Figure 14) (Petz et al., 2002; Petz et al., 2004). Thus the ability of RhoGDIα to affect differently the activity of ERα depends on the differences in the population of cis-elements and the trans-acting factors associated with various target genes.

The melatonin receptor is able to inhibit the proliferation of MCF-7 ER positive cell line (Hill et al., 1992; Kiefer et al., 2005) through a down-regulation of protein and mRNA

levels of ERα (Molis et al., 1994). In addition these effects are receptor specific as melatonin decreased the transcriptional activity of ERα both at ERE and AP-1 response elements, although these effects were not observed for ERβ. In fact it appears that the response to melatonin depends on the ERα /ERβ, as addition of ERβ results in MCF-7 insensitivity to melatonin (del et al., 2004).

On the other hand, signaling pathways known to be part of GPCR signaling can activate ERs. Brx, a guanine nucleotide exchange factor (GEF) can interact and stimulate the activity of both ERα and ERβ (Driggers et al., 2001) albeit through different pathways. While the cdc42-dependent pathway mediated ERα activation, ERβ's activity was stimulated by the p38 pathway. Moreover the chemokine receptor CXCR4 originally identified as an estrogen-regulated gene in ERα positive ovarian and breast cancer cells (Ali and Lazennec, 2007), has recently been reported by our laboratory to affect both the estrogen-dependent and independent transcriptional activates of ERα and ERβ. Indeed, the activation of CXCR4 with its ligand stromal cell-derived factor (SDF-1) promoted ERβ activity at ERE and AP-1 sites involving the phosphorylation of the receptor at ser-87 in the AF-1 domain by the MAPK pathway (Sauvé et al., 2009) (Figure 13).

5.5 ERs at the chromatin; a direct (ERE) and indirect (AP-1/ sp/1) relationship with DNA

ER genomic signaling can be divided into two distinct categories, the classical and the non-classical pathways. Classical signaling involves ERs binding directly to a specific DNA sequence and recruiting cofactors in order to initiate transcription (Figure 14). ERE-induced changes in ER conformation were predicted to alter ER affinity for coregulatory complexes (Klinge et al., 2001; Klinge et al., 2004). Although ERα and ERβ have been shown to display similar affinity for different EREs (Klinge, 2001) there are however conformational differences in ERs depending on both ligand and the bound ERE sequence (Klinge, 1999; Klinge et al., 2001; Bowers et al., 2000; Ramsey and Klinge, 2001;

Tyulmenkov and Klinge, 2001; Tyulmenkov et al., 2000). The steroidogenic factor-1 response element (SFRE) was found to interact with ERα not ERβ and even if heterodimers are preferential, ERα was not able to drive ERβ to the promoter (Yi et al., 2002) In fact, anti-estrogen treatment stimulated ERα, not ERβ, transcription at EREs in uterine cells through its N-terminal domain, which contributed to the agonistic activity of the anti-estrogen (McInerney et al., 1998; Hall and McDonnell, 1999).

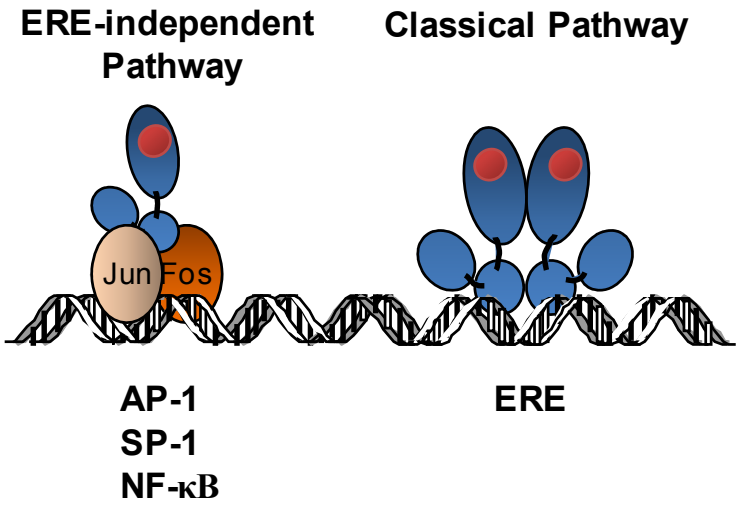

Figure 14 Classical vs non-classical ERβ pathways of target gene expression.

Alternatively non-classical signaling of ERα and ERβ involves altering the transcription of genes without directly binding to an ERE (Figure 14). Studies reporting estrogen-ER induction of genes containing no apparent ERE-like sequence led to the discovery that ligand-activated ERs can be tethered to other transcription factors and interact in an indirect manner with the regulatory regions of target genes. Both receptor subtypes are able to regulate gene expression indirectly via protein–protein interactions with c-Jun and c-Fos complex that bind to AP-1 (Matthews et al., 2006; Teyssier et al., 2001) as well as Sp1 and NF-κB (Pearce and Jordan, 2004). There are several genes regulated by ERα in a non-classical pathway such as *EGFR* (Salvatori et al., 2003), *c-myc* (Dubik and Shiu, 1992), *IGF-1* (Umayahara et al., 1994) and *Hsp27* (Porter et al., 1997).

Specifically, ERα-mediated expression of the *collagenase* and *IGF-1* genes is mediated through the interaction of the receptor with Fos and Jun at AP-1 binding sites, whereas several genes containing GC-rich promoter sequences are activated through ERα-Sp1 complexes (Figure 14) (Kushner et al., 2000). The AF-1 domain of each receptor plays a vital role in the outcome of their activity, although at times ligands determine their biological actions. ERα and ERβ can show opposite effects at AP-1 promoters in the presence of anti-estrogens (Webb et al., 1999). 4-hydroxytamoxifen can induce the activity of an AP-1 promoter in the presence of ERβ, whereas E_2 blocks transcription (Paech et al., 1997). ERβ was also able to regulate transcription of the *quinone reductase* gene in response to anti-estrogens but not E_2 (Montano et al., 1998). ERβ was not able to induce reporter activity driven by the *hRARα-1* promoter in the presence of estrogen however it did elicit transcriptional activation in the presence of 4-hydroxytamoxifen. Other ER antagonists including raloxifene, ICI-164,384 and ICI-182,780 also acted as agonists through ERβ via the *hRARα-1* promoter (Zou et al., 1999).

The availability and ability for second messenger signaling used to regulate ERα and ERβ and the subtype-specific promoter elements of target genes begin to account for the differences in ERs action in the various cells. Yet recent evidence has emerged establishing coactivators as points of convergence between ERs and growth factor signaling pathways by being targets of phosphorylation an event thought to enhance their transcriptional activities (Hall et al., 2001). In addition to activating ER protein directly, kinase-mediated growth factors signaling also modulate ER activity indirectly by targeting coregulatory complexes that interact with ERs.

6 Coregulatory complexes

In recent years, a large number of nuclear and steroid coregulators have been cloned and characterized to regulate receptor transcriptional activity. Following ligand binding to the receptor these coregulators are recruited to promoter regions through protein-protein

interaction either enhancing or impairing ERs mediated transcription (Klinge, 2000) by using their histone-modifying abilities to alter local chromatin structure. Acetylation on lysine residues was first discovered as a post-translational modification on histones and has long been considered as a direct regulator of chromatin structure and function. In fact the rate of gene transcription can be generally correlated with the degree of histone acetylation, where hyperacetylated regions of the genome are more actively transcribed than hypoacetylated regions (Pazin and Kadonaga, 1997).

Lysine residues are also used as sites for other post-translational modifications, such as ubiquitination, methylation, SUMOylation, biotinylation or neddylation (Yang, 2005), however their acetylation chemically locks the residue from any other modification. Lysine acetylation provides a great regulatory potential by creating a docking site to promote protein-protein interaction or interfere with binding of specific partners (Caron et al., 2005; Caron et al., 2003; Yang, 2004). Two types of enzymes control acetylation; histone acetyl-transferases (HATs), which use acetyl-CoA to transfer an acetyl group to the substrate. The other type of enzymes is histone deacetyl-transferases, (HDACs) which reverse the aforementioned modification. The discovery that mammalian HDAC was a homolog of the yeast corepressor, RPD3 (Taunton et al., 1996) gave rise to the hypothesis that regulated activation events could involve the potential exchange of complexes containing HDAC function with those containing HAT activity.

Biochemical and cloning techniques have uncovered a large number of factors that interact with nuclear receptors in either a ligand-dependent or ligand-independent approach. Many of these factors have been shown to increase the activity of nuclear receptor activity in transient transfection assays, suggesting that they are used by nuclear receptors as coactivators of transcription. Many of these proteins function as components of large, multiprotein complexes but as the number of potential regulators exceeds by far the capacity to directly interact with a single receptor, it is logical to suggest that transcriptional

activation of nuclear receptors involves multiple factors that act in both a sequential and combinatorial manner to reorganize chromatin templates, modify and recruit basal factors and RNA polymerase II (Pollard and Peterson, 1998). HATs and HDACs include distinct families in which members are considered as regulators of transcription (Kouzarides, 2000; Caron et al., 2003; Yang, 2004; Sterner and Berger, 2000; Glozak et al., 2005) (Figure 15).

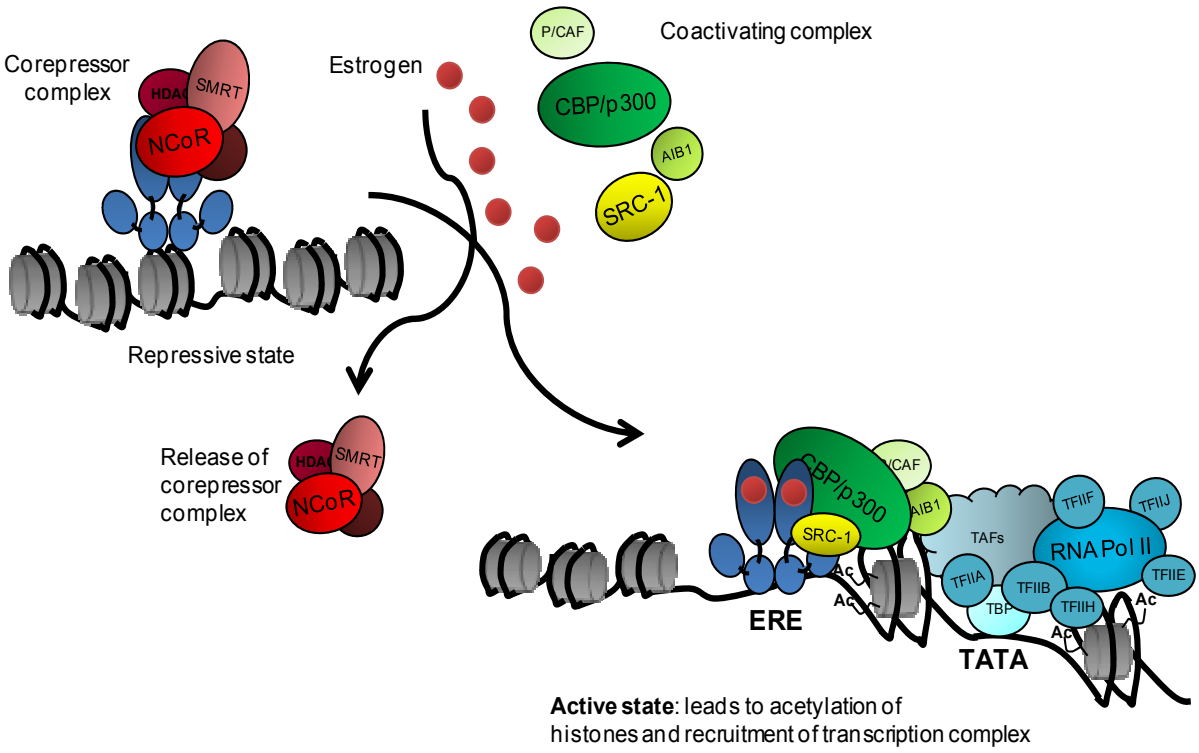

Figure 15 Mechanism of receptor activation through a change of coregulatory complexes in the presence of estrogen.

6.1 Coactivators

Coactivators are a specific group of chromatin-remodeling complexes that change the organization of nucleosomes in the vicinity of promoters, making the core elements of promoters accessible for binding to general transcription factors (Figure 15) (Naar et al., 2001; Lemon and Tjian, 2000; Taatjes et al., 2004). Several coactivators are able to bind to nuclear receptors however this section will focus on the more popular coactivators having a role on ERs transcriptional activity. Steroid receptor coactivator-1 (SRC-1), amplified in breast cancer1 (AIB1/ACTR/SRC-2), glucocorticoid receptor interacting protein 1/transcriptional intermediary factor 2/SRC-3 (GRIP1/TIF-2/SRC-3), p300/CBP and p300/CBP-associated factor (p/CAF) (Fu et al., 2002; Fu et al., 2003; Xu et al., 1999) are but a few members of coactivators known to regulate the activity of ERα and ERβ.

6.1.1 SRC-family

The SRC family of coactivators contains not only LXXLL motifs that allow for ER binding but also contain C-terminal activation domains (AD1 and AD2) and N-terminal basic-helix-loop-helix/PAS (bHLH/PAS) domains, which associate with factors involved in chromatin remodeling. Specifically, AD1 recruits the histone acetyltransferases p300 and Creb Binding Protein (CBP), while the AD2 interacts with protein arginine methyltranferases (PRMTs) such as coactivator-associated arginine methyltransferase 1 (CARM1) and PRMT1. The existence of these secondary complexes allows for amplification of ER responses: SRC-1 together with CBP and GRIP1 together with PRMTs were shown to function in a synergistic manner to potentiate the transcriptional activity of ER (Stallcup et al., 2000).

Both ERα and ERβ have a strong affinity preference for particular coactivators, which may be mediated through subtype specific utilization of different LXXLL motifs for their interaction with the SRC family of proteins (Li et al., 2003b; Wong et al., 2001).

Furthermore, GF stimulation enhances phosphorylation of the murine ERβ AF-1 and promotes SRC-1 binding and transcriptional activation (Tremblay et al., 1999b). SRC-1, GRIP1, and AIB1 are phosphorylated by MAPK, which enhances their activities (Smith and O'Malley, 2004). More elaborate control of coregulator function was demonstrated with the identification of six phosphorylation sites in AIB1 and different combinations of phosphorylation sites on AIB1 are required for mediating the activation of NF-κB compared to phosphorylation patterns on AIB1 required for oncogenic transformation of MEFs (Wu et al., 2004). High levels of SRC-1 in breast and uterus enhance the agonistic activity of tamoxifen (Shang and Brown, 2002). SRC-1 can also interact with CBP to activate ERβ-mediated transcription in a ligand-independent manner (Tremblay and Giguere, 2001). In tamoxifen treated women with breast cancer, high AIB1 expression, activated by ErbB2 signaling pathway counteracted the antagonistic effects of tamoxifen on ERs and was associated with a poor prognosis indicating the development of a tamoxifen resistant phenotype (Osborne et al., 2003). In fact, compared to SRC-1 and GRIP1, AIB1 is restricted to a few tissues including the uterus, mammary glands and the testis (Suen et al., 1998). Despite its restricted expression pattern, its disruption in mice severely affects growth and reproduction of mammary gland development (Xu et al., 2000). Crosstalk between ERα and ErbB2 has been shown to result in phosphorylation of SRC-3 thereby enhancing its coactivating capacity (Font de Mora and Brown, 2000).

6.1.2 CBP and p300

The coactivator p300 and its related ortholog CBP are transcriptional integrators regulating NR function. The relative abundance of both is considered rate-limiting in diverse signaling pathways involved in metabolism and cellular differentiation (Goodman and Smolik, 2000). The coordination of these activities involves a scaffold function of the protein to tether transcription factors to the basal transcription apparatus and both possess an intrinsic HAT activity which not only modifies local chromatin to alter the access of

transcription factors to their cognate DNA binding site but are able to directly acetylate transcription factors and thereby alter their function (Kalkhoven, 2004; Fu et al., 2004).

6.1.2.1 CBP and p300- Two of the same?

While CBP was isolated as a coactivator of the transcription factor CREB (Chrivia et al., 1993) and p300 was discovered as a protein interacting with the transforming adenoviral E1A protein (Eckner et al., 1994), both were shown to have similar functions (Arany et al., 1995; Lee et al., 1996). The recognition that both CBP and p300 acted as coactivators for CREB, AP-1 (Bannister and Kouzarides, 1995) as well as nuclear hormone receptors (Kamei et al., 1996; Chakravarti et al., 1996) initiated a large number of studies revealing CBP and p300 as essential coactivators for several other transcription factors (Vo and Goodman, 2001; Goodman and Smolik, 2000). Transient overexpression of both proteins resulted in interchangeable functions, however using hammerhead ribozymes to lower the expression of CBP or p300 indicated that although both proteins are necessary for apoptosis and G1 arrest of F9 embryocarcinoma cells, differentiation was dependent upon p300. Interestingly, Kawasaki *et al.* further observed that p300 specifically regulated the expression of the cell cycle inhibitor $p21^{Cip1}$, however expression of $p27^{Kip1}$ was regulated by CBP (Kawasaki et al., 1998) revealing how treatment of F9 cells with retinoic acid (RA), which normally decreases the rate of proliferation, did not significantly reduce proliferation when p300 or CBP-directed ribozymes were expressed. Similar methods also demonstrated that p300 was necessary for the cellular response following ionizing radiation in MCF-7 cells, not CBP (Yuan et al., 1999a; Yuan et al., 1999b), and CBP and p300 were unable to mutually complement each other during the differentiation of 3T3-L1 preadipocytes into mature adipocytes (Takahashi et al., 2002).

One of the major paradoxes in CBP and p300 activity is that these two proteins are able to contribute to completely different cellular processes. Their importance was elucidated by the group of Yao (Kung et al., 2000) who after performing null mutations in

one allele of CBP in mice, rodents developed a variety of hematological abnormalities, including extramedullary myelopoiesis and erythropoiesis, lymph node hyperplasia and splenomegaly which are conditions associated with bone marrow failure. As mice grew older they developed a high incidence of hematological malignancies which include histiocytic sarcomas, monomyelocytic leukemia and lymphocytic leukemia. In addition, patients who suffer from Rubinstein-Taybi syndrome (RTS), due to CBP heterozygosity, also have increased incidence of malignancy (Miller and Rubinstein, 1995). Surprisingly, the hematological defects and cancer predisposition were not observed in mice that contained the identical mutation in one p300 allele although its role as a tumour-suppressor cannot be dismissed as reports have shown that p300 missense mutations is associated with loss of heterozygosity in tumours of patients suffering from colorectal and gastric carcinomas (Gayther et al., 2000; Muraoka et al., 1996).

6.1.2.2 Functional domains

CBP is an evolutionarily highly conserved protein. The human CBP cDNA shares 89% homology at the DNA level with its murine homolog, while sharing 95% homology at the protein level (Giles et al., 1997). CBP shares 63% identity at the amino-acid level with p300 (Figure 16). Greater similarity is observed in specific regions such as the CREB binding site (KIX), the E1A binding site (C/H_3) and the bromodomain (Arany et al., 1995). Because they have very similar cellular functions, the literature has often referred to these two proteins as CBP/p300. The modular structure of p300 and CBP facilitates these diverse functions through distinct domains.

The bromo domain regulates protein-protein interactions and facilitates association with chromatin. Three cysteine histidine rich domains (CH) serve as docking modules for transcription factors and the glutamine rich carboxyl terminus interacts with the NR coactivators, including the steroid receptor coactivators. Located between the amino acids 1004 and 1044, just before the bromo domain (figure 16), is a region referred to as the CRD (cell cycle regulatory domain). This domain was named due to the fact that it was a target of $p21^{CIP1}$ activity. Recent studies identified the CRD1 domain as the key site of p300 sumoylation (Girdwood et al., 2003) which dampens its coactivating potential.

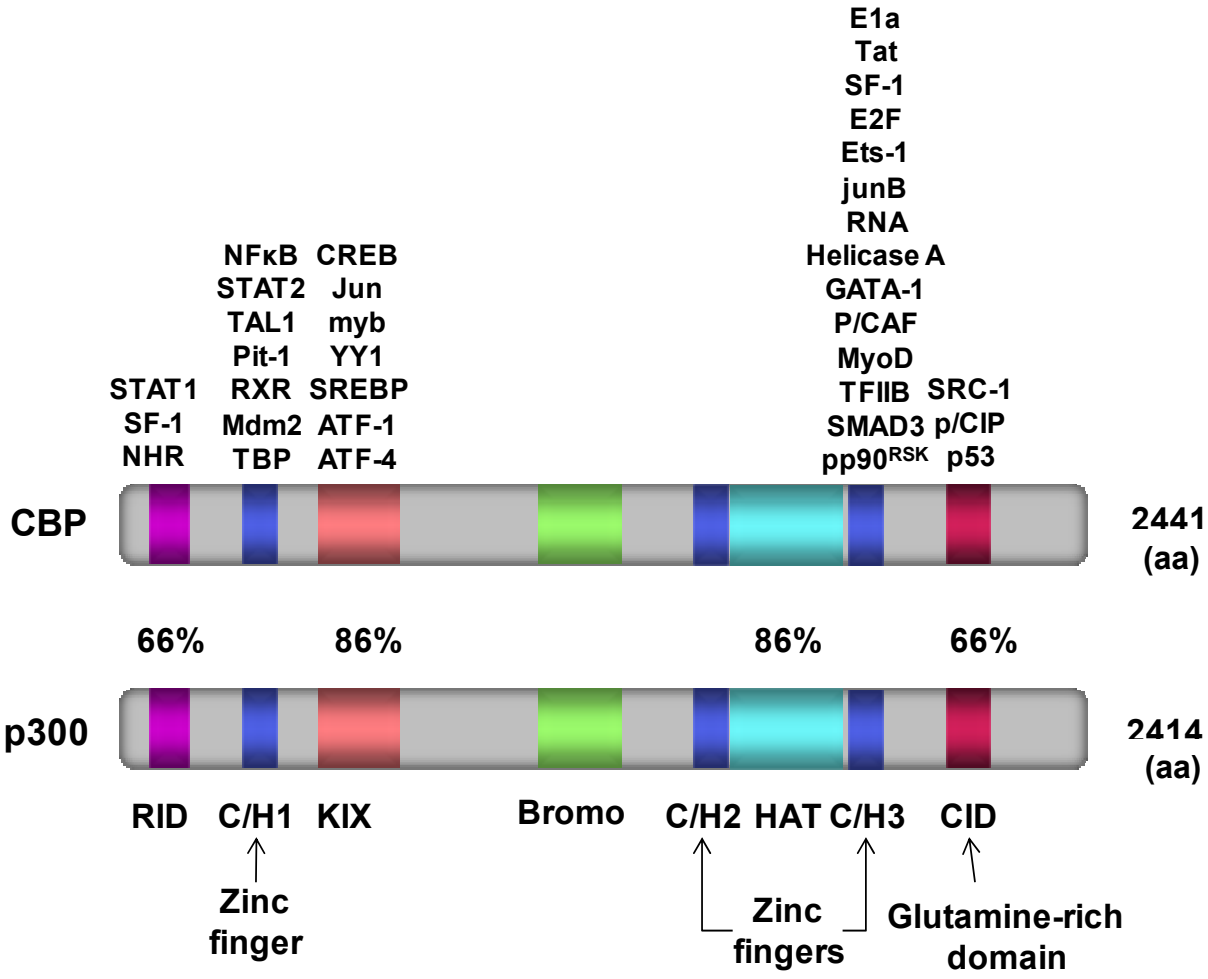

Figure 16 CBP and p300 functional domains. The different domains and the factors known to associate with CBP and p300 are labeled above CBP. aa: amino acids.

6.1.2.3 CBP/p300- impact on ER activity

ERα interacts functionally with p300 and CBP (Shibata et al., 1997) which are 'cointegrators' as they form complexes with TBP and a variety of activator proteins (McKenna et al., 1999). CBP can stimulate unliganded ERα and ERβ transcription on ERE promoters. The finding that CBP enhanced estrogen-induced ERα transcription only from some EREs is consistent with experiments showing that CBP shows weaker interaction with the ERα LBD than SRC-1 *in vitro* (Heery et al., 2001). In contrast to ERα findings, CBP enhanced estrogen-induced ERβ transcription from *pS2* and the distal *PR* (1148) EREs. Therefore, CBP interacts differently with ERα and ERβ depending on the ERE sequence as well as the cell type (Jaber et al., 2006). Acetylation of ERα within its hinge region was first described to occur in the presence of p300 which decreased its estrogen-dependent activity (Wang et al., 2001). However, in the absence of estrogen, a previous study did demonstrate that p300 cooperated with unliganded ERα to stimulate the transcription of the *pS2* promoter (Kraus and Kadonaga, 1998; Kobayashi et al., 2000a). In addition, only CBP and not the p160 family of coactivators was able to interact with ERα even in the presence of the pure receptor antagonist ICI 182,780 however transcriptional activation of target genes was not achieved (Jaber et al., 2006).

Most proteins involved in the control of cell growth are regulated by phosphorylation and CBP and p300 are not an exception to this modification. MAPK phosphorylates CBP (Janknecht and Nordheim, 1996; Liu et al., 1999) to maximize its intrinsic HAT activity (Ait-Si-Ali et al., 1999). Similarly cyclinE/cdk2 and the PI3-K/Akt pathway were able to phosphorylate CBP/p300 to also enhance its activity in order to encourage transcription of certain substrates and promote cell cycle progression (Sanchez et al., 2007; Ait-Si-Ali et al., 1998; Huang and Chen, 2005).

6.2 Corepressors

The fact that all known natural ligands of ERs are agonists suggests that the cellular role of NRs is to elevate transcription from target gene promoters; hence, the existence of coactivator proteins that amplify these responses is reasonable. Physiologically, however, there are fluctuating levels of circulating estrogens, and in tissues such as ovary, chronically high levels of estrogen could provide for sustained ER activation and overstimulation of ER biological pathways. Thus, as coactivators enhance ER activity, there has to exist pathways in which the activation is controlled by decreasing the transcriptional activity of nuclear receptors (Figure 15).

Corepressor proteins have been identified because they reduce the agonist effects of estrogens. A recently described corepressor of ERα and ERβ, termed repressor of estrogen receptor activity (REA), decreases ER activity by interfering with SRC-1 access to the receptor (Martini et al., 2000). RIP140, an LXXLL-motif containing protein, associates with the estrogen-bound ERs through the AF-2 domain, occluding access of AF-2 coactivators, such as SRC and CBP. In addition, RIP140 can decrease basal ER target gene expression by associating with histone deacetylase (HDAC) complexes, which repress transcription by catalyzing the condensation of chromatin (Smith and O'Malley, 2004). Interestingly, there is a direct repressive effect of RIP140 on ERβ activity which appeared to be stronger than on ERα. ERβ interacts with RIP140 more efficiently than ERα suggesting that RIP140 can differentially affect ERα and ERβ transactivation. Thus, the existence of corepressors that moderate the agonist activities of ERs provides an additional mechanism for fine-tuning the expression of ER target genes.

Corepressors function with unliganded nuclear receptors to silence gene expression. These corepressors include nuclear receptor corepressor (N-CoR), silencing mediator of retinoid and thyroid hormone receptor (SMRT), HDACs and BRCA1 (Fu et al., 2003; Xu

et al., 1999). It is also apparent that cell signaling pathways can facilitate coactivator-corepressor exchange, as cAMP stimulation of mammalian cells promotes dissociation of NCoR and SMRT from antagonist-bound PR, allowing for coactivator access (Wagner et al., 1998). Similarly, we found that SMRT inhibited basal transcription by ERα or ERβ, but SMRT inhibited estrogen-induced transcription for both ERα and ERβ, although the effect was more pronounced for ERα than ERβ. Notably, NCoR enhanced the anti-estrogen 4-hydroxytamoxifen inhibition of ERα activity, but not ERβ activity whereas SMRT increased 4-hydroxytamoxifen inhibition of ERβ, not ERα (Keeton and Brown, 2005).

Thus, estrogen and extracellular signaling pathways may enhance ERs action by coordinately enhancing the recruitment and activity of coactivators while decreasing the association and functionality of corepressors. Overall, posttranslational modification of cofactors appears to provide a mechanism to integrate extracellular signaling pathways, regulate assembly and dissociation of coregulators, and enhance or decrease the transcriptional efficacy of ER-cofactor complexes.

7 Other Estrogen Receptor modifications

In addition to phosphorylation and acetylation, other post-translational modifications have been identified influencing receptor function. Methylation of CBP by CARM1 is important for strong CBP coactivation of ER and GRIP1 complexes, and SUMOylation of SRC-1 or GRIP1 enhances coactivation functions by retaining these cofactors in the nucleus (Smith and O'Malley, 2004; McKenna and O'Malley, 2002). Acetylation and ubiquitination can decrease the half-life of not only ERs but cofactors involved in ERs transcriptional complexes by catalyzing their dissociation or degradation. The regulation of protein synthesis and degradation is a critical and highly regulated process.

7.1 The Ubiquitin-Proteasome pathway

Protein degradation is an important step in many cellular functions. Not only are misfolded and damaged proteins destroyed to avoid toxicity but concentrations of regulatory proteins are adjusted by degradation at the appropriate time. In eukaryotic cells, an ATP-dependent protease known as the proteasome is responsible for a big part of protein targeted degradation. Different proteasomal complexes exist in the cell (Finley, 2009); however the type that will be focused on is the 26S proteasome. This form is composed of a 20S core particle capped at one or both ends by a 19S regulatory particle (Groll et al., 1997; Tanaka, 2009). The 19S regulatory particle, containing ATPase subunits, guards the entrance to the degradation channel and plays a role in substrate recognition, unfolding and translocation onto the 20S particle (Tanaka, 2009; Finley, 2009).

In order to be targeted towards degradation, proteins must be attached covalently to a tag that consists of several copies of the small protein ubiquitin (Figure 17) (Thrower et al., 2000; Weissman, 2001). Ubiquitin is attached to proteins by a series of three enzymatic activities. Subsequently, these substrates can either bind directly to the 19S subunit of the proteasome by interacting with the non-ATPase Rpn10 (Deveraux et al., 1994) and Rpn13 (Husnjak et al., 2008) and/or the ATPase regulatory particle Rpt5 (Lam et al., 2002) otherwise ubiquitinated substrates can be brought to the proteasome by adaptors that bind both the proteasome and the ubiquitin chain on the substrate to deliver it for degradation (Elsasser et al., 2004; Kim et al., 2004; Verma et al., 2004a) however the complete picture has yet to be put together (Elsasser and Finley, 2005).

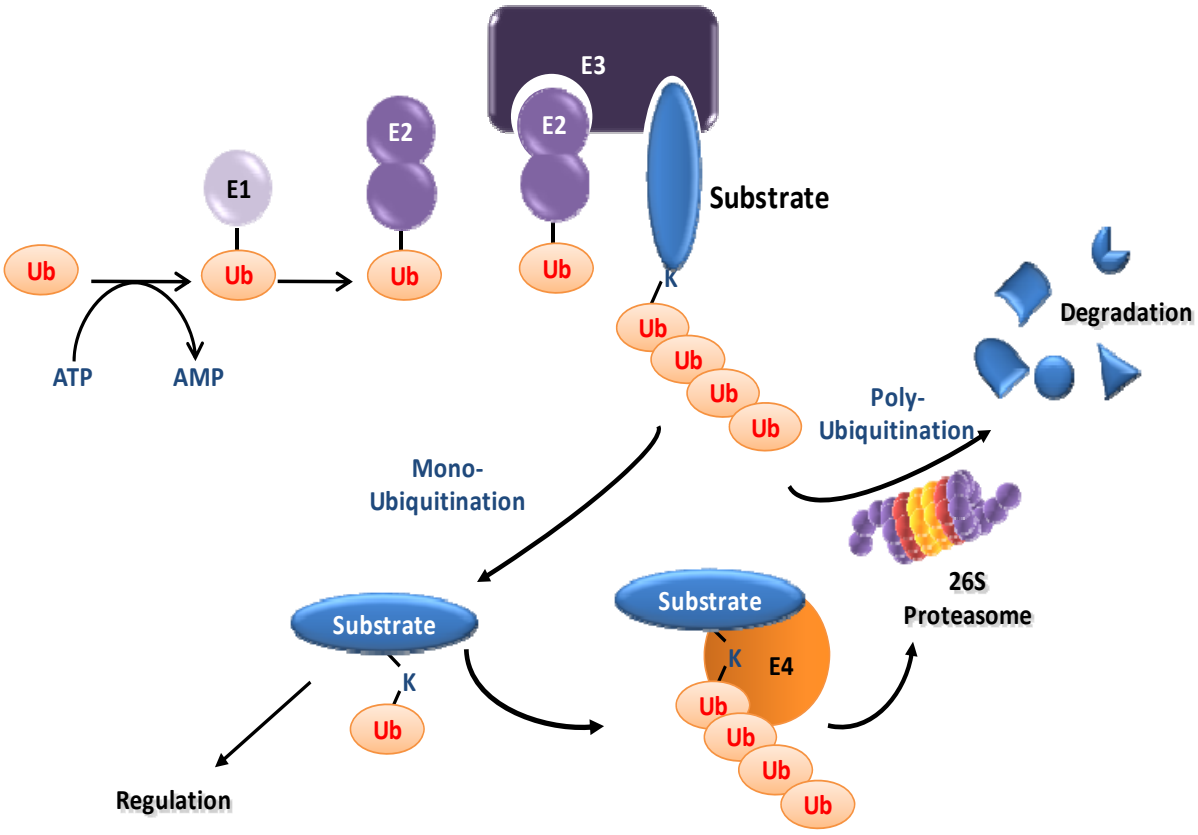

Figure 17 Ubiquitin-Proteasome Pathway.

Ubiquitin molecules are activated by ubiquitin activating enzymes (E1) using ATP and transferred to ubiquitin conjugating enzymes (E2). Subsequently, the ubiquitin is transferred to the substrate which is recognized by ubiquitin protein ligase enzymes (E3) (Weissman, 2001). The modification reaction does not stop after the addition of a single ubiquitin but continues so that an additional ubiquitin moiety is attached to a lysine of the first ubiquitin and so forth, generating a long ubiquitin chain formed on the substrate. Occasionally, an additional enzyme, called E4, is involved in this reaction (Hoppe, 2005).

The ubiquitin-proteasome pathway is an essential process for estrogen-dependant transcriptional activity of ERα and ERβ (Nawaz and O'Malley, 2004; Lonard et al., 2000; Nawaz et al., 1999; Reid et al., 2003; Picard et al., 2008; Tateishi et al., 2006). Studies have demonstrated that the requirement of the 26S proteasomal regulation of ERα and ERβ to maintain a continuous receptor turnover is essential in order to sustain or limit a hormonal response. In fact a component from the 26S proteasome subunit SUG1/TRIP1 interacts with several nuclear receptors in a ligand-dependant manner, including ERs (Masuyama and Hiramatsu, 2004; Tateishi et al., 2006; Zhang et al., 2006). In the presence of estrogen, ERβ interacts with SUG1 and the overexpression of SUG1 promotes the degradation of ERβ (Masuyama and Hiramatsu, 2004). Furthermore, the F domain of ERβ seems to protect the receptor from proteolysis by abrogating the binding of ERβ to the proteasome 26S through SUG1 (Tateishi et al., 2006).

7.1.1 E3-Ubiquitin Ligases

CHIP (Carboxyl Terminus of Hsc70-Interacting Protein) is a chaperone-dependent U-box E3 ubiquitin ligase (Figure 17) known to mediate the degradation of ERα and attenuates receptor-mediated gene transcription (Fan et al., 2005; Tateishi et al., 2004). Recently, CHIP was shown to interact with N-terminal region of ERβ (Tateishi et al., 2006). Even after ligand withdrawal, CHIP selectively eliminates the active form of ERβ providing evidence that receptor degradation is involved in transcriptional attenuation. The recruitment of another E3-ubiquitin ligase, E6-AP (Li et al., 2006), to ERα was dependent upon the phosphorylation of Ser-118 (Valley et al., 2005). Furthermore, E6-AP has been shown to participate in the regulation of the cellular levels of ERβ by degrading the receptor through the 26S proteasome (Picard et al., 2008). The recruitment of E6-AP on ERβ is also triggered by phosphorylation of AF-1 domain Ser-94 and Ser-106 (Figure 13). During activation of ERβ by MAPK, the recruitment and the action of E6-AP was

estrogen-independent and regulated by MAPK demonstrating the importance of activation-degradation cycling for the activity of the receptor.

Absence of the BRCA1 tumour suppressor gene highly predisposes women to develop breast and ovarian cancer (Perou et al., 2000; Sorlie et al., 2001). BRCA1 is implicated in a broad spectrum of biological processes including cell proliferation, cell cycle progression and DNA repair/recombination (Starita and Parvin, 2003; Parvin, 2004). BRCA1, together with BARD1 (BRCA1-associated RING domain), act as a ring E3 ubiquitin ligase (Hashizume et al., 2001; Wu et al., 2008). A recent study shows that this transcriptional regulation is processed by the non-classical pathway of ERs since BRCA1 promoter lacks EREs (Hockings et al., 2008). ERβ may play an important role in its regulation, since BRCA1 associated tumours show significantly higher expression of ERβ compared to ERα (Litwiniuk et al., 2008).

The human ubiquitin-conjugating enzyme 7 (UbcH7), which was found to play a role in nuclear receptor transactivation, is another interesting target for the regulation of ERβ by the proteasome pathway (Verma et al., 2004b). UbcH7 interacts with SRC-1 which is essential for the coactivation function of UbcH7. Furthermore, immunoprecipitation assay in MCF-7 and T-47D demonstrated a hormonal-dependant recruitment of UbcH7 on ERα promoter. Another estrogen-inducible RING E3 ligase is EFP (estrogen-responsive finger protein) (Chen et al., 2006). Upon estrogen treatment, EFP transcription is enhanced by both ERs and breast cancer cell lines positive for EFP protein simultaneously express either ERα or ERβ protein (Ikeda et al., 2004). In ovarian cancer tissues a strong correlation was found between EFP and ERβ.

7.1.1.1 MDM2

Mdm2 belongs to a large family of (really interesting gene) RING-finger-containing proteins, and as most of its members, Mdm2 functions an E3 ubiquitin ligase (Figure 17)

(Jackson et al., 2000) and targets various substrates for mono and/or poly ubiquitination affecting substrates compartment localization, and/or concentration levels by proteasome-dependent degradation. Mdm2 was identified as an amplified gene on a Murine Double-Minute chromosome, in a spontaneously transformed derivative of the BALB/c cell line 3T3DM, tumourigenic in nude mice (Fakharzadeh et al., 1991). The human homolog, Hdm2 also contains an oncogenic potential. The importance placed on Mdm2 over the past years is mostly due to its function as the major inhibitor of the tumour suppressor p53. Their interdependence was proved by the complete rescue of lethality of embryos lacking *mdm2* by elimination of p53 (Iwakuma and Lozano, 2003).

Additional functions for Mdm2 have been identified in cell cycle control, differentiation, cell fate determination, DNA repair, basal transcription and other biological processes which can contribute to its oncogenic potential (Ganguli and Wasylyk, 2003). Studies show that increased expression of Mdm2 is due to gene amplification, increased transcription, or enhanced translation (Oliner et al., 1992; Watanabe et al., 1994)., in fact, *Mdm2* amplification is seen in approximately 7% of all human tumours (Momand et al., 2000) In general, overexpression of Mdm2 is linked to a worse prognosis and a more advance stage of the disease (Cordon-Cardo, 2004). On the other hand, elevated levels of Mdm2 expression in ERα-positive breast carcinoma (40%) (Quesnel et al., 1994; McCann et al., 1995) is associated with a favourable prognosis (Onel and Cordon-Cardo, 2004; Cordon-Cardo, 2004).

Mdm2 is induced in response to growth and survival factors and mediates their signal into the nucleus. The expression of *mdm2* is also induced by IGF-1 treatment activation of the PDGF-β receptor (Fambrough et al., 1999; Leri et al., 1999). The PI3K/Akt survival pathway is pivotal in the cellular response to serum and IGF-1. Activation of this pathway induces association between Akt and Mdm2 with subsequent phosphorylation of serines 166, 186 and 188 (Meek and Knippschild, 2003; Milne et al.,

2004; Feng et al., 2004). Akt induces phosphorylation of Mdm2 which promotes its stabilization through the inhibition of its self-ubiquitination (Feng et al., 2004), which permits its association with p300 and lowering its interaction with p53 (Mayo and Donner, 2001; Zhou et al., 2001). This provides a mechanism where Akt can counteract p53 induced apoptosis.

Strict control of the steroid receptor superfamily signaling is paramount to the maintenance of regulated cell function. Susceptibility to Mdm2 is common to these receptors and represents a critical node of regulation (Sengupta and Wasylyk, 2004). From results generated on the *pS2* promoter (Metivier et al., 2004), Mdm2 when recruited to unliganded ERα at the endogenous ERE *pS2* promoter encourages a fruitless cycle with a fast turnover of about 20 min. The fate of the polyubiquitinated receptor is proteasomal destruction. However in the presence of estrogen, ERα binds the ERE which then interacts with RNA polymerase II followed by E6AP and then Rpt6. The turnover of ERα under these conditions is of 45 min which favours gene transcription. In addition, ERα also modulates the p53/Mdm2 regulatory loop. In the presence of ERα and p53, the ERα protects p53 from Mdm2 (Liu et al., 2000). In turn, the accumulated p53 inhibits the ERα directed transcription by preventing its binding to the ERE of its target genes (Liu et al., 2000). Interestingly, estrogen-bound ERα can be recruited to the *mdm2* promoter and enhance Mdm2 protein synthesis, suggesting that ERα can participate in the regulation of Mdm2 transcription (Kinyamu and Archer, 2003).

Overall, Mdm2 performs a vital role in mediating the regulation of hormone receptors such as ER, by controlling its stability and activity in response to levels of its ligand, which shows that continuous elevated levels of Mdm2 in cells responding to steroid hormones can lead to oncogenic transformation.

7.2 SUMOylation

Another post-translational modification is the attachment of Small Ubiquitin-like MOdifier (SUMO) proteins to target proteins. Although SUMO modification resembles that of ubiquitin, the consequences are distinct (Ulrich, 2005). SUMO proteins can alter the target localization, activity and stability mainly by modulating protein-protein interactions and DNA binding (Geiss-Friedlander and Melchior, 2007) and although this modification correlates mostly with the inhibition of transcription factors and/or their cofactors activity (Gill, 2005) it appears ERα is an exception (Sentis et al., 2005). Overexpression of SUMO-1 stimulates the activity of ERα due to its sumoylation within its hinge region. In addition, SUMOylation of SRC-1 stimulates its activity promoting furthermore the activity of ERα. Although the present work focused on ERα, it will be of interest to observe how SUMOylation affects ERβ transcriptional activity as diverse coregulator complexes modulate ERβ activity and are, for the majority, regulated by SUMO as well (Karamouzis et al., 2008).

7.3 Methylation

Coactivator-associated arginine methyltransferase 1 (CARM1) is one of the most studied methyltransferases implicated in nuclear receptor transcriptional regulation. CARM1 was initially identified as a partner of p160 coactivators (Chen et al., 1999), CARM1 is considered a secondary coactivator to ER only being able to function in the presence of p160 coactivators. Its importance has been shown in CARM1 null fibroblasts and embryos exhibiting aberrant expression of estrogen-responsive genes (Yadav et al., 2003). In addition CARM1 overexpression in breast cancer has been observed by the increase on cyclin E1 gene (El et al., 2006). In breast cancer cell lines MCF-7, addition of estrogen lead to the activation of *ps2* and *E2F* through the recruitment of CARM1 which methylated histones at the gene promoter (Bauer et al., 2002; Frietze et al., 2008).

7.4 Glycosylation

Another dynamic modification of nuclear and cytosolic proteins is Ser(Thr)-O-β-GlcNAcylation. O-GlcNAc and phosphorylation have similar cycling rates and generally occur on the same proteins. Several site mapping studies have also shown that O-GlcNAc and phosphorylation can be alternatively attached to the same serine or threonine residue within a protein (Zachara and Hart, 2006). The reciprocal site leads to different functional properties or activities of proteins, including the c-*myc* oncogene, RNA polymerase II, and ERβ (Cheng and Hart, 2001; Zachara and Hart, 2006).

The competitive interplay between these two modifications is important in nutrient/stress sensing, transcription, and signaling, and the balance between them on signaling proteins is key to normal cellular metabolism and functions. Prior work established that ERβ is alternately O-GlcNAcylated or phosphorylated at Ser-16 within the N-terminal of the protein (Cheng and Hart, 2001). O-GlcNAcylated renders ERβ less active in stimulating target gene expression, yet is more stable within the cell. Phosphorylation of Ser-16 renders ERβ more active in stimulating target gene expression, but paradoxically is rapidly degraded.

Overall, posttranslational modifications appear to provide a mechanism to integrate extracellular signaling pathways, regulate assembly and dissociation of complexes to enhance or decrease the transcriptional efficacy of ER-cofactor complexes.

7.5 Non-Genomic actions of ER

7.5.1 ERs at the membrane

The initial school of thought was that ERs were nuclear transcription factors which required the interaction with DNA in order to exert their effects. Nonetheless recent evidence shows that not only ERs but other nuclear receptors, such as AR and PR are able to exert extranuclear/non-genomic activity (Madak-Erdogan et al., 2008). The non-genomic actions of ERs are characterized by rapid responses that do not require DNA interaction by the receptors themselves, but are mainly regulated by ER-dependent activation or repression of intracellular signaling kinases (Figure 18) (Ordonez-Moran and Munoz, 2009). Extranuclear signaling of ERs has been shown to activate important signaling pathways including s-Src, PI3-Kinase/Akt and MAPK pathways which result in Ca^{2+} mobilization from the endoplasmic reticulum, induction of nitric oxide production, rearrangement of the cytoskeleton and proliferation all of which are dependent on cell-type specificity.

ERs do not contain a transmembrane domain nor an intrinsic kinase domain, therefore post-translational modifications such as myristoylation (Rai et al., 2005), palmitoylation (Galluzzo et al., 2007; Acconcia et al., 2005) and protein-protein interaction, are probably involved to recruit ERα and ERβ to the plasma membrane. Studies demonstrated the presence of ERα in lipid rafts through its interaction with calveolin-1 and palmitoylation on cysteine (C447) of ERα (Acconcia et al., 2005). Shc and IGF-IR were shown to play a role in the recruitment of ERα to the membrane observed by siRNA knockdown (Song et al., 2004) which was inducible by tamoxifen but inhibited through the treatment of fulvestrant (ICI) (Huynh and Pollak, 1993) or inhibitors of MAPK (Kahlert et al., 2000). In addition, ERα can also interact with ErbB2 directly protecting breast cancer cells from tamoxifen-induced apoptosis (Chung et al., 2002).

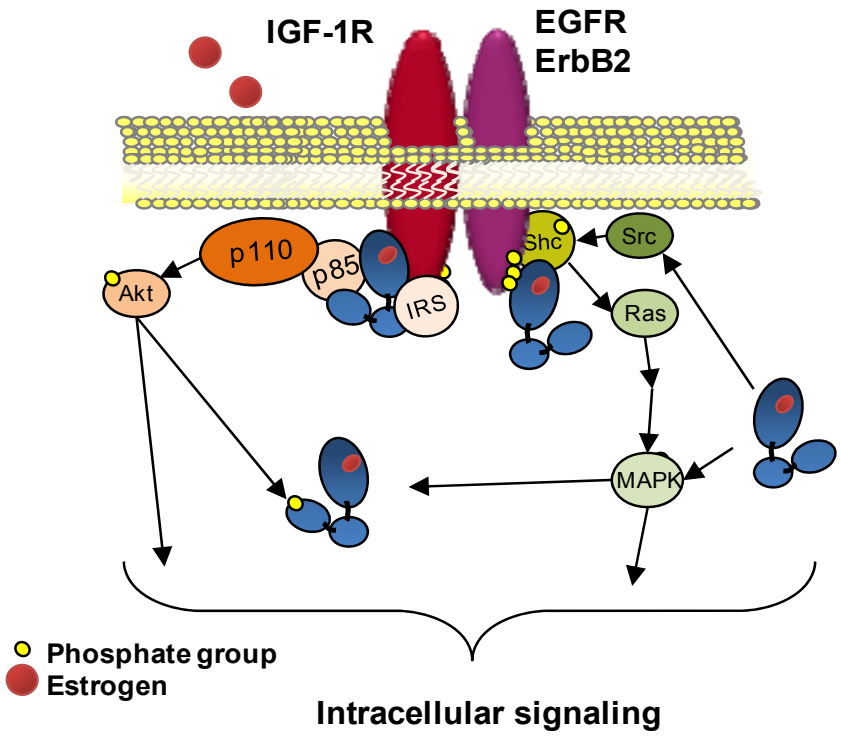

Figure 18 Non-genomic effects of ERs.

ERs can interact with several other signaling molecules such as c-Src (Wong et al., 2002), Shc (Song et al., 2002), and the p85α subunit of PI3K (Sun et al., 2001) leading to the activation of secondary signaling messengers and downstream kinase pathways, such as MAPK and Akt. These events have been well documented in the cardiovascular system where estrogen exerts rapid modulation of the vascular endothelium through nitric oxide production (Schlegel et al., 2001; Schlegel et al., 1999). Also, MCF-7 cells exhibit E_2-dependent activation of MAPK through the association of ERα with Shc, Src and Ras resulting in cell cycle progression (Migliaccio et al., 1996). Furthermore these kinases are then capable of activating nuclear ERs and coregulatory proteins thus promoting ER-dependent transcription. This bidirectional crosstalk between ERs and growth factors enhances the survival potential of breast cancer cells which can potentially create a greater resistance to single forms of molecular therapy.

7.5.2 GPR30

It wasn't until recently that another receptor responsive to estrogen, in the absence of ERα and ERβ (Filardo et al., 2000) led to the identification of a new member of the estrogen receptor family. Unlike its transcription factor counterparts, GPR30, also known as G protein-coupled estrogen receptor 1 (GPER1) is a seven transmembrane-domain G protein-coupled receptor (GPCR) considered until recently an orphan receptor. In 2005 two groups (Revankar et al., 2005; Thomas et al., 2005) showed that GPER1 could bind E_2 and activate intracellular signaling by coupling with Gs proteins, stimulating cAMP production and transactivate EGFR by cleaving heparin-bound EGF. Although a receptor agonist (G-1) has been developed for GPER1 which discriminates its actions from the classical ERs, several studies report that E_2 acts independently of GPER1, leaving its action as an ER not entirely accepted (Pedram et al., 2006) (Madak-Erdogan et al., 2008) (Otto et al., 2008; Ahola et al., 2002).

The cellular localization of GPER1 is ambiguous as reports place it within the endoplasmic reticulum (Revankar et al., 2005) and at the plasma membrane (Filardo et al., 2007; Funakoshi et al., 2006). The receptor is expressed in the uterus (Otto et al., 2009), ovaries (Owman et al., 1996) and mammary glands (Otto et al., 2009), specifically the theca and granulose cells (Wang et al., 2007). However GPER1 deficient mice did not exhibit any abnormalities in uterine function, reproduction or mammary gland (Otto et al., 2009) (Wang et al., 2008; Martensson et al., 2009; Isensee et al., 2009; Windahl et al., 2009) which in contrast to ERα and ERβ knockouts, demonstrate that GPER1 is not required for reproduction or for normal female reproductive organ physiology. Considering however the available biological and pharmacological data, E_2 is likely a physiological ligand but maybe not the main ligand and the establishment of a broader pharmacological spectrum may reveal other ligands as it also appears to be a target for estrogenic endocrine disruptors (Pandey et al., 2009).

8 Implications of ER action

8.1 ER subtypes in tumour progression

ER signaling is required for normal functioning and maturation of the mammary gland by promoting DNA synthesis, however aberrant signaling can lead to abnormal cellular proliferation, promoting the progression of breast cancer. ERα is the dominant isoform and correlates with most of the prognostic factors in breast cancer (Fuqua et al., 2003). Several studies report an increase ERα/ERβ ratio in breast cancer compared to benign tumors and normal tissues (Roger et al., 2001; Shaw et al., 2002), suggesting that ERα aberrant signaling is most likely involved in tumour development and progression while ERβ is likely to act as a tumour suppressor as proposed by studies reporting that overexpression of ERβ can inhibit ERα-positive breast cancer (Strom et al., 2004; Speirs et al., 2002; Paruthiyil et al., 2004; Williams et al., 2008; Speirs, 2002). However it would be unique among tumour suppressors to be expressed in over 75% of lesions (Shaw et al., 2002).

8.2 ERβ; Friend or foe

8.2.1 ERβ, potential oncogene?

Differential signaling between ERα and ERβ has been demonstrated with estradiol and tamoxifen at the AP-1 response element in ER target genes (Paech et al., 1997) and reviewed in chapter 5.5, suggesting that the ratio of ERα/ERβ may result in alternate gene regulation and could consequently be important in determining the response to ER modulators.

Estrogen metabolism drastically changes with menopause, with a marked decrease of serum estrogens resulting in a relative increase of androgens. In postmenopausal women,

estrogen-metabolizing enzymes aromatase, steroid sulfatase and 17β-HSD play important roles in the pathobiology of breast cancer (Honma et al., 2006; Vermeulen et al., 1986). Androstenediol, one of the major sex steroid hormones present in postmenopausal breast tissue (Szymczak et al., 1998; Vermeulen et al., 1986) exhibiting estrogenic function, is known to preferentially bind to ERβ over ERα (Kuiper et al., 1998). This suggests a relatively more important role for ERβ in the pathobiology of breast cancer in postmenopausal than premenopausal women. Early reports on ERβ mRNA expression in breast cancer described significant associations with the rate of tumour recurrence while on endocrine therapy (Speirs et al., 1999b; Speirs et al., 1999a); suggesting ERβ is a poor prognostic indicator. In addition, ERα negative/ERβ positive tumours have revealed a proliferative role for ERβ (Skliris et al., 2006) due to its correlation with proliferation markers Ki-67 and CK5/6, although these tumours represent 10-20% of diagnosed tumours of the breast (Murphy et al., 2003).

Two studies have examined the clinical importance of ERβ in tamoxifen-treated patients with breast cancer using the same monoclonal antibody (Nakopoulou et al., 2004; O'Neill et al., 2004). One of the studies reported better survival in women with ERβ-expressing tumours (Nakopoulou et al., 2004) compared to the second study which used a much higher concentration of ERβ antibody and a different cutoff value, revealing opposite results (O'Neill et al., 2004). Such reports have left the role of ERβ in tumour progression controversial. Few large studies have been performed analyzing ERβ protein expression in normal breast, early lesions and invasive cancers. Antibodies directed against the N-terminal domain of ERβ, detecting both full-length ERβ and various C-terminal truncated isoforms, (Fleming et al., 2004) found no correlation between ERβ expression and tumour grade, proliferation, S-phase fraction or DNA ploidy, while others found ERβ status to be a significant predictor of response to endocrine therapy (Myers et al., 2004; Mann et al., 2001).

8.2.2 **Tumour-suppressor activity?**

Although not all reports agree on how ERβ impacts cancer development, more studies are leaning towards the possibility that ERβ may function as a tumour suppressor, and that loss of ERβ could promote tumourigenesis. The balance between ERα and ERβ plays a role in the development of hormone-dependent cancers in various organs such as the ovary (Pujol et al., 1998), colon (Konstantinopoulos et al., 2003), and prostate (Horvath et al., 2001). Indeed reduction in ERβ protein expression and up-regulation of ERα (Bardin et al., 2004) have been associated with the development of invasive phenotype (Skliris et al., 2003). In the study by Skliris *et al.* antibodies directed against the C-terminal were used and examined the expression of ERβ in relation to established clinical parameters of breast cancer. Results demonstrated a positive association between ERβ protein expression and disease-free survival (DFS), which was further supported by a significant inverse relationship between ERβ and the proto-oncogene ErbB2 (chapter 5.2).

The finding of a positive influence of ERβ expression on the outcome of breast cancer patients treated with tamoxifen is supported by several reports where ERβ was detected by mRNA or IHC staining (Esslimani-Sahla et al., 2004; Gruvberger-Saal et al., 2007; Folgiero et al., 2008). Positive ERβ protein staining was invariably almost always associated with a favorable response to anti-estrogen treatment, consistent with its potential anti-proliferative and anti-invasive properties, also observed in ERβ-expressing cell lines (Lazennec et al., 2001). When only the studies examining ERβ protein expression are considered, there is a surprisingly high degree of similarity in terms of assigning frequency of expression (Skliris et al., 2008). Though there are different conclusions with respect to the correlations between ERβ and prognostic markers, two types of prognostic studies have been performed to date, those evaluating the levels of ERβ mRNA levels and studies that examined protein expression. The studies defining ERβ as a poor prognostic marker have examined its RNA level by quantitative or semi-quantitative PCR, however primers used

may amplify alternative spliced RNA variants, potentially increasing the false-positive rate or skewing the results towards a higher expression rate. Protein analyses however, appear to be less contradictory suggesting that ERβ protein expression is a favorable prognostic indicator although cut-off values to determine ERβ-positivity vary (Jensen et al., 2001; Miyoshi et al., 2001; Mann et al., 2001; Omoto et al., 2002; Omoto et al., 2001), thus a more uniformly adopted classification of ERβ expression is required to help clarify the potential role of ERβ in cancer progression. The use of ERβ protein expression levels as a tissue biomarker, in addition to protein expression levels of ERα, has the potential of more successful indication of therapeutic responses and the development of the disease in ER-positive tumours.

8.3 Tackling ER-dependent cancers

The initial therapeutic strategies of using selective estrogen receptor modulators (SERMs) such as tamoxifen, which inhibit the action of ERs, has proven to reduce the prevalence in early breast cancer and improve patient outcomes. Nonetheless resistance to all forms of endocrine therapy remains a major obstacle. Continuous work into ERs biology and the present progress of the field to understand resistance mechanisms, including molecular cross-talk between ERs and the various GF signaling pathways, and the coregulatory complexes involved, are generating greater options to offer better and specific treatments in order to target resistance and improve breast cancer outcome. Endocrine therapies can exert pressure on breast cancer cells pushing them to adapt to a new environment reflecting their distinctive plasticity. Under these circumstances, patients may have the misfortune of developing a resistance to anti-estrogen therapy, as observed with tamoxifen, due to developing hypersensitivity to the estrogenic properties of tamoxifen. In addition, upregulation of growth factor pathways involving ErbB2, IGF-IR and ErbB3 play an important part in the progression of this process (McMahon et al., 2005). Blockade of the downstream effects of the IGF-IR, ErbB3 and ErbB2 pathways would also be beneficial and allow continuing responsiveness to anti-estrogen treatments.

8.3.1 ER interacting compounds

8.3.1.1 Selective ERs modulators (SERMs)

Tamoxifen, raloxifene and toremifene are estrogen-like compounds harbouring agonistic and antagonistic properties known as selective estrogen receptor modifiers (SERM). SERMs compete for the same AF-2 binding site within the c-terminal domain as E_2 of both ERs. Binding of tamoxifen will lead to dimerization but will affect the positioning of helix 12 partially occluding the coactivator-binding sites as a mean to dampen the transcriptional activity of ERs and instead promote corepressors binding (Brzozowski et al., 1997; Shiau et al., 1998; Privalsky, 2004; Nettles and Greene, 2005). The agonistic properties of SERMs are carried out by the AF-1 domain of ERs (Smith et al., 1997; McDonnell, 1999). These effects are tissue-dependent relying on the cellular availability of coregulatory complexes (Smith and O'Malley, 2004; Jordan and O'Malley, 2007). Tamoxifen showed agonistic activity in endometrial cell lines where SRC-1 is highly expressed, but is antagonistic in mammary cell lines where the expression of SRC-1 is much lower (Shang and Brown, 2002). In addition on AP-1 and sp-1 regulated genes, where E_2 is an agonist to ERα and antagonist to ERβ, tamoxifen acts as an agonist for both receptor isoforms (Kushner et al., 2000; Paech et al., 1997; Webb et al., 1995; Saville et al., 2000; Zou et al., 1999).

In addition, elevated levels of ERβ are not involved in tamoxifen-stimulated growth of tamoxifen resistant tumours (Chen et al., 2005). The ratio of ERβ/ERα can alter the estrogen like properties of tamoxifen. In the absence of BRCA1, genistein (phytoestrogen) decreases the expression of ERα and increases ERβ, shifting the ratio in favour of ERβ, promoting tamoxifen's antagonistic activity. In cells with functional BRCA1, genistein increases the expression of ERβ, but does not change ERα level. Here again ERβ/ERα ratio increases making the cells more sensitive to tamoxifen. So genistein may render cells sensitive to antiestrogen tamoxifen irrespective of BRCA1 status (Thasni et al., 2008).

8.3.1.2 Selective ER down-regulators (SERDs)

Much like E2 and SERMs, SERDs such as fulvestrant (ICI 182780) bind the LBD of ERs but acts as a pure antagonist by inhibiting dimerization and DNA binding (Wakeling et al., 1991; Bowler et al., 1989; Pike et al., 2001; Fawell et al., 1990). In addition, fulvestrant affects ER localization and triggers ERα, not ERβ, degradation by the 26S proteasome by interacting with cytokeratins drawing the receptor close to nuclear-matrix associated proteasomes (Carlson, 2005) (Long and Nephew, 2006; Peekhaus et al., 2004).

8.3.1.3 Estrogen agonist-like compounds

Understanding how activated ERs elicit organ-specific effects is crucial in order to design pharmacological agents reproducing estrogenic effects in specific target tissues. As the endogenous agonist 17β-estradiol binds to both ER subtypes in tissues (Shughrue et al., 2002) two approaches have been envisioned to accomplish selective modulation of ER activity: SERMs and selective ligands capable of binding with higher affinity for either ER subtype. Since the discovery of ERβ in 1996, a major effort was undertaken to develop compounds acting specifically on either ER subtype.

Although several agonists exist for both types of ER subtypes, few have been studied *in vivo*. Selectivity of ER-specific agonists has been measured in *in vitro* screens with the use of transcriptional assays and competitive radioligand binding assays. The beauty of selective agonists is the fact that they represent a tool to assess the respective functions of ERα and ERβ. Comparative data on the selectivity for both types of ER-selective compounds on classic estrogenic signaling are, to date, consistent. However there are few studies on the ERβ agonist *in vivo* positive effects. Both academic and industrial groups have developed ERβ ligands and have been quite successful in generating high affinity selective templates due to its differential tissue distribution and selective

estrogenicity supported by the diverse and often opposing functional roles of ERβ compared to ERα, described in earlier chapters. Designing ER-specific compounds was achievable once the crystal structure of the LBD ERβ bound to genistein was first obtained. In combination with the earlier structure of ERα LBD, it was discovered that the polar interactions of both ERs were identical. Crucially their binding pockets vary by two amino acids M336/L384 and I373/MM421 producing topological and pocket size differences that must be the basis for subtype selectivity. In search for selective agonists, several non-steroidal families of compounds have been developed, inspired by the structure of genistein.

The first ERβ-selective agonist diarylpropionitril (DPN) was reported in 2001 (Meyers et al., 2001). It is a potent ERβ agonist with 30 to 70- fold selectivity over ERα (Mewshaw et al., 2005). On the other hand, Propylpyrazole (PPT) is approximately 400-fold more potent on ERα than on ERβ (Hillisch et al., 2004) (refer to Table 1). These synthetic ER agonists induce specific ER conformations exposing interaction surfaces for coregulator recruitment, which can differ from estrogen (Shughrue et al., 1998). Following the elaboration of DPN, the next synthetic peptides to appear were ER-041, WAY-202041, WAY-200070 and 8β-VE_2 in 2004 (Malamas et al., 2004) followed by WAY-202196 in 2005, (Mewshaw et al., 2005) (Table 1). Since 2007, phytoestrogenic compounds have also been added to the list including MF101, an extract from 22 different herbs (Cvoro et al., 2007) and liquiritigenin (LIQ) (Mersereau et al., 2008) isolated from individual plants that constitute the MF101 extract. These agonists have been well characterized *in vitro* however their biological activities have not been equally tested *in vivo.* Although DPN is the most commonly used agonist in rodent studies, comparative studies have shown that each compound produces distinct biological actions *in vivo.* DPN, ERβ-041, WAY-202196, WAY-20070 and MF101 alongside LIQ (Cvoro et al., 2007; Mersereau et al., 2008) do not increase significantly uterine weight at comparable doses, however 8β-E_2 increases uterine weight at a dose 100-fold lower than the other agonists (Harris, 2007). Other examples

include vasomotor instability (hot flashes) in rats, which can be regulated by DPN but not ERβ041 (Malamas et al., 2004). In addition, a randomized-placebo-controlled study in 2009 published that MF101 was able to also reduce hot flashes in postmenopausal women (Grady et al., 2009).

Table 1. Affinity Selectivity of ER-subtype agonists.

	RBA*	Fold Selectivity
ERα selective	**hERα**	**ERα**
PPT	49	410
16α-LE_2	57	250
ERβ selective	**hERβ**	**ERβ**
DPN	18	72
8β-VE_2	83	180
ERβ-041	72	225
WAY-202196	180	78
WAY-200070	133	68

*RBA: Relative Binding Affinity

These results suggest that ERβ selective agonists use different mechanisms to regulate gene expression. Additional studies have demonstrated that even though agonists could regulate a common subset of genes, each agonist could also uniquely regulated other genes, indicating that these uniquely activated genes might contribute to the differing biological effects observed *in vivo*. When three different cell lines were studied, Caco-2, Ishikawa and HeLa, all engineered to express ERβ, there was very little overlap in the genes regulated by MF101 and LIQ in each cell line, demonstrating remarkable cell-type specificity in the gene expression response. This strongly suggests that more comparative studies will be indispensable to evaluate these agonists for potential therapeutic use in

addition to the variety of molecular mechanisms used by ERβ to regulate transcription (Paruthiyil et al., 2009).

The treatment of menopausal symptoms and osteoporosis in postmenopausal women has largely relied on estrogens, however the Women's health Initiative (WHI) trial (Chlebowski et al., 2003; Manson et al., 2003; Rossouw et al., 2002; Shumaker et al., 2003; Wassertheil-Smoller et al., 2003) discovered that the risks outweighed the benefits of hormone therapy (HT). Therefore the discovery of estrogens-like compounds that can selectively regulate ERα and ERβ would be most beneficial. From what we currently know of ER subtype activity, selective estrogen towards ERβ could be more advantageous for HT as ERβ is generally thought to counteract ERα-dependent cell proliferation and tumour formation (Lazennec et al., 2001; Paruthiyil et al., 2004; Strom et al., 2004). The lack of proliferative effects of ERβ was demonstrated with ERβ041, which did not exhibit any proliferative effects on the mammary glands and uterus of rats (Harris et al., 2003a). In addition MF101 and LIQ were not able to stimulate uterine growth or breast cancer tumour formation in xenograft models (Cvoro et al., 2007; Mersereau et al., 2008).

Studies performed on cell lines revealed that ERβ-selective agonists activated genes that were not normally induced by estrogen (Paruthiyil et al., 2009). In addition the ligands would not always activate the same genes in different cell-types, which leads to believe that availability and differential recruitment of coactivators could explain the variations and also it is possible that the drugs are not metabolized equally in the different cell types. If the metabolites are active, this might account for the differences in the regulated genes.

8.3.2 Targeting alternative pathways to control ER

8.3.2.1 Aromatase Inhibitors (AI)

Blocking aromatase has the benefit of decreasing the levels of circulating estrogen, which is more relevant in post-menopausal women producing estrogen by peripheral aromatization of androgens. Two classes of AI are presently in use for women diagnosed with ER-positive breast cancer, a steroidal-based inhibitor, exemestane, which binds aromatase irreversibly, and non-steroidal-based inhibitor which block reversibly aromatase (Smith and Dowsett, 2003). Clinical trials with these inhibitors investigated three different treatment strategies; substitution of tamoxifen with AI; sequential treatment with tamoxifen followed and AI during the first five years after surgery; and extended adjuvant treatment using AI after five years of tamoxifen. Results from these studied showed that AIs were more effective than tamoxifen in preventing the recurrence of a tumour when used in substitution or sequential strategies (Howell et al., 2005; Baum et al., 2003; Baum et al., 2002; Jakesz et al., 2005; Thurlimann et al., 2005; Coombes et al., 2004). In addition, the tolerability of AIs is similar to tamoxifen, although adverse events with AI are more manageable as they prevent estrogen biosynthesis, and do act as inhibitors to estrogen receptors as opposed to tamoxifen which competes with estrogen for binding at the receptor acting as an inhibitor or an activator.

8.3.2.2 Signal transduction inhibitors (STIs)

These inhibitors can act at two levels; they delay significantly the development of anti-estrogen resistance and/or affect hormone-resistant cancers. In ER-positive breast cancer, the combination of tamoxifen or AI with farnesyltransferase inhibitors (FTI) was able to inhibit cell growth and promote apoptosis (Ellis et al., 2003). Monoclonal antibodies raised against the ErbB family work by either preventing ligand binding to the receptor or interfering with ligand-independent receptor signaling. Amplification of EGFR, although only observed in 0.8-6% of breast cancer cases (Bhargava et al., 2005) is blocked by gefitinib (anti-EGFR) which given together with tamoxifen or fulvestrant, the anti-proliferative effect and the trigger of apoptosis was greater in combination compared to either drug alone (Gee et al., 2003). ErbB2 is amplified in 20-30% of breast cancers and correlates with increased proliferation, higher metastatic potential and poor prognosis, as such several antibodies have been raised in order to inhibit its signaling potential (Ross and Fletcher, 1998; Slamon et al., 1987). The antibody lapatinib, a dual EGFR/HER2 inhibitor

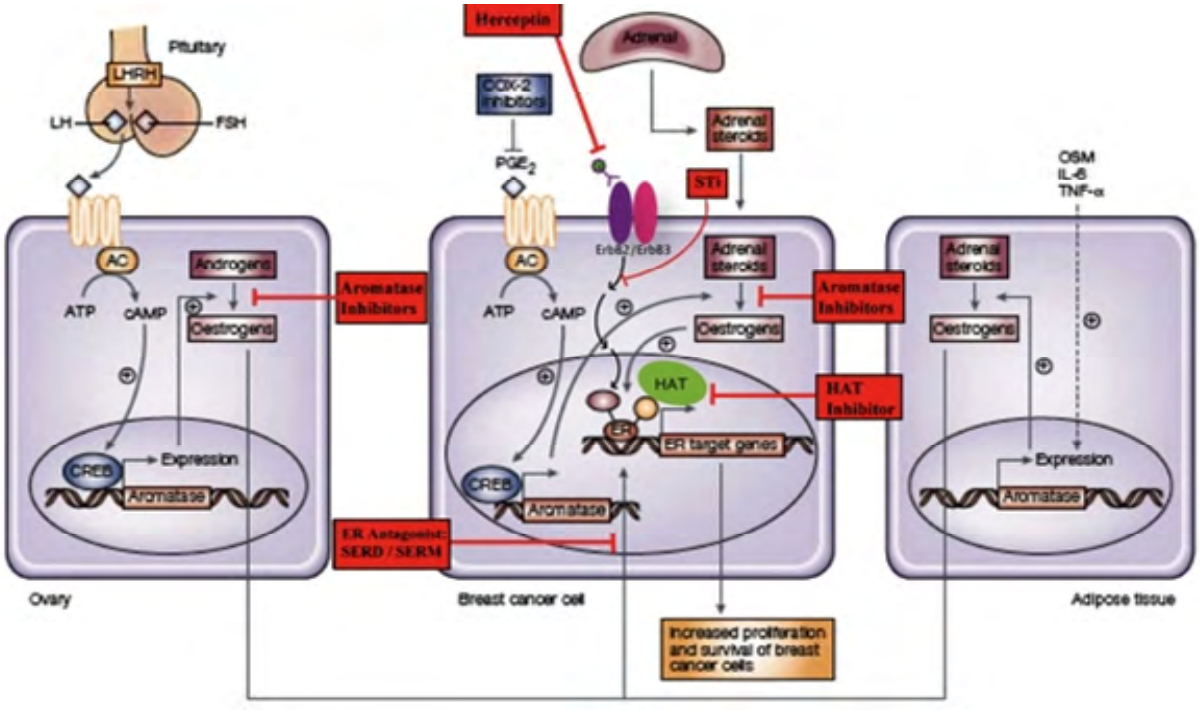

Figure 19 Mechanisms of action of therapeutic agents presently used to treat hormone-dependent cancers- Adapted from (Ali and Coombes, 2002).

collaborates with tamoxifen to reduce levels of cyclin D1, inhibit cyclin E-cdk2 and increase p27 kinase inhibitor in order to disrupt cell proliferation (Chu et al., 2005). Furthermore, ER-positive breast cancer xenograph models overexpressing ErbB2 treated with trastuzumab, pertuzumab (both anti-ErbB2) and gefitinib, in order to completely block all ErbB pairs, with tamoxifen, responded better than treating with a single antibody demonstrating the outstanding plasticity of cancer cells (Arpino et al., 2007).

8.3.2.3 Small-molecule HAT inhibitors

As mentioned previously, HATs can acetylate histones and non-histone proteins affecting enzymatic activity and protein-protein interactions. Bisubstrate inhibitors, described for PCAF and p300, are remarkably selective and bind to the enzymatic region of these two HAT proteins inhibiting their enzymatic activity (Lau et al., 2000). In addition, there is a selection of natural compounds; curcumin inhibits the acetylation of histone H3 and H4 specifically by p300/CBP since no effect was observed by PCAF, which also contains the functional HAT domain (Balasubramanyam et al., 2004). Indeed, curcumin addition to tumour cells promoted apoptosis. The natural product garcinol inhibits PCAF by also promoting apoptosis (Mantelingu et al., 2007). Anarcadic acid, which can inhibit both PCAF and p300 activity, targets the NF-κB activation pathway. Although the idea of targeting HATs that play a role in cancer progression could potentially be a promising strategy in combination with other therapies, considering the fact that HATs are part of large multiprotein complexes creates a challenge when trying to validate HATs as drug targets.

8.3.2.4 The proteasome as a drug target

Inhibitors of the 26S proteasome as drug candidates came from studies done in different leukemia and lymphoma derived cells, where apoptosis was induced (Imajoh-Ohmi et al., 1995; Orlowski et al., 1998). Most synthetic inhibitors are short peptides that

mimic substrates. One method utilized by proteasome inhibitors is to activate JNK-mediated apoptosis. Activation of JNK leads to the phosphorylation of 14-3-3 proteins, translocation of bax into the mitochondria and release of cytochrome c initiating the cascade leading to apoptosis (Lopes et al., 1997). Another inhibitor targets NEDD8-activating enzyme, which controls the activity of RING ubiquitin ligases that regulate the cell cycle and signal transduction pathways. Initial experiments have demonstrated that the inhibitor induces apoptotic cell death in different human tumour models (Soucy et al., 2009).

Knowing the specific molecular mechanisms or resistance adopted by hormone-dependent tumors is vital in order to define the optimal timing and sequence of treatment. The molecular characterization of the complex signaling networks active in hormone resistant cells will allow to further develop pharmaceutical compounds targeted at the various components of these pathways to hopefully overcome or delay the onset of resistance to endocrine therapy in breast cancer. Our current knowledge, although growing, is still insufficient to identify groups of patients that will benefit from the different endocrine agents, because by profiling individual tumors, we could predict the most appropriate endocrine therapy for each patient.

9 Hypothesis and Objectives

ERα and ERβ are important for the development, growth, and maintenance of the female and male reproductive systems yet aberrant regulation of these receptors is implicated in the initiation and progression of cancers. Similarly deregulated signaling by ErbBs has been associated with a strong mitogenic potential and correlation between ErbBs and ERα status has served as a predicting factor in the response to endocrine treatment. Nonetheless the response of ERβ to ErbBs signaling remains undefined.

The studies accomplished during the progression of my doctoral degree were developed in order to improve our comprehension of the mechanisms regulating hormone-independent activation of ERs, in particular ERβ, which its function (ERβ) as a definitive marker for either a tumour–suppressor or an oncogene remains unconvincing. Thereupon it was deemed crucial to investigate how the activity of ERα and ERβ was influenced following the activation of ErbB2/ErbB3 heterodimer and the consequent outcome on cellular proliferation.

The objectives of my doctoral studies were:

I) To determine the impact of ErbB2/ErbB3 activation on the transcriptional activity of ERs (1st article) by:

   a) Evaluating the effect of ErbB2/ErbB3 activation upon the transcriptional activity of ERβ in comparison to ERα.

   b) Establishing which regulatory pathways are elicited following the activation of ErbB2/ErbB3 and how they affect ERs.

c) Examining the implication of ERβ regulation of target genes following ErbB2/ErbB3.

II) To understand the mechanisms implicated in the transcriptional regulation of ERβ as a result of ErbB2/ErbB3 signaling (2nd article) by:

a) Determining the events which lead to transcriptional inhibition of ERβ.

b) Studying the implications of Ser-255 phosphorylation in the degradation of ERβ.

c) Evaluating the importance of CBP in the degradation of ERβ.

d) Examining the impact of ERβ degradation on the proliferation of breast cancer cell lines.

CHAPTER 2 : RESULTS

1 The Hormonal Response of Estrogen Receptor β is Decreased by the PI3K/Akt Pathway via a Phosphorylation-Dependent Release of CREB-Binding Protein

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Running title: CBP-mediated regulation of ERβ by Akt

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Foreword

The development of a hormone-independent status in reproductive cancers as observed for the breast has been generally attributed to a deregulated function of ErbB2 and ErbB3 receptor tyrosine kinases. In addition these tumors often express high levels of ERα for which its activity has been extensively studied. ErbB2/ErbB3 signaling has been shown to activate ERα by triggering pathways which promote its phosphorylation. Phosphorylation of ERα activates several pathways leading to cell proliferation and tumour progression. Despite the fact that ERβ is also expressed in several breast tumors, very few studies have examined the involvement of ERβ during the development of a hormone-independency by breast tumors. Phosphorylation of ERβ, by intracellular signaling pathways has been observed to increase its activity (Tremblay et al., 1999a). However recent evidence shows that ERβ is inhibited following the activation of ErbB2/ErbB3 by the activation of the MAPK p38 signaling pathway (St Laurent et al., 2005b). From these results, the following publication was developed in order to improve our knowledge of the mechanisms leading to the down-regulation of ERβ transcriptional activity. We discovered that the activation of the PI3K/Akt pathway lead to the downregulation of ERβ even in the presence of estrogen and the coactivator CBP. The repression was due to the phosphorylation of an Akt-consensus site within the hinge domain of ERβ, Ser-255. These results are shared by other nuclear receptors tested harboring an Akt consensus site within their hinge region, similar to ERβ. Unlike ERβ, ERα was activated under these conditions demonstrating a molecular mechanism by which the activation of the PI3K/Akt pathway can discriminate the activity of ERβ from other nuclear receptors.

Contributions of authors:

Mélanie Sanchez : As first author of this article I designed the experiments with Dr Tremblay and performed all of them. I also created the mutant for ERβ at Ser-255 and proceeded to clone the mutant into the vectors with tags used in the article. I also wrote the first draft of the manuscript and took care of the corrections that ensued.

Karine Sauvé: As second author, Karine was able to bring her expertise in ErbB signaling in critical discussions and participated in the correction of the manuscript.

Nathalie Picard: As third author, Nathalie also participated in active discussions on the topic and participated in the corrections of the manuscript.

André Tremblay: As supervisor, Dr Tremblay supervised and participated in the design of the experiments. He also corrected the article

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THE HORMONAL RESPONSE OF ESTROGEN RECEPTOR β IS DECREASED BY THE PI3K/AKT PATHWAY VIA A PHOSPHORYLATION-DEPENDENT RELEASE OF CREB-BINDING PROTEIN

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ABSTRACT

The hormonal response of estrogen receptors ERα and ERβ is controlled by a number of cofactors including the general transcriptional coactivator CREB-binding protein (CBP). Growing evidence suggests that specific kinase signaling events also modulate the formation and activity of the ER coactivation complex. Here we show that ERβ activity and target gene expression are decreased upon activation of ErbB2/ErbB3 receptors despite the presence of CBP. This inhibition of ERβ involved activation of the PI3K/Akt pathway, abrogating the potential of CBP to facilitate ERβ response to estrogen. Such reduced activity was associated with an impaired ability of ERβ to recruit CBP upon activation of Akt. Mutation of serine-255, an Akt consensus site contained in the hinge region of ERβ, prevented the release of CBP and rendered ERβ transcriptionally more responsive to CBP coactivation, suggesting that Ser-255 may serve as a regulatory site to restrain ERβ activity in Akt-activated cells. In contrast, we found that CBP intrinsic activity was increased by Akt through threonine-1872, a consensus site for Akt in the C/H3 domain of CBP, indicating that such enhanced transcriptional potential of CBP did not serve to activate ERβ. Interestingly, nuclear receptors sharing a conserved Akt consensus site with ERβ also exhibit a reduced ability to be coactivated by CBP, while others missing that site were able to benefit from the activation of CBP by Akt. These results therefore outline a regulatory

mechanism by which the PI3K/Akt pathway may discriminate nuclear receptor response through coactivator transcriptional competence.

INTRODUCTION-

Estrogen mediates many aspects in growth, development and reproduction, through its interaction with estrogen receptors ERα and ERβ1. While encoded by unique genes, the two ERs share the functional domains characteristic of the nuclear hormone receptor family (1). These consist of a N-terminal region (also termed AB region) which confers ligand-independent activation of ERs through its activation function AF-1, a highly conserved DNA-binding domain (C) that allows specific binding to genomic response elements, a flexible hinge region (D) that includes signals for nuclear localization and the binding of heat shock proteins, and finally a C-terminal region (EF) that contains the ligand binding domain, and the AF-2 function which mediates hormone-dependent activation.

Increasing evidence suggests that, beside hormonal activation, ER function can be modulated by phosphorylation-dependent mechanisms, involving a wide variety of protein kinases that mostly target the AF-1 domain (2,3). In particular, direct phosphorylation of ERα AF-1 by MAPK/Erk in response to EGF was shown to induce ERα transactivation in absence of ligand (4,5). Similarly, phosphorylation of Ser-167 by $pp90^{RSK1}$ was described to promote ERα AF-1 activity (6). Activation of phosphatidylinositol 3-Kinase (PI3K) and Akt/PKB (protein kinase B) also contributed to phosphorylate ERα and mediate its ligand-independent activation, an effect shown to oppose the tamoxifen-induced apoptosis in breast cancer cells (7). Although, phosphorylation of ERβ has not been examined in detail, ERβ has been proposed as a potential target for intracellular kinases that modulate its transactivation properties. It was found that the ability of EGF and oncogene ras to activate ERβ resulted from the MAPK-directed phosphorylation of Ser-106 and Ser-124 within the AF-1 domain leading to favored recruitment of coactivators SRC-1 and CBP (8,9). Furthermore, the ligand-dependent activation of ERβ by proto-

oncogen Brx was shown to involve phosphorylation of ERβ in a p38 dependent manner, although the exact site(s) were not described (10). More recently, we reported that activation of ErbB2 and ErbB3, which belong to the EGFR/ErbB receptor tyrosine kinase family, by growth factor heregulin resulted in a decrease in the estrogen-dependent cell growth and activity of ERα and ERβ in breast cancer cells (11). However, unlike ERα, this transcriptional repression of liganded ERβ by heregulin was dependent upon ERβ AF-1 function, thereby supporting a repressive role for kinase-mediated pathways in regulating ERβ AF-1 and AF-2 functions. Taken together, the regulation of estrogen receptor activity by phosphorylation is intricate and could dictate receptor function, whether it involves activation or repression.

Recent evidence has emerged that nuclear receptor coactivators may also serve as points of convergence between ER and growth factor signaling pathways. Phosphorylation of SRC coactivators has been described to modulate their intrinsic activities in mediating nuclear receptor transcription (12). Coregulatory proteins are often present in limiting concentrations in the nucleus so that modifications of their level of expression as well as their activity can lead to alterations in nuclear receptor signaling. The transcriptional coactivators CREB binding protein (CBP) and p300 are evolutionary highly conserved proteins and genetic evidence supports their availability to be critical. In humans, loss of one functional copy of *cbp* leads to Rubenstein-Taybi syndrome, a haploinsufficiency disorder resulting in mental retardation (13). Through their extremely versatile ability in bridging numerous transcription factors, including most nuclear receptors, with the basal transcription machinery, recruitment of CBP/p300 is important to maintain appropriate transcriptional events (14). One of the likely mechanism responsible for CBP/p300 recruitment involves phosphorylation. It was reported that phosphorylation of CBP promotes its interaction with several transcription factors, including CREB, Smad3, NFκB p65 subunit, and p53 (15,16). We have recently shown that MAPK-dependent phosphorylation of ERβ also facilitates the

recruitment of CBP to potentiate the ligand-independent activation of ERβ in response to growth factors (9). Given such diversity in the signaling pathways integrated by CBP, it is believed that phosphorylation-mediated events may compete at various levels for the limited availability of CBP.

Here we describe a molecular mechanism by which ErbB2/ErbB3 and PI3K/Akt signaling impairs the activity of ERβ by reducing its ability to recruit and use CBP as a coactivator. The repression by Akt was also found for other nuclear receptors, for which a conserved Akt site may also participate in a manner similar to ERβ. In contrast, nuclear receptors that do not share such homology yielded increased responsiveness to CBP and benefit from the enhanced intrinsic activity of CBP by Akt.

EXPERIMENTAL PROCEDURES

Plasmid Constructs - Expression pCMX plasmids coding for ERα, ERβ, CBP, ErbB2, its constitutive variant V659E and ErbB3 receptors, and luciferase reporter constructs vitA_2-ERE$_{tk}$Luc and UAS$_{tk}$Luc have been described previously (8,9,11). ERβ fragments corresponding to the AB (aa1-167) and DEF (aa234-549) regions were obtained by PCR amplification and fused in-frame with the Gal4 DNA binding domain. The ERβ serine-255 to alanine and the CBP threonine-1872 to alanine mutants were generated by PCR mutagenesis using pfu polymerase (Stratagene). All constructs were verified by automated sequencing. The expression plasmid coding for constitutively active PI3K p110α catalytic subunit was a kind gift from J. Downward, and plasmids expressing Akt and K179M kinase dead Akt were generously provided by T. Chan and P. Tsichlis.

Cell Culture, DNA Transfection and Luciferase Assay - Human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS). The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2. For transient

transfection, cells were seeded in phenol red-free DMEM supplemented with 5% charcoal dextran-treated FBS, and plasmid constructs were introduced into cells using the calcium phosphate precipitation method as described (11). Typically, 50-60% confluent cells were transfected with 2μg of DNA per well which include 500ng of reporter plasmid, 100ng receptor expression vector, 250ng CMX-βgal, 100ng each of PI3K and Akt expression vector, and 30ng CBP plasmid when indicated. After 5-8h, medium was changed and cells were stimulated with 10nM estradiol (E_2; Sigma) and/or 50ng/ml heregulin-β (R&D Systems) for 16-20h or left untreated. For luciferase assay, cells were lysed in potassium phosphate buffer containing 1% Triton X-100, and light emission was measured using a luminometer (Wallac) after the addition of luciferin. Luciferase assays are performed in duplicates from at least three independent experiments, and values are expressed as relative light units (RLU) normalized to the β-galactosidase activity of each sample.

Western Analysis and Immunoprecipitation Assay - Western analysis for the determination of phosphorylated and total Akt was performed as described with minor modifications (11). Briefly, transfected 293T cells were treated with 50ng/ml heregulin-β for 20min, washed in ice cold PBS, and lysed in PBS containing 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton-X100, 1mM sodium orthovanadate, 1mM sodium fluoride, 1mM phenylmethanesulfonylfluoride and protease inhibitors (Roche). Cell lysates were then subjected to SDS-PAGE and proteins transferred to nitrocellulose for immunoblotting. Membranes were incubated at 4°C with blocking reagent (Roche) in TBS, probed with either a rabbit polyclonal antibody against phosphorylated Akt (Santa Cruz) or a mouse anti-Akt monoclonal antibody (Cell Signaling Technology), and signals revealed by ECL using appropriate HRP-conjuguated secondary antibodies. The same procedure was used to determine the levels of ERβ, except that cells were transfected with HA-tagged ERβ (wt or

S255A) and analyzed by Western using an anti-HA antibody (12CA5). For immunoprecipitation assay, transfected cells were washed in ice cold PBS and lysed as described above. Cell lysates were precleared before incubation with an anti-CBP antibody (Santa Cruz) and protein A-Sepharose beads at 4°C. Immunoprecipitates were then washed in lysis buffer, resolved by SDS-PAGE, and analysed by western blotting using an anti-HA antibody. Membranes were also probed with an anti-CBP antibody for standardization of CBP levels in each well.

Generation of Hs-ER stable clones and RT-PCR – ER-negative Hs578t breast cancer cells were maintained in DMEM containg 10% FBS, and transfected with expression vectors for ERα and ERβ as previously described (11), and resistant clones were isolated in the presence of G418 (0.6 mg/ml; Invitrogen) to generate respectively Hs-ERα and Hs-ERβ cell lines. Stable clones were functionally validated for their respective expression of ERα or ERβ by Western analysis and for their estrogenic response by luciferase assay, compared to mock-transfected Hs-578t cells. Total RNA was isolated from cells using TRIzol reagent (Invitrogen) and RT-PCR analysis was performed as described (17). The relative signal intensity was analyzed (Alpha Innotech, San Leandro, CA) from three separate experiments.

In Vitro Phosphorylation Assay - Bacterially expressed and purified GST fusions of wild type and S255A mutated ERβ were prepared as described (18). For *in vitro* phosphorylation assay, GST-ERβ fusions immobilized on glutathione-Sepharose 4B beads were resuspended in kinase buffer containing [γ-^{32}P]ATP (Amersham Biosciences) and active Akt1 (Cell Signaling), and incubated at 30°C for 30 min according to the manufacturer instructions. Beads were then washed twice in kinase buffer and twice in PBS, and ^{32}P incorporation was determined following SDS-PAGE and autoradiography. Gels were stained with Coomassie blue to monitor for equal loading.

Fluorescence Microscopy - Cells were seeded on coverslips in a six-well plate overnight prior to transfection in phenol red-free DMEM supplemented with 5% charcoal dextran-treated FBS. Transient transfections were carried as above using the expression plasmids YFP-CBP and CFP-ERβ. 20h after transfection, cells were washed twice with cold PBS and fixed in 4% formaldehyde. The coverslips were mounted on microscope slides and examined in fluorescence with excitation/emission filters of 435/470 nm (for CFP), and 480/535 nm (for YFP), using a Nikon TE-2000 inverted microscope.

RESULTS

ErbB2/ErbB3 receptor dimer activation impairs the hormonal response and coactivation of ERβ by CBP - Activation of the epidermal growth factor receptor EGFR/ErbB1, a member of the ErbB receptor tyrosine kinase family, is well recognized to promote ERα and ERβ transcriptional activation (4,8). However, we have recently reported that activation of the ErbB2/ErbB3 heterodimer combination lead to a decreased transcriptional activity of ERβ (11). Given the ability of CBP to associate and promote the activation of ERβ by growth factor such as EGF (9), we addressed how CBP could modulate ERβ activity in response to ErbB2/ErbB3 activation. ER-negative human embryonic kidney 293T cells were transfected with an EREtkLuc reporter and an ERβ plasmid. Cotransfection with CBP conferred a 2-fold increase in ERβ basal activity, and a 7-fold increase in the presence of hormone (Fig.1A). As previously reported (11), the activation of the ErbB2/ErbB3 heterodimer by growth factor heregulin-β, which binds ErbB3, resulted in a reduced activation of ERβ by estrogen. Such impaired response was also mimicked using a constitutive variant of human ErbB2 (V659E), which corresponds to the natural mutation found in the rat Neu oncogene (19). However, while CBP strongly transactivates ERβ in control cells, it is unable to prevent the inhibition

of the hormonal response of ERβ when the ErbB2/ErbB3 heterodimer is not only expressed but also stimulated by heregulin-β (Fig.1A). Despite the presence of CBP, the transcriptional activity of ERβ was decreased in both a hormone-independent and dependent manner. This inhibition was even more pronounced in cells expressing the constitutive ErbB2 V659E mutant.

Signaling of the EGFR/ErbB family members involves the activation of a variety of kinase pathways. More specifically, activation of the ErbB2/ErbB3 heterodimer has been shown to efficiently couple with the PI3K/Akt pathway, mainly through the intrinsic ability of numerous SH2 binding motifs within ErbB3 that recognize the p85 regulatory subunit of PI3K (20,21). In order to evaluate the impact of ErbB2/ErbB3 activation on the Akt pathway, the activity of endogenous Akt was determined by Western analysis using a phospho-specific antibody against Ser-473. While treatment of mock-transfected 293T cells with heregulin-β did not lead to activation of Akt, indicating that endogenous expression of ErbB3 is negligible, if not absent, an increase in phosphorylated Akt was observed in cells expressing ErbB2/ErbB3 and treated with heregulin-β (Fig.1B). Similarly, cells expressing the ErbB2 V659E variant in presence of ErbB3 also showed increased levels of phosphorylated Akt.

Activation of the PI3K/Akt pathway mimics the inhibition of ERβ response to hormone in the presence of CBP through the C-terminal region of ERβ - The possibility that ErbB2/ErbB3 activation by heregulin-β decreases ERβ activity and its coactivation by CBP by enhancing the activity of Akt was further tested by transient expression of a membrane bound and constitutively active p110α subunit (CAAX) of PI3K. Expression of the p110α mutant was sufficient to activate endogenous Akt in 293T cells, which was further enhanced when cells were cotransfected with a plasmid for wt Akt, as determined by Western blot analysis (22, data not shown). Under these conditions, we found that the estrogen-dependent activation of

ERβ in the presence of CBP, which reached almost 12-fold compared to untreated cells, was strongly impaired dropping to a 3-fold response in Akt-activated cells (Fig.2A). A previously observed in ErbB2 V659E-expressing cells in response to Akt activation (Fig.1A), addition of CBP reduces further the response of ERβ to estrogen when compared to cells without exogenous CBP. These results suggest that ectopic expression of CBP could not relieve the inhibition of ERβ by the PI3K/Akt pathway, therefore mimicking the results in ErbB2/ErbB3-expressing cells. The repression of ERβ by activated Akt in the presence of CBP was partially relieved in cells expressing a dominant negative form of Akt (K179M), suggesting that the effects of activated PI3K on ERβ mainly transit through Akt (Fig.2A). We next performed Western analysis to ascertain whether the modulation of ERβ activity was not a direct effect of its protein concentration under the conditions used. As shown in Fig.2B, activation of the Akt pathway lead to an accumulation of ERβ in untreated cells. A similar increase was also observed in the presence of estrogen, although the levels of ERβ were slightly lower compared to untreated cells, probably reflecting an increase in ER turnover in response to hormone as previously reported (23). These results suggest that the inhibition in ERβ activity to Akt activation is not related to a decrease in ERβ protein levels.

CBP is known to transactivate estrogen receptors through both its AF-1 and AF-2 activities (9,24). In an attempt to identify the functional domain within ERβ responsible for its impaired ability to be coactivated by CBP in response to Akt, we used Gal4 fusions of truncated forms of ERβ for which each respective AF-containing domain has been removed. Fig. 2C shows that in Akt-activated cells, the activation of a Gal4-ABβ (corresponding to ERβ aa1-167) on a UAStkLuc reporter was further enhanced by CBP, reaching a near 5-fold increase compared to control cells. The N-terminal domain of ERβ is known to contain several serine residues which are conserved within recognition motifs for Ser/Thr kinases of the MAPK family, and phosphorylation of specific

residues was shown to allow for coactivators such as CBP to be recruited and potentiate ERβ AF-1 activity (8,9). However, none of the potential phosphorylation sites within ERβ AB region belongs to a consensus Akt site, suggesting that the enhanced activity of ERβ AF-1 by CBP in response to Akt might possibly result from other kinase pathways activated by Akt or direct effects on CBP itself. We next tested the role of the C-terminal region of ERβ in the same conditions. Cells transfected with a Gal4-DEFβ (aa234-549) showed a reduced hormone-dependent activity to Akt activation in the presence of CBP, mimicking the response observed with full length ERβ (Fig. 2C, *right panel*). These results indicate that the repressive effect of activated Akt on CBP-mediated transactivation of ERβ is mediated through a region contained in the C-terminal portion of ERβ, which in the context of the full length receptor, seems to counteract the positive effect on the AF-1 activity.

Serine-255 in the hinge region mediates ERβ inhibition to ErbB2/ErbB3 signaling - Our examination of the C-terminal sequence of mouse ERβ revealed a consensus sequence RQRSAS^{255} in the hinge region of ERβ that corresponds to the recognition motif RxRxx(S/T) for the kinase Akt (Fig. 3A). To determine whether Ser-255 is a direct target for Akt-mediated phosphorylation, we used site-directed mutagenesis to convert the serine at position 255 into an alanine, and performed an in vitro kinase assay. Fig. 3B shows that disruption of Ser-255 strongly abolished the phosphorylation of ERβ by Akt compared to wild type, indicating that Ser-255 can be efficiently phosphorylated by Akt. We then tested whether Ser-255 was involved in the inhibition of ERβ activity to ErbB2/ErbB3 activation as observed in Fig.1A. Using the S255A mutant in luciferase assay, we found that the inhibition observed for wt ERβ by either ErbB2/ErbB3 dimer expression or its activation with heregulin-β, was completely abrogated by disruption of Ser-255 (compare Figs.1A and 3C). Noticeably, the hormonal response of

S255A was enhanced upon ErbB2/ErbB3 activation, and further potentiated by CBP. This enhanced response to hormone by the S255A mutant was also observed in response to Akt activation using the constitutively active p110α PI3K construct in transfection (Fig. 3C). Therefore, the results indicate the hinge region of ERβ contains a specific site that not only can be targeted by Akt but also dictates responsiveness of ERβ to CBP coactivation in response to Akt signaling pathway. In order to determine whether Ser-255 is involved in the regulation of ERβ in terms of protein levels, we next performed Western analysis on cells expressing the ERβ S255A mutant. As compared to wild type ERβ (Fig. 2B), the disruption of Ser-255 completely abrogated the accumulation of ERβ in response to Akt activation (Fig. 3D), indicating that Ser-255 is a critical site in the regulation of ERβ levels by the PI3K/Akt pathway.

ERβ modulates the intranuclear behaviour of CBP in an Akt-dependent manner through Serine 255 - Studies using fluorescent tagged proteins have demonstrated that expression of estrogen receptor, in particular ERα, affects the intranuclear organization of coactivators of the SRC/p160 family in response to hormone or antiestrogens (25-27). Based on our results on the transcriptional response of ERβ to CBP in Akt activated cells, we investigated whether ERβ could modulate the intranuclear behaviour of CBP in response to Akt activation. Expression plasmids encoding a YFP-tagged full length CBP and a CFP-fused ERβ were generated and functionally validated in luciferase coactivation assay (data not shown). We first determined the intranuclear distribution of CBP by transfecting cells with YFP-CBP in absence of ERβ. In these conditions, CBP mainly localized into discrete clustered nuclear regions or foci, with a subpopulation showing a more diffuse pattern throughout the nucleus (Fig.4A). This particular behaviour of CBP has been observed in different cell types under basal or non activated conditions, and although not fully characterized, such pattern was associated to poorly transcribing or transcriptional inactive compartments

devoid of nascent mRNA transcription and active RNA polymerase II (28-30). Given our results on the effects of CBP on ERβ activity, we tested whether ERβ could modulate the intranuclear distribution of CBP by cotransfecting cells with YFP-CBP and CFP-ERβ. Both proteins were shown to colocalize to the nucleus but the ectopic expression of ERβ strongly diminished the formation of CBP-related speckles, resulting in a more dispersed distribution of CBP throughout the nucleus (Fig.4A). Interestingly, when the Akt pathway was activated in cells expressing both YFP-CBP and CFP-ERβ, CBP appeared to readopt the formation into speckles, while the dispersion of ERβ remained unaffected, indicating that Akt can induce a relocalization of CBP within the nucleus in the presence of ERβ (Fig.4A). Given the role of ERβ Ser-255 to impair CBP-mediated coactivation of ERβ in response to Akt, we next tested a CFP-ERβ S255A construct on CBP intranuclear distribution. We observed that as opposed to wt ERβ, expression of the S255A mutant did not favor CBP to fully reform into speckles, but instead CBP remained in a more diffuse pattern (Fig.4A). This distinct behaviour of CBP in response to wt vs S255A ERβ expression was also observed in the presence of hormone (data not shown), indicating that both unliganded and liganded receptor affect in a similar manner CBP nuclear distribution to Akt activation. These results suggest that CBP relocalizes into the nucleus in response to Akt activation and that this behaviour depends on the presence of ERβ in a manner specific of Ser-255.

Cellular activation of Akt releases CBP from ERβ through Serine-255 - The observation that CBP could relocalize within the nucleus in a manner dependent of ERβ, and that Ser-255 seems to modulate that behaviour in response to Akt activation, prompted us to determine the effect of activation of the PI3K/Akt pathway on the interaction of ERβ with coactivator CBP. We found that under basal conditions CBP potently coimmunoprecipitated with ERβ and that this interaction was further stabilized in the presence of estradiol (Fig.4B), thus correlating with the enhanced activation of

ERβ by hormone and CBP (Figs.1A and 2A). However, such interaction was strongly disrupted in Akt-activated cells independently on the presence of hormone (Fig.4B). We then tested the S255A mutant using similar conditions and found that, as opposed to wt ERβ, CBP could efficiently immunoprecipitate the mutant ERβ in absence or in presence of estradiol despite activation of Akt in cells (Fig.4B). These results therefore provide a role for ERβ Ser-255 to induce a release of CBP from ERβ in response to Akt activation.

Akt promotes the intrinsic transcriptional activity of CBP through Thr-1872 - CBP has been described as being targeted by several kinase signaling pathways, of which for example cyclin dependent kinase or MAPK/Erk-directed phosphorylation of CBP lead to upregulate its histone acetyltransferase activity, and therefore its intrinsic potential to activate transcription (31). In order to determine how CBP could affect transcription by ERβ in response to Akt, we generated a Gal4 fusion of full length CBP which, by interacting onto a UAStkLuc reporter, allows to monitor directly CBP transcriptional activity in a luciferase assay. Cells transfected with Gal4-CBP showed a 4-fold activation in luciferase activity compared to cells expressing an empty Gal4 plasmid (Fig.5A), indicating that CBP was able to promote transcription under these conditions. A further increase in CBP activity, reaching 8-fold activation compared to control, was observed upon expression of constitutive p110α PI3K and Akt in cells, suggesting that CBP intrinsic ability to promote transcription can be enhanced by Akt. By looking at the sequence of CBP, only one putative site (Thr-1872) is contained within the consensus motif for Akt. Interestingly, this site is homolog to Ser-1834 of p300 which was recently described as a target of Akt that promotes p300 activity (32). We therefore substituted Thr-1872 by an alanine residue and tested a Gal4-CBP T1872A mutant for its activity. We found that not only was the response to Akt activation completely abrogated by the mutation, but the basal activity was also severely impaired (Fig.5A), indicating that Thr-1872 is a crucial regulatory site for

CBP activity. The T1872A mutation did not significantly affect the steady state levels of CBP expressed in cells and Akt activation did not modulate wild type or mutated CBP levels as shown by Western analysis (Fig.5B). Given the ability of CBP to respond to Akt through Thr-1872, we next tested whether this site was involved in the response of ERβ and of ERα to Akt. We found that, although the CBP T1872A mutant was less efficient in promoting ERβ response to estrogen, it behaves similarly as wt CBP in the inhibition of ERβ by Akt, indicating that these effects were independent of CBP Thr-1872 (Fig.5C). However, the activation of ERβ S255A by Akt in the presence of wt CBP was lost when CBP T1872A mutant was expressed in cells. Similar results were obtained with ERα (Fig.5C), suggesting that in contrast to ERβ, ERα seems to benefit from the enhanced activity of CBP to Akt in a manner dependent of Thr-1872.

Estrogen-responsive genes are regulated differently by heregulin in ER-expressing stable clones – Based on our results on the apparent difference between ERα and ERβ response to CBP when Akt is activated and to delineate each ER contribution, we generated ERα and ERβ-expressing stable clones using ER-negative Hs578t breast cancer cells. Hs578t cells are an appropriate model to study the effect of Akt since they exhibit high basal Akt activity through ErbB receptor signaling and mutated active ras (33,34). In addition, Akt can be further activated by heregulin-β in each Hs-ER stable clone in a time-dependent fashion (Fig.6A), indicating that these cells maintain the ability to respond to heregulin-β (35). Stable expression of ERα or ERβ also confers enhanced estrogen-dependent activation of Akt compared to negative cells (Fig.6A). Using RT-PCR analysis on Cathepsin D1 (CatD1) and progesterone receptor (PR), two recognized estrogen-responsive genes, we found their expression were enhanced by estradiol in both ER stable clones, compared to negative control cells (Fig.6B). However, these increases were severely impaired by the addition of heregulin-β to Hs-ERβ cells, therefore correlating with results obtained in luciferase assays. In contrast, treatment of

Hs-ERα cells with heregulin-β further potentiated the estrogen-stimulated expression of both genes (Fig.6B). This suggests that the regulation of CatD1 and PR expression by ERβ was more dependent on the effect of heregulin-β than the one through ERα (Fig.6B). Under these conditions, the CBP steady-state levels were not significantly modified in Hs-ERα and Hs-ERβ cells (Fig.6C).

A conserved Akt site can predict the transcriptional response of nuclear receptors to CBP - Based on our results on the critical role of Ser-255 in regulating the response of mouse ERβ to Akt and CBP coactivation, we checked whether the Akt motif containing Ser-255 was conserved within the nuclear receptor family. It should be noted that Ser-255 is located within the hinge region of ERβ which is generally more conserved between ERs and orphan estrogen-related ERR receptors, than with other nuclear receptors. As such, the sequence alignment in Fig. 7A showed that while ERα and all three isoforms of ERR contain the necessary arginine residue at position –3, and the less stringent arginine/lysine residues at position –5 of the canonical site for Akt (36) in their respective hinge regions, only ERRβ possesses the expected phosphorylated serine (Fig.7A). It is interesting to note that as opposed to the mouse and rat isoforms, human ERβ does not contain a serine at the corresponding position, but rather has a negatively-charged aspartic acid residue. In addition, human, mouse and rat forms of ERα do not share the conserved serine residue, having a glycine or leucine when aligned with ERβ Ser-255 (mERα is shown in Fig.7A). Although no perfect consensus site for Akt could be found in glucocorticoid (GR) and progesterone receptors (PR), a putative Akt site conserved in mouse and human GR was found with the required arginine residue at position –3 and was aligned with ERβ. In order to address how other nuclear receptors responded to Akt and CBP-mediated coactivation and to find a possible correlation in respect to their sequence homology with ERβ Ser-255, we tested various nuclear receptors in luciferase assay. Using an ERE-driven luciferase reporter known to bind and

respond to ERRs as dimers (37), we found that coexpression of CBP increased the activity of the three ERR isoforms by 2- to 3-fold in 293 cells (Fig.7B). Interestingly, when Akt activation was induced with p110α PI3K expression, CBP only failed to further transactivate ERRβ, whereas ERRα and ERRγ reached respectively 3- and 5-fold activation compared to controls. Under these conditions, the response of ERRβ to Akt activation and the inability of CBP to potentiate transactivation strongly correlates with what we observed with ERβ, and therefore points to a shared role for the putative Ser-191 Akt site of ERRβ that overlaps with Ser-255 of ERβ. This observation also applies to GR for which CBP-mediated coactivation was severely abrogated in response to Akt. Conversely, among the receptors tested that do not share homology with ERβ Ser-255, we found that ERα, PR and peroxisome proliferator-activated receptor PPARγ were all further activated by CBP in Akt-activated cells in the presence of their respective ligand (Fig.7B). CBP, which has originally been described to directly interact with cAMP responsive binding protein CREB, also potentiated CREB activation to Akt. Hence, the impaired ability of CBP to transactivate ERβ in response to Akt can be transposed to other receptors that share an apparent homology with ERβ Ser-255 containing motif. Accordingly, at least for those receptors tested that do not fit into that category, they seem to benefit from the enhanced intrinsic activity of CBP in response to activation of the PI3K/Akt pathway.

DISCUSSION

Increasing evidence suggests that besides ligand activation, nuclear receptors are responsive to kinase signaling mechanisms, and for estrogen-responsive tissues in particular, this may represent a mean to regulate the different ER-mediated transcriptional pathways (2,3). More recently the idea that signaling pathways can also mediate transcriptional repression of estrogen receptors has lead to further investigate how these pathways are tightly controlled (11,38). Here we show that activation of ErbB2/ErbB3 receptors and the PI3K/Akt pathway can impair the

transcriptional response of ERβ to estrogen and its coactivation by CBP. The mechanism underlying ERβ inhibition involves Ser-255 which upon its phosphorylation by Akt prevents CBP to interact with ERβ, therefore abrogating ERβ activity.

Dimerization of ErbB3 with its preferred partner ErbB2 is considered the most potent combination of ErbB receptors in terms of cellular growth and transformation (39). Deregulated signaling by ErbB2/ErbB3 has been associated with detrimental mitogenic potential in a number of reproductive cancers and the correlation of ErbB2 with ERα status has served as a predictive factor in endocrine-based therapy (40,41). However, the response of ERβ to ErbB2/ErbB3 activation is not clearly defined and the exact role of ERβ in tumorigenesis remains uncertain. We found that the transcriptional response of ERβ to estrogen was diminished upon activation of ErbB2/ErbB3 with ErbB3 ligand heregulin or the constitutive ErbB2 variant V659E derived from the Neu oncogene. These results have been transposed to ERβ-expressing stable breast cancer cells, therefore altering endogenous ER-responsive genes, as observed with the downregulation of CatD1 and PR. Both conditions were associated to increased cellular activation of Akt. Intriguingly, while coactivator CBP potently contributes to enhance basal and estrogen-dependent response of ERβ, it became inefficient to render optimal activation of ERβ in Akt activated cells. These effects seem to be specific to CBP since we showed that coactivator SRC-1 was able to relieve ERβ inhibition to heregulin signaling (11). Based on our observations that SRC-1 and CBP can trigger ERβ response to growth factors in an AF-1 dependent manner (8,9), it was predicted that both coactivators would behave similarly. In an attempt to delineate the role of the AF-1 domain, we found using an N-terminal form of ERβ that CBP promoted ERβ activation to Akt, suggesting a positive effect of the Akt pathway that obviously did not correlate with the response of the full length receptor. Although the N-terminal region of ERβ contains phosphorylation sites described to be directly phosphorylated by

MAPK conferring AF-1 activity of the receptor in response to EGF or ras (8,18), it does not have consensus site for Akt, and therefore upregulation in AF-1 activity by Akt might relate to possible indirect effects, including activation of CBP itself, as predicted in Fig.5A. Removal of the AF-1 region demonstrated a similar inhibitory pattern as observed with the full length ERβ, and further identifies Ser-255 as a functional site responsible for the inhibition of ERβ to ErbB2/ErbB3 and Akt signaling. Together, these findings clearly demonstrate that many signaling events converge to ERβ to regulate cofactor assembly and transcriptional activity either in a positive or negative manner.

Our observation that ERβ cellular levels were augmented by the PI3K/Akt pathway in the presence or absence of estrogen raised the possibility that ERβ turnover is regulated by Akt. Interestingly and consistent with this idea is the apparent opposite regulation of the S255A mutant in the same conditions, suggesting that Ser-255 is a determinant involved in ER recycling in response to Akt signaling. Studies using ERα have integrated the response to estrogen with the cellular degradation of the receptor, thus supporting a means by which target cells can sustain or limit a hormonal response through a continuous receptor turnover. ERα has been shown to be degraded through the proteasome pathway in a ligand-dependent manner (23,42), and blocking proteasome activity impaired the ability of ERα to mediate a transcriptional response to hormone (43-45), suggesting that ER turnover is necessary for receptor activity. Similarly, activation of the PI3K/Akt through PDGF stimulation of smooth muscle cells was shown to target CREB for degradation in a phosphorylation-dependent manner (46).

Recent studies derived from fluorescent-based approaches have revealed the dynamic nature of ERα within the nucleus and its behaviour with transcriptional coactivators in response to hormonal stimuli (26,44,47,48). Under basal conditions and in the absence of ERβ, CBP adopted a speckled pattern with a subpopulation being more diffuse within the nucleus. The reason for such behaviour is unclear, but the ability of

CBP to form speckles has been observed in different cell types under non activated conditions, and was associated to poorly transcribing or transcriptionally inactive compartments devoid of nascent mRNA transcription and active RNA polymerase II (28-30). The speckled clustering of CBP has also been shown to not segregate with regions of histone hyperacetylation, suggesting a decreased activity of CBP (49). However, such compartmentalized pattern of CBP was not always related to transcriptional inactivity, as the promyelocytic protein PML was identified as a nuclear receptor coactivator that segregates CBP into nuclear bodies (50). Interestingly, the expression of ERβ resulted in a marked decrease in speckle formation and a more diffuse pattern of CBP thoughout the nucleus which overlapped with the distribution of ERβ. This colocalization of ERβ and CBP occured in absence or presence of estrogen, therefore correlating with the enhanced activation of ERβ by CBP in luciferase assays. However, the activation of the PI3K/Akt pathway has the distinct effect of driving CBP to readopt a speckled pattern while ERβ remained diffused, coinciding with a reduced ERβ activity. Although these studies did not allow assessing directly the interaction between CBP and ERβ, it is interesting to note that while the S255A mutant was tested, the formation of CBP-related foci was greatly reduced in Akt activated cells. The expression of ERβ therefore allows for a redistribution of CBP in the nucleus which implicates Ser-255 as a determinant in the response to Akt. Consistent with these observations, activation of Akt led to a release of CBP from ERβ even in the presence of estrogen as determined in coimmunoprecipitation assay, whereas disruption of Ser-255 was found to stabilize such interaction. These observations emphasize the role of Ser-255 in mediating CBP release from ERβ in a phosphorylation-dependent process. While phosphorylation provides an important mechanism by which steroid hormone receptors can be activated (3), increasing evidence suggests that phosphorylation also mediates nuclear receptor inhibition or repression involving various mechanisms and different kinase

pathways. Phosphorylation of serine 236 by PKA was reported to impair ERα dimerization and hence transcriptional activity (51). Phosphorylation of AF-1 Ser-112 by MAPK reduced the ligand binding affinity and activity of PPARγ (52). In the case of the androgen receptor, Ser-210 and Ser-790 were identified as phosphorylation sites for Akt which inhibited the association of AR with coactivator ARA70 (53). Our results therefore provide a mechanism by which ErbB2/ErbB3 and Akt signaling impairs ERβ activity through a phosphorylation-dependent release of coactivator CBP.

CBP/p300 are general signal integrators common to many transcription factors and evidence suggests that part of the mechanism that regulates their function involves direct phosphorylation (14). Interestingly, phosphorylation of Ser-1834 by Akt was shown to promote p300 histone acetyltransferase activity and its transcriptional potential (32). By mutating the corresponding site within CBP, we observed that Thr-1872 is essential to promote CBP enhanced transcriptional capacity in response to Akt activation. However ERβ was not able to benefit from this improved activity as opposed to the S255A mutant, suggesting that phosphorylation of ERβ at Ser-255 may prevail in the response of ERβ to Akt. Indeed, phosphorylation of Ser-255 impaired CBP recruitment to ERβ and did not allow for proper CBP-mediated coactivation, therefore preventing any potential of CBP to activate ERβ. A similar mechanism was described in the inhibition of C/EBPβ-targeted gene expression by insulin, except that the phosphorylation of Ser-1834 in the C/H_3 domain of p300 by Akt prevented p300 to interact with C/EBPβ (54). CBP Thr-1872 is also contained in the C/H_3 domain, which is described to mediate the recruitment of many transcription factors to CBP/p300 (14). However, our results suggest that Thr-1872 of CBP does not regulate the transcriptional response of ERβ to Akt. A recent report has described the interaction of ERα with CBP C/H_3 domain in the presence of an antiestrogen, as opposed to the previously recognized N-terminal interaction domain of CBP for agonist-bound nuclear receptors, but

whether phosphorylation of CBP was involved has not been determined (55).

Genetic studies have established that the cellular availability of CBP is critical for normal physiologic functions and as a coactivator that integrates the effects of several transcription factors, this may represent a mean by which CBP can discriminate between various regulatory pathways (16,56). As such, while testing other members of the nuclear receptor family, we found that unlike ERβ, the activation of ERα by Akt was potentiated in the presence of CBP and further contributed to enhance the expression of known ER target genes such as CatD1 and PR, in stably ERα-expressing breast cancer cells. ERα does not contain the corresponding Ser-255 found in ERβ, but an Akt site within ERα AF-1 domain, which is absent in ERβ, has been described to functionally activate ERα (7,57). Such isoform-selective coactivation of ERs by CBP may represent a mechanism by which CBP can discriminate between ERα and ERβ regulated pathways in response to Akt signaling. This mechanism can become important in pathologic conditions such as early breast cancer, in which activation of Akt is extremely frequent as a consequence of ErbB2 amplification (58). Clinically, Akt activation strongly correlates with ERα in breast tumours, while the prognostic value of ERβ is not established (40,59). It seems therefore interesting to propose that the negatively-charged aspartic residue that corresponds to mouse Ser-255 could predict for a reduced response of human ERβ to CBP coactivation. Clearly, further studies are needed to unravel these distinctions.

The ER isoform-specific effect of CBP by the PI3K/Akt pathway has also been observed between ERR members. As opposed to ERRα and ERRγ, ERRβ contains a consensus for Akt found within the same region as ERβ, and was found negatively regulated by Akt in the presence of CBP. Although structurally closely related to the ERs, the ERRs do not exhibit estrogen binding and are still considered orphan receptors without a known endogenous ligand. However, our results predict that ERRs can be selectively regulated by kinase signaling

pathways such as PI3K/Akt, and with the emerging role of ERR isoforms in modulating ER functions and target gene expression (37,60), it will be of interest to investigate whether such regulation might influence these aspects.

The present findings demonstrate a molecular mechanism by which the PI3K/Akt pathway may dictate the activity of ERβ and other nuclear receptors, through their selective ability to use CBP as a coactivator. With the impact of ErbB2 signaling and/or Akt activation pathways to also affect CBP intrinsic coactivation properties, elucidation of the various regulatory signals that dictate nuclear receptor-coactivator functions might provide insights into their integrative function.

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FOOTNOTES

Acknowledgments - We wish to thank J. Downward, T. Chan, P. Tsichlis, and V. Giguere for providing plasmids. We acknowledge the technical assistance of J. Martin and thank members of the lab for critical reading and useful comments. A.T. is a New Investigator of the Canadian Institutes of Health Research (CIHR). This work was supported by the CIHR, the Cancer Research Society Inc., and the Canadian Foundation for Innovation.

[1] The abbreviations used are: ER, estrogen receptor; PI3K, phosphatidylinositol 3-kinase; CBP, CREB binding protein; C/H_3, cysteine and histidine-rich 3; SRC, steroid receptor coactivator; ERR, estrogen-related receptor; GR, glucocorticoid receptor; PR, progesterone receptor; PPAR, peroxisome proliferator-activated receptor; Hrg, heregulin; EGFR, epidermal growth factor receptor; ERE, estrogen response element

FIGURE LEGENDS

Fig. 1. ErbB2/ErbB3 signaling impairs the hormonal response and coactivation of ERβ by CBP.

A, 293-T cells were transfected with an ERβ plasmid and an EREtkLuc reporter, and expression vectors encoding ErbB2 and ErbB3, or a constitutively active ErbB2 (V659E) mutant in the presence or absence of CBP. Cells were then treated with 10nM estradiol (E2) and/or 10ng/ml heregulin (Hrg-β) for 20h and harvested for transcriptional activity. Luciferase values were normalized to β-galactosidase activity and expressed as fold activation compared to untreated cells set at 1.0. B, 293-T cells were transfected with ErbB plasmids and treated with 10ng/ml heregulin for 20 min as indicated. Total cell extracts were analyzed by electrophoresis, and endogeneous Akt phosphorylation was monitored by Western blotting using a specific anti-phospho Akt. Cell lysates were also analyzed for Akt content using an anti-Akt antibody.

Fig. 2. The effect of Akt activation on ERβ response to CBP coactivation is mediated by the C-terminal region of ERβ.

A, 293T cells were transfected with the EREtkLuc reporter and expression plasmid for ERβ in the presence or absence of CBP. Cells were also transfected with plasmids for Akt

or its kinase dead K179M mutant form, in the presence of the constitutively active p110α subunit of PI3K, as indicated. Cells were then treated with 10nM E2 for 20h and harvested for transcriptional activity. Luciferase values were normalized to β-galactosidase activity and expressed as fold activation compared to untreated cells set at 1.0. B, Western analysis of ERβ in response to Akt activation. 293T cells were transfected with ERβ in absence or presence of p110α PI3K and Akt plasmids to trigger the Akt pathway. Cells were then treated with 10nM E2 or left untreated for 20h, and harvested for Western analysis using an anti-ERβ antibody. Loading was monitored with β-actin for each sample. C, Cells were transfected with a UAStkLuc reporter and truncated forms of ERβ corresponding to the N-terminal or AB region (*left*), or the C-terminal or DEF region (*right*) fused to the Gal-4 DNA binding domain. Cells were also transfected with p110α and Akt plasmids, and treated with 10nM E2 for 20h prior to luciferase assay. Luciferase values are expressed an in (A).

Fig. 3. Serine-255 in the hinge region of ERβ is a target for Akt phosphorylation and mediates the inhibition of ERβ activity to ErbB2/ErbB3 signaling.

A. Schematic representation of serine-255 that resides within a consensus recognition motif for Akt in the hinge region of ERβ. B, In vitro phosphorylation assay of partially purified wt and S255A GST fusions of ERβ using activated Akt. ^{32}P-labeled proteins were analyzed by autoradiography (upper panel). Proteins were stained with Coomassie to ensure equal loading (lower panel). C, 293T cells were transfected with an EREtkLuc reporter and an expression vector encoding ERβ S255A mutant. Transcriptional activity was assessed in cells expressing either ErbB2/ErbB3 receptors activated by heregulin-β, or p110α/Akt, in presence or absence of CBP. Treatments were 10nM E2 and/or 10ng/ml heregulin-β for 20h. Normalized luciferase values are expressed as fold activation compared to untreated cells set at 1.0. D, Western analysis of ERβ S255A mutant in response to Akt activation. Cells transfected with S255A mutant with or without p110α/Akt plasmids, were treated or not with 10nM E2 and then harvested for Western

analysis using an anti-ERβ antibody. Loading was monitored with β-actin for each sample.

Fig. 4. Serine-255 of ERβ modulates the intranuclear behaviour of CBP and its release from ERβ in response to Akt activation.

A, Subnuclear localization of CBP and ERβ by fluorescence microscopy. 293T cells were transfected with YFP-CBP in absence or presence of CFP-ERβ or CFP-ERβ S255A plasmids. Akt was activated by cotransfecting cells with p110α and Akt plasmids. Fluorescence signals were visualized using filters for YFP and CFP shown alone and merged. Cell nuclei were also stained with DAPI. B, CBP is released from ERβ through Ser-255 in Akt-activated cells. Cells were cotransfected with HA-tagged wt or S255A ERβ in the presence of CBP, and then treated or not with 10nM E2 for 20h. To activate the Akt pathway, cells were also transfected with PI3K p110α and Akt plasmids. Immunoprecipitation was carried out with an anti-CBP antibody, and ERβ was analyzed by Western. CBP was also monitored in each sample by Western analysis.

Fig. 5. Akt promotes the intrinsic transcriptional activity of CBP through Thr-1872.

A, The intrinsic ability of CBP to activate transcription was assessed by transfecting cells with a UAStkLuc reporter in the presence or absence of Gal4 fusions of CBP or CBP T1872A mutant, and p110α/Akt plasmids. Cells were harvested 20h after transfection and analysed for luciferase activity. B, Western analysis of CBP and T1872A mutant in response to Akt activation. Loading was monitored with β-actin for each sample. C, Thr-1872 of CBP is not involved in the response of ERβ to Akt. 293T cells were transfected with a ERE$_{tk}$Luc reporter and expression vectors encoding ERβ, ERβ S255A mutant, or ERα in the presence of CBP or CBP T1872A plasmid. The p110α/Akt plasmids were used to trigger Akt in cells. After transfections, cells were treated with 10nM E2 for 20h or vehicle and transcriptional activity was measured. Normalized luciferase values are expressed as fold activation compared to control cells set at 1.0.

Fig. 6. Estrogen-responsive genes are regulated differently by ERα and ERβ in response to heregulin-β.

A- Activation of Akt in stable Hs-ERα and Hs-ERβ clones in response to heregulin-β and estrogen. ERα and ERβ-expressing stable clones have been generated using ER-negative Hs578t cells (control) and were treated with 10ng/ml heregulin-β for the indicated time or 10nM E2 for 60min. Endogeneous Akt phosphorylation was monitored by Western blotting using a specific anti-phospho Akt. Cell lysates were also analyzed for Akt content using an anti-Akt antibody. B- RT-PCR analysis of ER responsive genes from Hs-ER clones and Hs control cells treated with 10ng/ml heregulin-β and/or 10nM E2 for 20h prior to RNA isolation. Representative images are shown from at least three separate experiments. GAPDH expression was used to normalize samples. C- Western analysis of CBP in Hs-ER stable clones and Hs control cells treated as above. Samples were normalized for protein loading with β-actin.

Fig. 7. A conserved Akt site dictates the transcriptional response of nuclear receptors to CBP.

A- Sequence alignment for predicted Akt phosphorylation site of nuclear receptors. Shown are the predicted phosphorylated serine (arrow) with the obligatory arginine residue (R) at position –3, and the less stringent arginine/lysine (K) at position –5 of receptor sequences aligned with mouse ERβ. The predicted Akt site is conserved in human and mouse ERRβ and GR. B, Response of nuclear receptors to Akt and CBP coactivation. 293T cells were transfected with expression plasmids for the indicated nuclear receptors with their respective luciferase reporter as follows : EREtkLuc for ERα and the estrogen-related receptor ERR isoforms; GREtkLuc for glucocorticoid (GR) and progesterone receptor (PR); PPREtkLuc for peroxisome proliferator-activated receptor (PPARγ); and CREtkLuc for cAMP responsive binding protein CREB. Cells were also transfected with CBP and/or p110α/Akt plasmids, and treated with ligands as follows : 10nM E2 (ERα), 10nM dexamethasone (GR), 10nM progesterone (PR), or

1μM troglitazone (PPARγ) for 20h prior to determination of luciferase activity. Normalized luciferase values are expressed as fold activation compared to untreated cells set at 1.0 for each receptor.

FIGURES

Fig.1

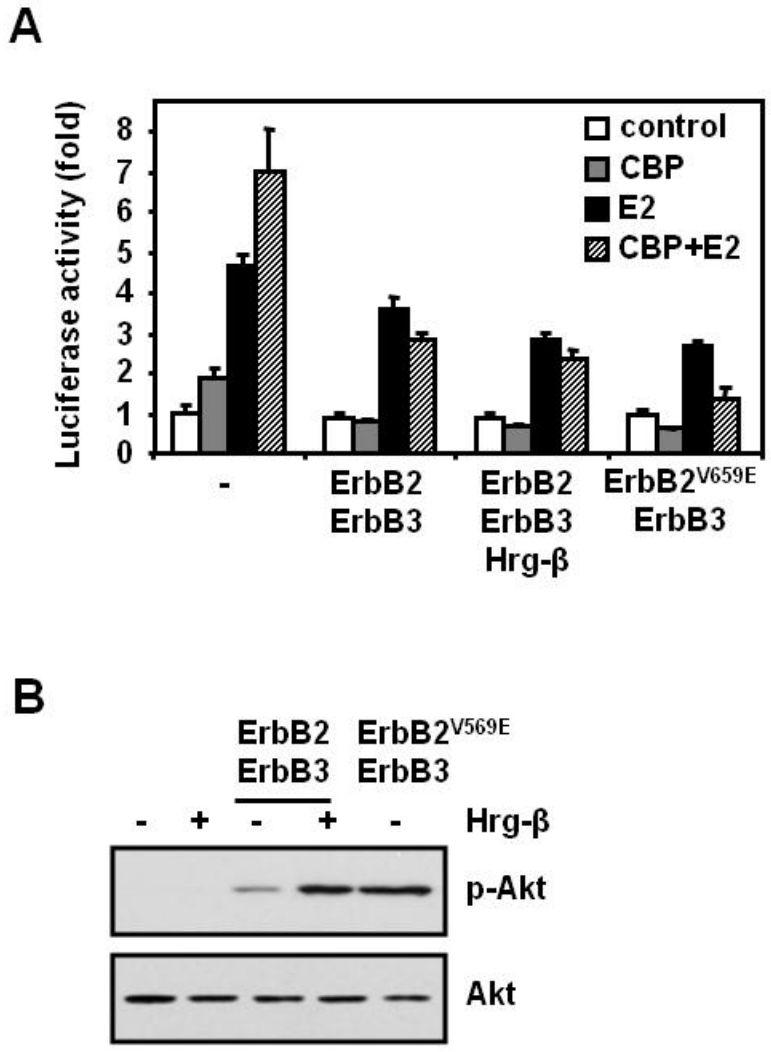

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Fig.2

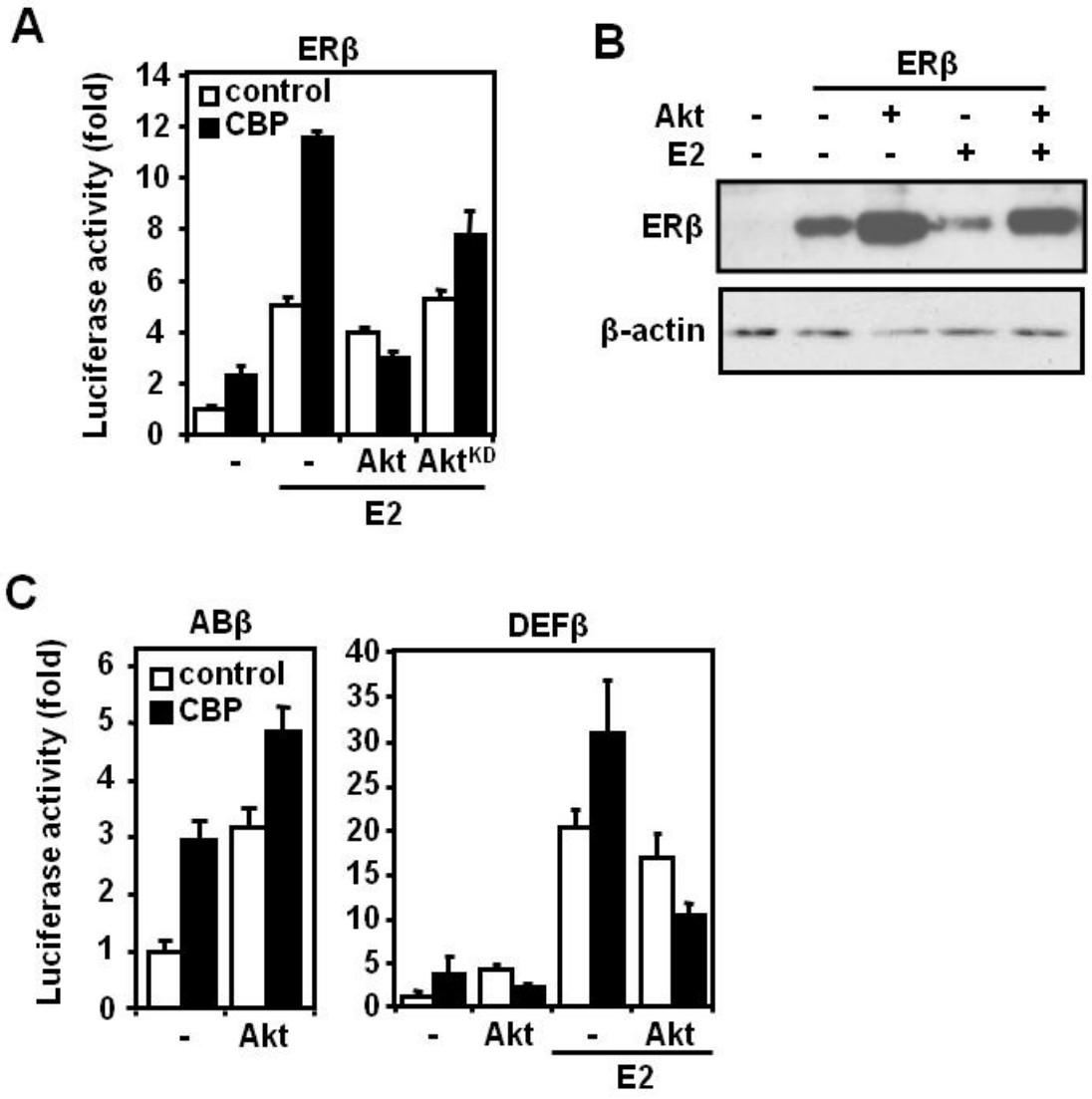

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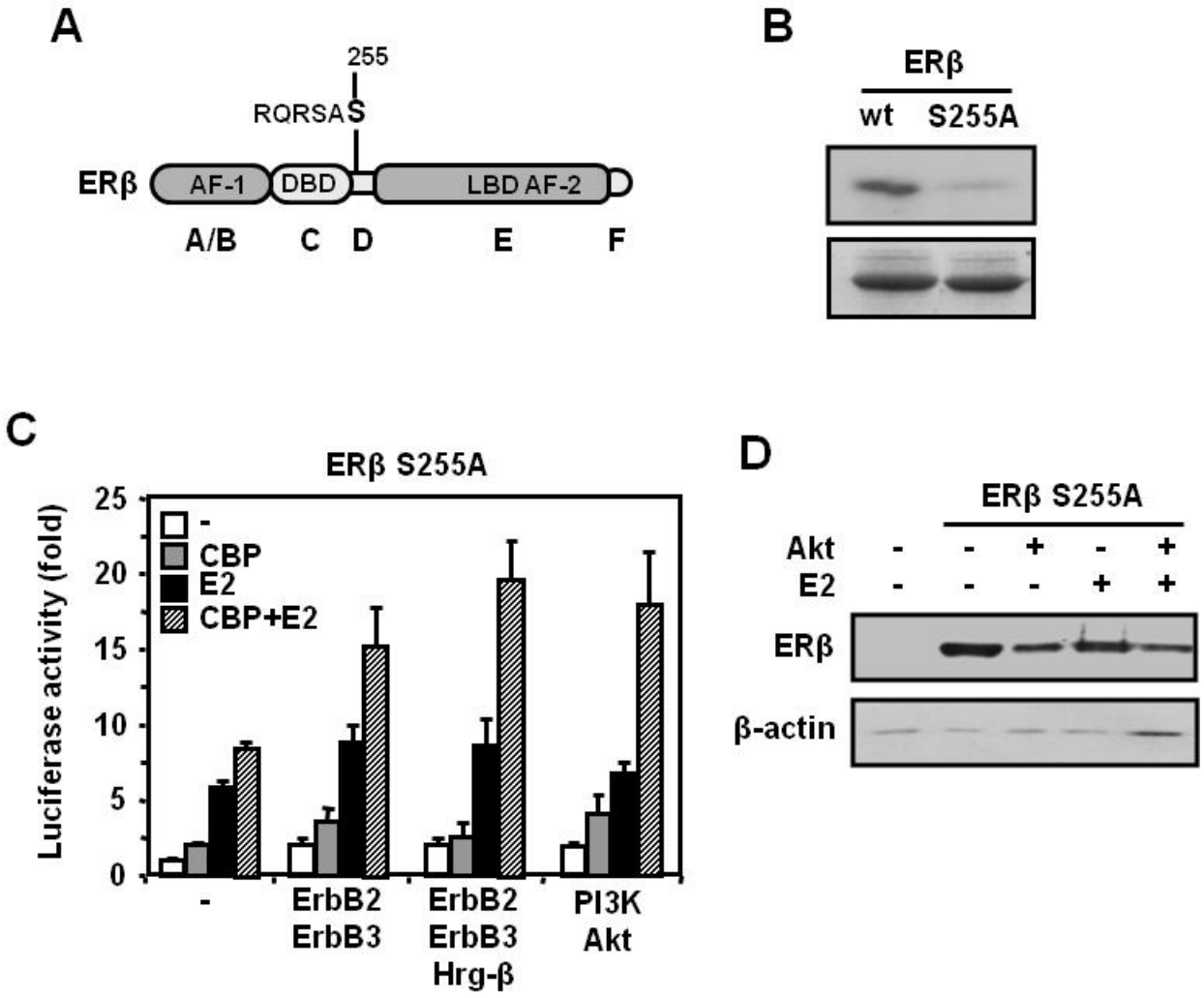
Fig.3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A/B
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wt
S255A
C
ERβ S255A
Luciferase activity (fold)
25
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15
10
5
0
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CBP
E2
CBP+E2
-
ErbB2
ErbB3
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ERβ
β-actin

Fig.4

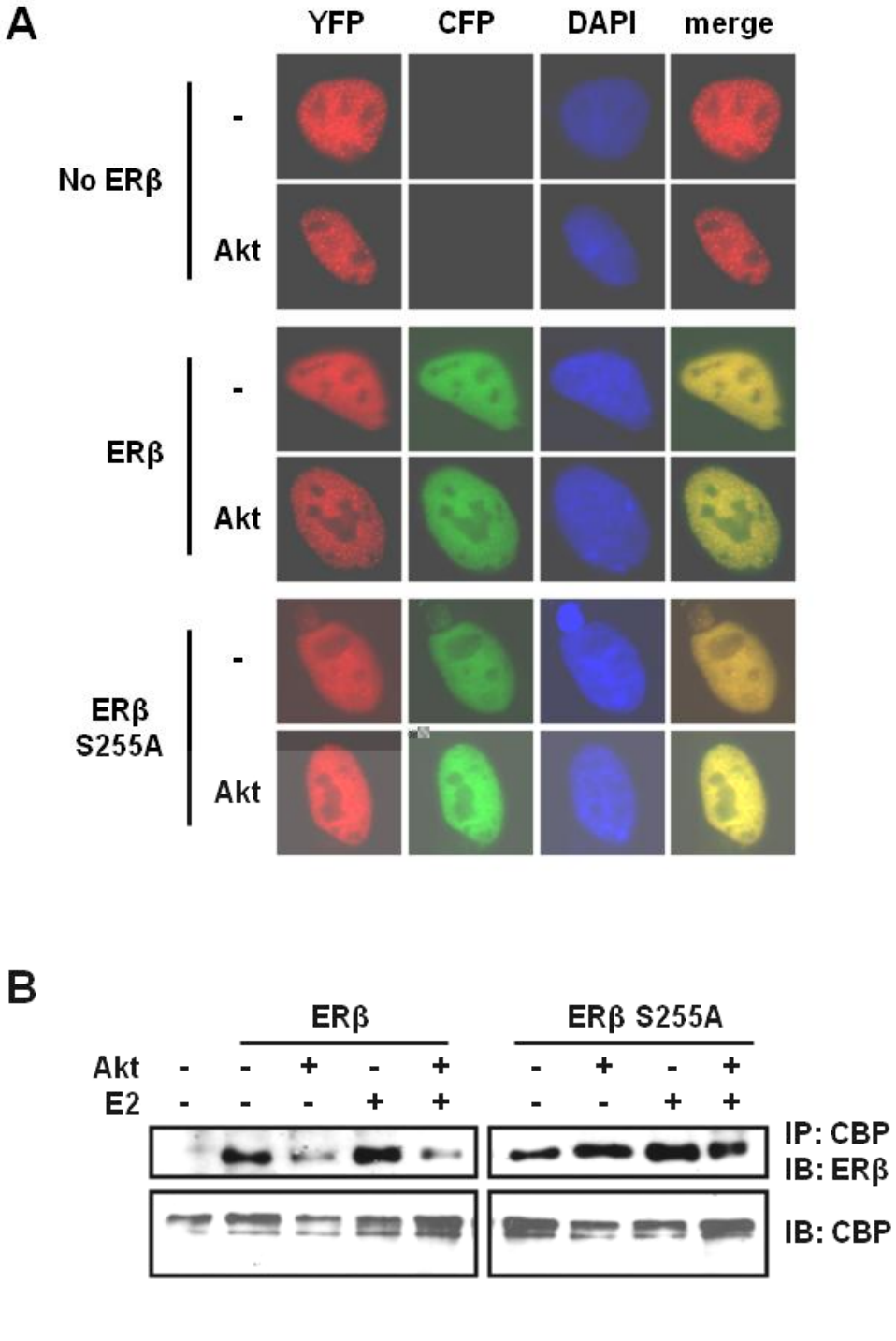
A
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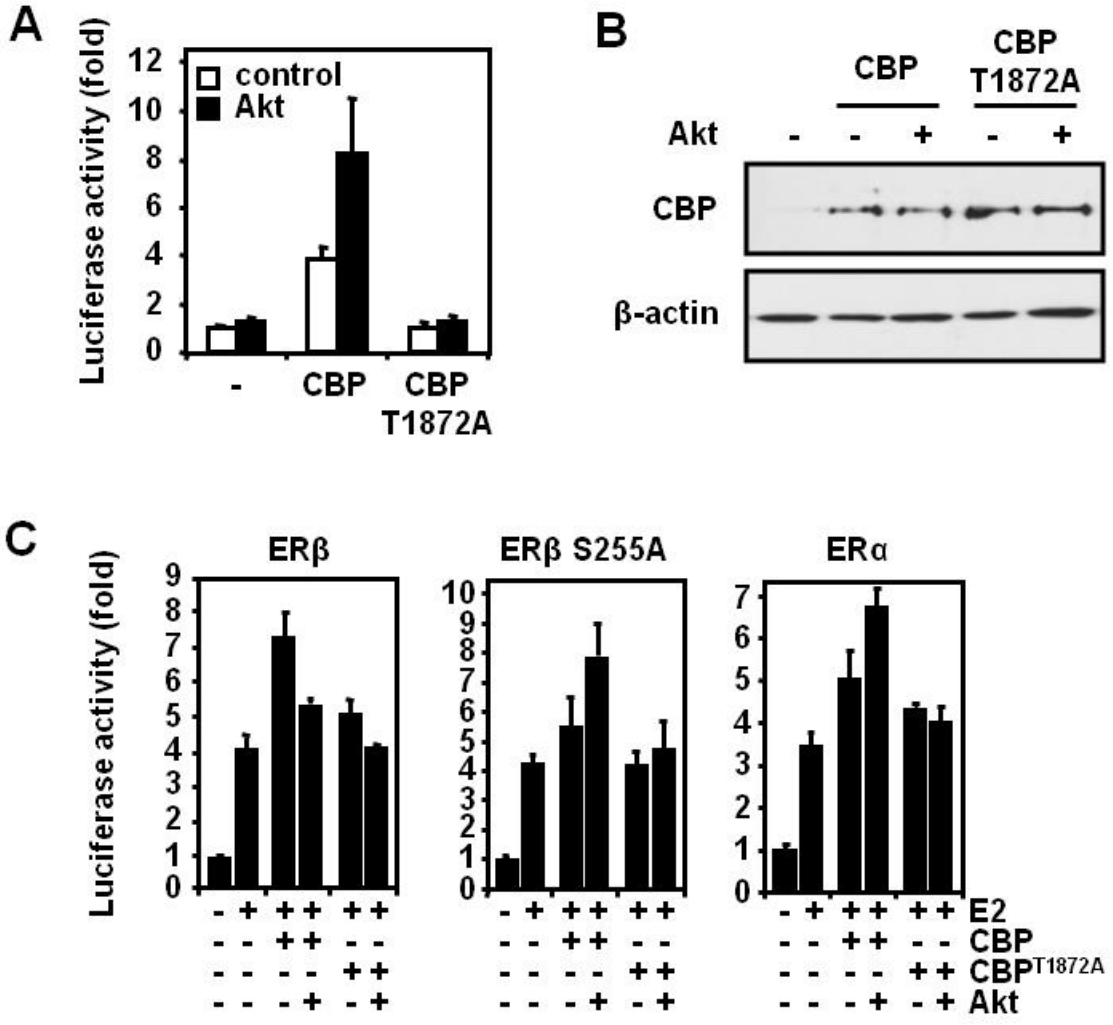
Fig.5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Akt

Fig.6

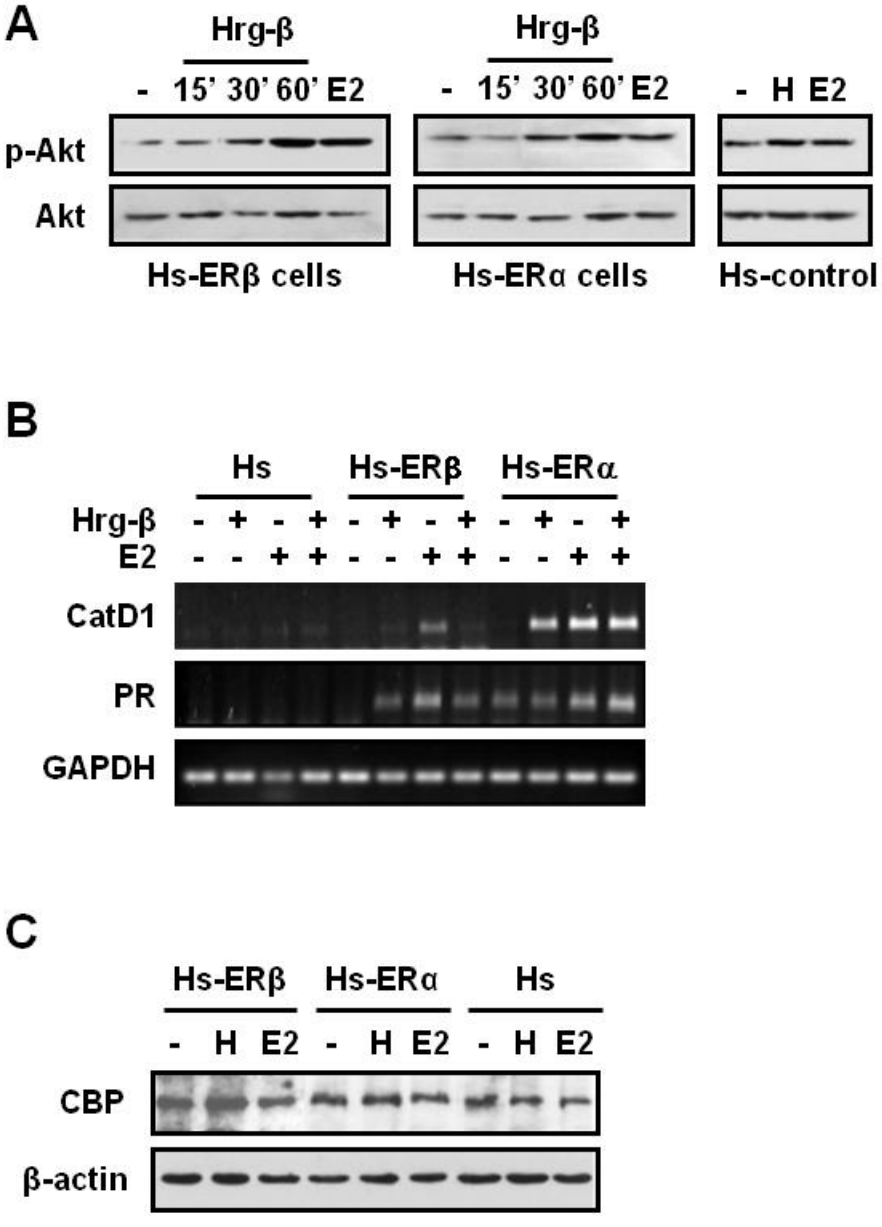

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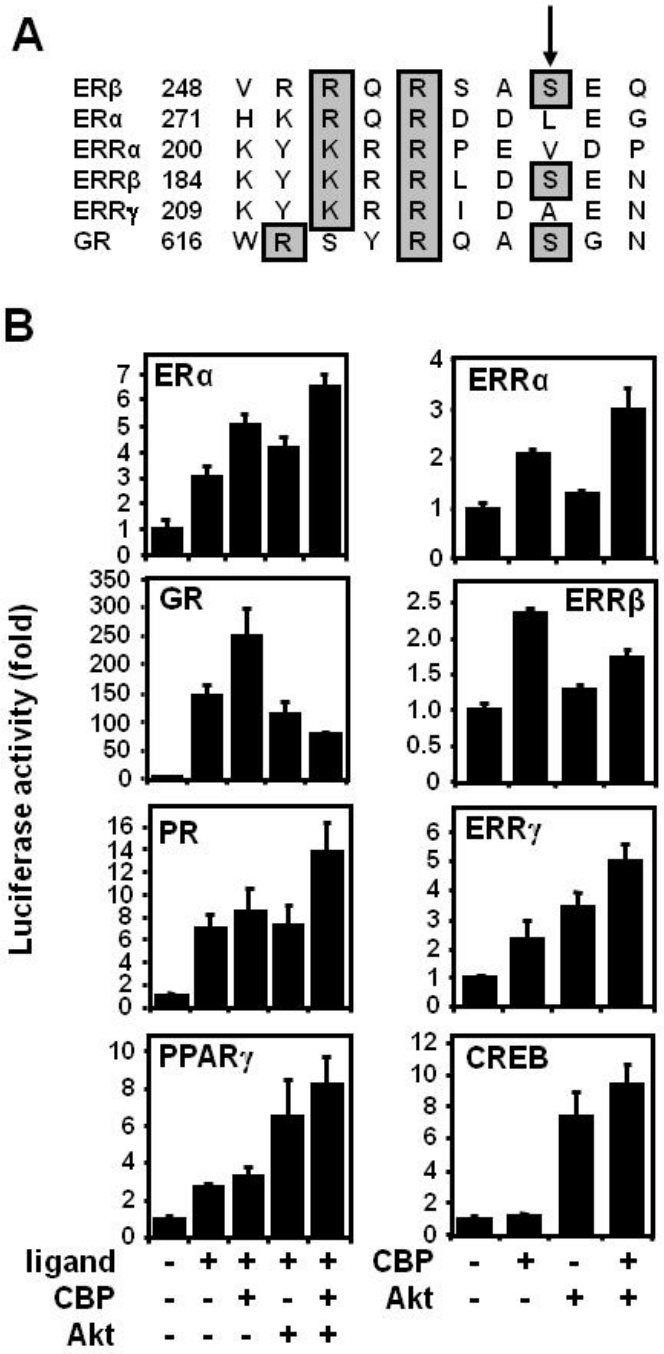
Fig.7



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2 Mdm2 and CBP cooperate in targeting Estrogen Receptors β proteasomal degradation in response to growth signals

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(Accepted with modifications to Cell Research)

Keywords: estrogen receptor; ERα; ERβ; growth factors; EGF; heregulins; receptor tyrosine kinase; EGFR; ErbB2/HER-2/Neu; ErbB3; AF-1; AF-2; MAPK; SRC-1; CBP/p300; E3 ubiquitin ligase CHIP; E6-AP; 26S proteasome; ubiquitin; SUMOylation

DISCLOSURE STATEMENT: The authors have no competing interests.

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Foreword

The information available on the role of ERβ remains quite limited. Its regulation and its role in tissues are presently ambiguous. Some groups have considered ERβ to promote cellular proliferation and could be a good target as a prognostic marker in the establishment of hormone-dependent tumors (such as in the breast), while others support the concept that ERβ is a tumor-suppressor. Here we investigate how the activity of ERβ unlike that of ERα is inhibited following the activation of ErbB2/ErbB3 heterodimer. In our first study we identified the region within ERβ that was necessary for the inhibition. In addition, the presence of CBP was required in order to observe an inhibition of ERβ. Therefore the role played by CBP was also investigated. In this manuscript we have established a mechanism by which the activation of the PI3K/Akt pathway downregulates ERβ due to an increased degradation of ERβ that requires the 26S ubiquitin-Proteasome system. Furthermore we found that CBP was necessary to trigger ERβ degradation, a mechanism that is subtype specific as ERα degradation was not detected. We observed in breast cancer cell line MCF-7, which expresses both subtypes, a decrease in its proliferation when ERβ is present. These results strongly suggest that during ErbB2/ErbB3 deregulation, there is a targeted degradation of ERβ dependent on CBP which provides these cells with a stronger proliferation potential. Therefore the ratio of ERα/ERβ, under these conditions, is relevant in order to predict the fate of cell growth.

Contribution of authors:

Mélanie Sanchez: As first author of this article I conceptualized the project, designed and performed the experiments. I also created the mutant for ERβ and MDM2 and proceeded to clone the mutants into the vectors with tags used in the article. I also wrote the first draft of the manuscript and took care of the corrections that ensued.

Nathalie Picard: As second author, Nathalie was able to bring her expertise on the targeted degradation by the ubiquitin-proteasome system in critical discussions and will participate in the correction of the manuscript.

Karine Sauvé: As third author, Karine also participated in active discussions on the topic and will participate in the correction of the manuscript.

André Tremblay: As supervisor, Dr Tremblay supervised and participated in the design of the experiments. He also corrected the article.

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Mdm2 and CBP cooperate in targeting Estrogen Receptor β proteasomal degradation through growth signals

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Keywords: estrogen receptor; ERα; ERβ; growth factors; EGF; heregulin; receptor tyrosine kinase; EGFR; ErbB2/HER-2/Neu; ErbB3; AF-1; AF-2; PI3-K; Akt/PKB; SRC-1; CBP/p300; Mdm2; Hdm2; E3 ubiquitin ligase; 26S proteasome; ubiquitin

Running title: Concerted degradation of ERβ by Mdm2

DISCLOSURE STATEMENT: The authors have no competing interests.

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Abstract

Estrogen-regulated target gene expression is mediated through direct interaction with ERα and ERβ. Yet, alternative signaling events contribute to the activity of ERs. We show that heregulin-β stimulated ErbB2/ErbB3 activates the PI3-K/Akt pathway prompting ERβ degradation by the 26S proteasome, a mechanism involving the coactivator CBP. We found that CBP promoted ERβ degradation following Akt activation, which was relieved by the Akt-consensus site mutant ERβ^{S255A} supporting a role for a negatively charged hinge region in regulating ERβ turnover. Active Akt induced a stable interaction between ERβ and Mdm2 which was promoted by CBP leading to poly-ubiquitination of ERβ. Akt sites mutants CBP^{T1872A} or Mdm2$^{S186/188A}$ resulted in a dissociation of the ERβ-CBP-Mdm2 complex and reduced ERβ ubiquitination. Heregulin-β promoted of MCF-7 breast cancer cells proliferation with a decrease in ERβ levels, while ERα remained unchanged. Knockdown of Mdm2 restored endogenous levels of ERβ resulting in a reduced growth of MCF-7 cells. These studies identify an Akt-regulated phosphorylation switch involving CBP, dictating ERβ activity and turnover to growth factor signaling pathways through Mdm2-mediated ubiquitination.

Introduction

Estrogen regulates many aspects of reproductive physiology, development, and metabolism, but is also mitogenic in hormone-regulated cancers, such as breast and endometrial cancers. Its effects are mediated by the actions of estrogen receptors ERα and ERβ, which are homologous members of the nuclear receptor family of ligand-inducible transcription factors. Although encoded by unique genes, both isoforms recognize palindromic estrogen-responsive elements in target gene promoter/enhancer regions, share similar estradiol binding affinity, and undergo post-translational modifications in response to hormone and to various cellular kinase pathways [1–3]. Whereas an increased expression of ERα strongly correlates with the development and proliferation of breast carcinomas, therefore providing a basis for endocrine adjuvant therapy, the clinical value of ERβ remains uncertain.

Both ER isoforms are composed of distinct functional regions; a conserved C-terminal globular region harboring the ligand-binding domain (LBD) and the transcriptional coactivator interaction function AF-2, a centrally-located highly conserved DNA binding domain (DBD), a hinge region which links the DBD and LBD, and a poorly conserved N-terminal domain containing an autonomous transactivation function (AF-1) which exhibits ligand-independent activity and strong phosphorylation capacity. Much of the studies performed on the ligand-independent activation of both ERα and ERβ have revealed a diversity of potential mechanisms. Among these,

members of the EGFR/ErbB family were identified to regulate ERα and ERβ transcriptional activity through transduced kinase signaling pathways that conduct ER phosphorylation [3,4]. However, the outcome of ER phosphorylation by ErbB receptors not only differs according to the identity of the activator and the selectivity of targeted site(s), but also differently impacts the transcription potential of ERs. For example, phosphorylation of the AF-1 by MAPK/Erk in response to EGF was shown to induce ERα activity [5,6]. Likewise, activation of EGFR/ErbB1 resulted in ERβ AF-1 phosphorylation, recruitment of steroid receptor coactivator SRC-1 and CREB-binding protein (CBP), and subsequent receptor activation [7,8]. On the other hand, activation of the ErbB2/ErbB3 heterodimer by growth factor heregulin-β was able to repress ERβ response to estrogen in breast cancer cells, an effect relieved by promoting constitutive activation of the AF-1 [9] or by mutating an Akt site located within the hinge region of ERβ [10], indicating that phosphorylation of ERβ through the PI3-K/Akt pathway was involved in such response.

Several coactivators have been described to enhance the transcriptional activity of ERs, notably CBP and its heterologue p300, which mediate histone acetylation to remodel chromatin and facilitate transcription [11]. CBP/p300 have also been described to acetylate other transcriptional regulators, such as ERα [12], androgen receptor [13], and SRC-3/ACTR [14], as a mean to control transcriptional activity. The versatile role of CBP/p300 in transcription allows it to act as an integrative factor for diverse

transcription factors to bridge with components of the basal transcriptional machinery, allowing CBP/p300 to act as a scaffold for the formation of multi-component complexes [15]. The array of transcription factors, many of which have been implicated in cancer, that can interact with CBP/p300 suggests that competition for the limited intracellular pool of CBP/p300 must be achieved in a very dynamic process and through preferred recruiting signals. Elucidation of these mechanisms will certainly contribute to understand CBP/p300 specific activity and plasticity.

Studies on ERα have integrated the transcriptional response to estrogen with the 26S proteasome-directed degradation of the receptor [16,17], thus supporting a mean by which target cells can sustain or limit a hormonal response through a continuous receptor turnover. As such, components of the ubiquitin-proteasome system, including the 19S proteasome regulatory subunit Trip1/SUG1 [18], the E3 ubiquitin ligases E6-AP [16] and CHIP [19], and the estrogen-responsive finger protein EFP [20] were shown to enhance the hormonal response of ERα, suggesting that degradation of the receptor is closely related to its transcriptional competence. Given the potent ability of ERβ to respond to kinase signaling pathways, we recently reported that ERβ was ubiquitinated in response to the Mek1/Erk pathway [21], thereby providing evidence that mechanisms other than estrogen binding may dictate ER ubiquitination. Activation of Erk resulted in specific AF-1 phosphorylation, which regulates ERβ ubiquitination and activity through the recruitment of E6-AP [21], suggesting that phosphorylation-dependent mechanisms

determine the activation-degradation process of ERβ in order to integrate its response to changes in kinase-activated pathways.

In this study, we describe how ErbB2/ErbB3 receptor activation selectively commits ERβ towards degradation through the activation of the PI-3K/Akt pathway. We identified the E3 ubiquitin ligase Mdm2 as being phosphorylated and recruited to ERβ in a hormone-independent manner in order to mediate ERβ ubiquitination and turnover. This process requires a negatively charged hinge region of ERβ and an Akt-mediated phosphorylation of CBP, illustrating the capacity of CBP to couple ERβ activity and proteasomal degradation by integrating phosphorylation-directed kinase signaling pathways in breast cancer cells.

Results

Activation of the PI3-K/Akt pathway by heregulin-β directs ERβ degradation

We previously reported that activation of ErbB2/ErbB3 receptor heterodimer by heregulin-β was capable of decreasing the transcriptional response of ERβ to CBP coactivation [10]. In order to elucidate the possible mechanisms involved, we observed that, whereas the steady-state levels of ERβ increased following activation of ErbB2/ErbB3 by Heregulin-β or by expressing CBP in 293T cells, a significant decrease in the amount of ERβ was found when both conditions were combined (Fig. 1A). The

decrease in ERβ was dependent upon the 26S proteasome system as shown with the use of MG-132 inhibitor. Given the potent ability of ErbB2/ErbB3 to activate the PI3-K/Akt pathway [22], we observed that inhibition of Akt by transfecting cells with a dominant negative p85 regulatory subunit restored the levels of ERβ to those seen with CBP (Fig. 1A). The role of the PI3-K/Akt pathway was further established using a constitutively p110α active form of PI3-K which led to a decrease in ERβ content in the presence of CBP (Fig. 1B). A similar reduction in ERβ levels was observed in the presence of estrogen (Fig. 1C), indicating that these effects occur independently of the presence or absence of hormone.

Consistent with our previous observations that ERα responded differently from ERβ to Akt activation [10], no significant changes were observed in ERα levels in response to CBP expression and ErbB2/ErbB3 activation (Fig. 1D), indicating that this mechanism implicates ERβ. To determine whether the decrease in ERβ steady-state levels was consequent of its degradation, we performed cycloheximide chase experiments which showed that the half life of ERβ was increased from 5 to 8 h in the presence of CBP, confirming that CBP is able to stabilize ERβ (Fig. 1E). However, activation of Akt greatly reduced the turnover rate of ERβ ($t_{1/2}$= 2 h) in these conditions. These findings further demonstrate that induction of the Akt pathway through ErbB2/ErbB3 receptors induces the targeted degradation of ERβ by the 26S proteasome.

Ser-255 regulates the proteasomal degradation of mouse ERβ

Based on our previous findings that phosphorylation of Ser-255 located within the hinge region of ERβ was required in the repression of the receptor by the ErbB2/ErbB3 pathway [10], we next tested the role of Ser-255 on ERβ degradation. As opposed to wild-type receptor, the levels of ERβ S255A mutant were elevated in the presence of CBP and under ErbB2/ErbB3 activation (Fig. 2A). Such requirement of Ser-255 to mediate ERβ degradation was dependent on CBP, since accumulation of the S255A mutant to ErbB2/ErbB3 activation in the absence of CBP (Fig. 2A) was comparable to wild-type receptor (Fig. 1A). Similarly, S255A mutation prevented ERβ downregulation by constitutive p110α and CBP (Fig. 2B). In cycloheximide chase analysis, whereas basal turnover rates remained mostly unchanged between wt and S255A ERβ ($t_{1/2}$= ~5 h), the S255A mutation protected ERβ from degradation under Akt activation (Fig. 2C). To determine whether these effects were restricted to nuclear ERβ, we performed Western analysis on fractionated cell compartments (Fig. 2D). Nuclear ERβ levels were found markedly decreased by CBP and Akt activation (Fig. 2E), correlating with results obtained using whole cell extracts. It is interesting to note that cytoplasmic ERβ levels were mostly unregulated, except when both CBP and Akt were added which reduced its content. Again, Ser-255 mutation largely prevented the response of ERβ (Fig. 2E). These results identify Ser-255 as an important regulatory site that governs ERβ degradation by the PI3-K/Akt pathway.

The E3 ubiquitin-ligase Mdm2 regulates ERβ levels and activity in an Akt-dependent manner

Degradation through the 26S proteasome implicates the action of a family of E3 ubiquitin ligases, which covalently attach small ubiquitin moieties at specific lysine residues of target proteins. Interestingly, the E3 ligase Mdm2, which plays a key role in tumor suppressor p53 degradation, is tightly controlled by phosphorylation [23]. In particular, activation of Akt has been shown to enhance the ability of Mdm2 to poly-ubiquitinate p53 [24,25]. We therefore investigated the role of Mdm2 in mediating ERβ degradation in response to Akt activation. We found that the steady-state levels of ERβ were reduced upon expression of Mdm2 in 293T cells, an effect that was dependent on the E3 ligase activity of Mdm2 as the inactive C462A mutant was inefficient (Fig. 3A). Under these conditions, the ability of Mdm2 to reduce ERβ levels was not completely dependent on Ser-255, as levels of the S255A mutant were also affected by Mdm2 (Fig. 3B).

We next determined whether Mdm2 affected ERβ transcriptional activity. Luciferase assays using an estrogen-responsive EREtkLuc reporter revealed that expression of Mdm2 abolished the ligand-independent activation of ERβ by CBP (Fig. 3C), suggesting a repressive function of Mdm2. Also, Mdm2 impaired part of the activation of ERβ by estrogen in absence or presence of CBP. When Akt was activated, the expected decrease in ERβ response to CBP, as we previously reported [10], remained

unaffected upon Mdm2 expression. These effects were reliable upon the integrity of Mdm2 E3 ligase activity, as the use of a C462A ligase dead mutant did not result in ERβ repression, but rather increased ERβ response to Akt and CBP, thereby relieving their potential to inhibit ERβ (Fig. 3C). This effect suggests a role for endogenous Mdm2 in mediating the decrease in ERβ transcriptional potential in Akt-activated cells. The repressive effect of Mdm2 on ERβ activity was although not observed with the S255A mutant (data not shown), indicating that Ser-255 plays a role in ERβ transcriptional repression.

A cluster of Akt phosphorylation sites, which consist of Ser-166, 186 and 188, has been shown to activate Mdm2 in response to mitogenic/survival signals, resulting in enhanced p53 ubiquitination and degradation [24,25]. We thus set out to first determine whether activation of ErbB2/ErbB3 resulted in Mdm2 phosphorylation. We detected a strong increase in Mdm2 phosphorylation in 293T cells in response to ErbB2/ErbB3 activation with heregulin-β, which was significantly reduced upon PI3-K inhibition using LY-294,002 or dominant negative p85α subunit (Fig. 3D). Mdm2 phosphorylation was also induced with the constitutive p110α form of PI3-K. We next analyzed the contribution of Mdm2 phosphorylation on ERβ protein levels by generating serine to alanine mutations at position 166 (A1), and 186 and 188 (A2) of Mdm2. Western analysis showed that, whereas the A1 mutation still permitted Mdm2 to decrease ERβ levels in response to Akt activation, the A2 mutation abolished such effect (Fig. 3E),

suggesting that Mdm2 Ser-186 and -188 mediate ERβ downregulation by Akt. These effects were mostly reproduced using the S255A mutant, indicating that Ser-255 in these conditions plays a minor role.

Mdm2 interacts with and promotes ERβ ubiquitination

As Mdm2 was able to promote ERβ downregulation and transcriptional repression, we tested the interesting possibility that it could interact with ERβ in cells. Indeed, Mdm2 was able to coimmunoprecipitate with ERβ under basal conditions, and the interaction was enhanced by the presence of CBP or the activation of the PI3-K/Akt pathway (Fig. 4A). Interestingly, a stronger signal was further achieved when both CBP and constitutive p110α were added, suggesting that CBP and Akt contribute to stabilize the ERβ/Mdm2 complex. Consistent with these results, disruption of the Akt responsive Thr-1872 of CBP using a T1872A mutant strongly impaired the ERβ/Mdm2 interaction (Fig. 4A). These results demonstrate that Thr-1872, which was reported to direct CBP activation to Akt [10], is required to facilitate the interaction between Mdm2 and ERβ in Akt-activated cells. The interaction between Mdm2 and ERβ was partly dependent on Mdm2 ligase activity, as the C462A mutant exhibits a reduced ability to interact with ERβ (Fig. 4A). ERβ Ser-255 is also involved in the interaction with Mdm2, as the S255A showed an impaired recruitment of Mdm2 (Fig. 4A), and the additive effect of

Akt and CBP in inducing such recruitment was absent (Supplementary Fig. S1), as opposed to wild-type ERβ (Fig. 4A).

The formation of the ERβ/Mdm2 complex was mainly dependent upon the integrity of Ser-186/188 of Mdm2 (Fig. 4B), correlating with their role on ERβ degradation (Fig. 3E). It was reported that the cytoplasmic-nuclear shuttling of Mdm2 was induced by Akt, and that Ser-166 and -186 were involved [24]. In order to determine whether impairment of the A2 mutant to interact with ERβ was dependent on its cellular localization, we performed nuclear coimmunoprecipitation assays on isolated nuclei. Results show that wild type and mutated A1 and A2 forms of Mdm2 were still able to maintain an interaction with ERβ, although to a lesser extent with the A2 mutant (Fig. 4C). In fluorescent microscopy, we found that disruption of Ser-186/188 was more efficient in sequestering Mdm2 into the cytoplasmic compartment, as compared to Ser-166 (Supplementary Fig. S2). These results suggest that the activation of Akt not only targets ERβ to complex with Mdm2, but facilitates the entry of Mdm2 into the nucleus to enhance its interaction with ERβ.

Following on its role in reducing ERβ levels, we next determined whether Mdm2 was able to promote ERβ ubiquitination. We found that activation of Akt induced the ubiquitination of ERβ, an effect potentiated by the presence of CBP (Fig. 4D). Both CBP knockdown using a lentiviral-based shRNA, and expression of the Akt-defective T1872A CBP mutant led to a decrease in ERβ ubiquitination, supporting an important

role for CBP in promoting ERβ ubiquitination by Akt. Interestingly, knockdown of Mdm2 markedly impaired ERβ ubiquitination induced by Akt and CBP, establishing ERβ as a suitable target of Mdm2 in these conditions (Fig. 4D). Again, Ser-255 appears to be important but not essential in signaling ERβ ubiquitination by Mdm2 (Fig. 4E), which is consistent with the reduced capacity of S255A to be degraded in a CBP and Akt-dependent manner (Fig. 2).

CBP promotes the degradation of ERβ by Mdm2

Our results highlight an essential role of CBP in promoting ERβ ubiquitination and degradation by Mdm2. Consequently, we addressed the importance of CBP and whether it can recruit Mdm2 in conditions in which ERβ ubiquitination is favored. Using a one-hybrid reporter assay with a Gal4-Mdm2 fusion construct, we found that activating Akt enhanced by 1.5 fold the reporter activity, suggesting that phosphorylation of Mdm2 by Akt promoted a transcriptional response (Fig. 5A). However, addition of CBP increased by near 4-fold such response, whereas the T1872A mutant was less efficient, indicating that Akt activation promotes the recruitment of CBP to Mdm2 in a manner dependent on Thr-1872 (Fig. 5A). The critical role of CBP Thr-1872 was further demonstrated in Western analysis and cycloheximide chase experiments, showing that CBP T1872A mutant prevented ERβ degradation in Akt-activated cells (Fig. 5B and Supplementary Fig. S3). We next determined which region

of CBP was involved in recruiting Mdm2 using a two-hybrid assay. With the prominent role of CH_3 Thr-1872 in the regulation of ERβ degradation, it was surprising to observe that the CH_3 domain did not contribute significantly to Mdm2 recruitment (Fig. 5C). However, of the other domains tested, the KIX showed the strongest interaction with Mdm2 in response to Akt activation. Interestingly, CBP KIX domain has been described to interact with phosphorylated CREB through a direct contact with KIX Tyr-658 [26]. We found that disruption of Tyr-658 in the KIX domain abrogated the Akt-dependent interaction with Mdm2 (Fig. 5C), pointing toward a recruiting role of CBP Tyr-658 to mediate the interaction with phosphorylated Mdm2. We also found that the recruitment of Mdm2 to CBP was dependent on Ser-186/188, possibly reflecting again the importance of these sites in Mdm2 nuclear shuttling (Fig. 5D). In addition, of the other nuclear receptor coactivators tested for their possible implication to promote ERβ downregulation in response to Akt activation, none were able to mimic the effect of CBP, even its ortholog p300 and coactivators such as SRC-1, p/CIP and PCAF, known to share to different degree HAT enzymatic activity and co-interaction with ERβ (Supplementary Fig. S4).

A negatively charged hinge region dictates human ERβ degradation to the Akt pathway

When aligned with human ERβ, Ser-255 of mouse ERβ corresponds to Asp-236 in their respective hinge region, suggesting the interesting possibility that the negative charge provided by the aspartic residue in hERβ could mimic a phosphorylated state such as in the mouse isoform. Furthermore, the conserved glutamic residue at position 237 can also contribute to provide a negatively charged environment in the hinge region of human ERβ. We thus tested whether human ERβ was able to behave in a similar fashion as to the mouse isoform under conditions of Akt activation. Indeed, hERβ levels were decreased in response to ErbB2/ErbB3 activation and CBP expression in a manner dependent on the Akt pathway and the 26S proteasome (Fig. 6A). Ubiquitination of hERβ was also strongly induced by CBP and activation of Akt, an effect dependent upon Mdm2 ligase activity and CBP Thr-1872 phosphorylation (Fig. 6B), indicating that hERβ is a target of Mdm2. To ascertain these effects to the two negative sites in hERβ hinge region, we observed that mutating either Asp-236 (D/A) or in combination with Glu-237 (DE/AA) to alanine residues prevented the downregulation of ERβ by CBP and Akt, with a stronger effect using the double mutant (Fig. 6C). These results identify Asp-236 and Glu-237 as important sites in the regulation of human ERβ turnover by the Akt pathway, and support a role for a negatively charged hinge region in that process.

Mdm2 supports an anti proliferative role of ERβ in breast cancer cells

The role of Mdm2 was monitored on growth of human MCF-7 breast cancer cells in response to heregulin-β. Maintaining cells in the presence of 50ng/ml heregulin-β markedly increased their proliferation over a period of 5 days without the prior need of estrogen (Fig. 6D). However, Mdm2 knockdown in MCF-7 cells significantly reduced the effect of heregulin-β as compared to shLuc control, suggesting an important contribution of Mdm2 in the proliferative potential of MCF-7 cells to heregulin-β. Under the same conditions, there was a decrease in the content of ERβ during treatment with heregulin-β, which was restored upon Mdm2 knockdown (Fig. 6E). Meanwhile, the levels of ERα were not as much affected following heregulin-β treatment and Mdm2 knockdown. These results demonstrate that an enhanced MCF-7 proliferative state correlates with a decrease in ERβ content, and that increasing ERβ by interfering with Mdm2 activity results in MCF-7 growth retardation despite the presence of heregulin-β.

Discussion

In this study, we demonstrate that activation of cell-surface tyrosine kinase ErbB2/ErbB3 receptors by growth factor heregulin-β triggers the phosphorylation of E3 ligase Mdm2 and coactivator CBP by the PI3-K/Akt pathway, resulting in a concerted effort in promoting the ubiquitination and degradation of ERβ by the 26S proteasome. In addition, this mechanism is regulated by a negatively-charged cluster within the hinge region of ERβ, thus adding to the propensity of ERβ to be down-regulated by such incoming survival signals that can sustain breast cancer cell growth.

ERβ activity is tightly controlled by phosphorylation and several sites, residing mostly within the AF-1 region, were identified to mediate ERβ response to estrogen and growth factors [3]. This study further identified mouse Ser-255 and corresponding human Asp-236 to play a crucial role along with human Glu-237 in regulating ERβ turnover to Akt activation. Both these sites are located within in the hinge region of ERβ and confer a negatively charged nature to this region (once phosphorylated for mERβ), which seems essential in mediating receptor degradation. Interestingly, these sites are part of an Arg-X-X-Ser/Asp-Glu hinge motif of ERβ, which is conserved in higher vertebrates, including cervidae, birds and primates, suggesting a shared mechanism in ERβ regulation. However, such motif is not present in ERα, which might explain that unlike ERβ, ERα was not subjected to the downregulation by CBP in the same conditions, although other mechanisms such as lysine modifications have implied the

hinge region in ERα degradation [27–30]. Among the other nuclear receptors that share the motif with ERβ, the estrogen-related receptor ERRβ also exhibits a conserved Arg-X-X-Ser-Glu sequence in the hinge region and similar to ERβ, its activity was also inhibited by CBP and Akt [10]. Interestingly, with the emerging role of ERR isoforms in breast cancer progression, the clinical status of ERRβ has been shown to correlate with that of ERβ in human primary breast tumors [31], raising the possibility that ERRβ might serve as a phosphorylation target of Akt and be regulated in a similar fashion as ERβ by CBP and Mdm2.

We previously reported that activation of ErbB2/ErbB3 receptors was able to weaken the transcriptional activity of ERβ and that CBP was involved in such repression [10]. Here, we demonstrate that the inhibitory response of ERβ by ErbB2/ErbB3 is closely associated to its degradation, implicating CBP as an important determinant in ERβ turnover. This is in contrast with the ability of CBP to act as a transcriptional activator of ERβ, as it was shown in response to EGF and Erk activation for example [7]. In such case, the phosphorylation of ERβ by EGF signaling occurred within the AF-1 domain, which then triggered CBP recruitment and transcriptional activation without the prior need of hormone [7,8]. However, the repression of ERβ by ErbB2/ErbB3 signaling was observed in the presence of CBP and implicated phosphorylation of the hinge region of mouse ERβ [10]. This illustrates the capacity of CBP to integrate in a dynamic fashion various signaling pathways through a preferred use of interacting sites on ERβ in

order to translate a transcriptional response. In this case, by regulating ERβ response to the Akt pathway, and by being itself subjected to phosphorylation at Thr-1872 in that process, we show that CBP acts as a scaffold protein not only restricted to transcriptional regulation but that also favors ERβ polyubiquitination through the recruitment of Mdm2, thereby providing an important role of CBP in ERβ turnover. Such dual effect has been reported for other nuclear receptor coactivators in situation of agonist-induced transcription. Notably, AIB1 was reported to mediate the activation and degradation of ERα by estrogen [32], both processes being closely associated with each other through the 26S proteasome pathway [16,17]. The E3 ligase E6-AP was also shown to exhibit dual activity toward ERα by inducing both its activity and degradation in response to hormone [33]. Here we show that CBP is directly involved in promoting ERβ polyubiquitination to non-hormonal cellular signals. In that respect, our findings are in agreement with the reported role of p300 to participate in tumor suppressor p53 ubiquitination and on its apparent ability to serve as an E4 polyubiquitin ligase [34,35]. In the case of ERβ, the reason why p300 remained inefficient is unclear, but the attractive idea that CBP may behave as an E4 ligase of ERβ, promoting its polyubiquitination under cellular anti-apoptotic signals, warrants further investigation to explain how CBP exhibits such an intrinsic versatility to regulate transcription in seemingly opposite ways.

The role of Akt in ERβ degradation by ErbB2/ErbB3 signaling was primarily established with the use of selective inhibitors and expression of dominant negative components of the PI3-K/Akt pathway. Such implication led us to identify the E3 ligase Mdm2 in mediating ERβ ubiquitination and degradation, establishing ERβ as a novel target of Mdm2. Mdm2 is well recognized to perform ubiquitination of tumor suppressor p53, a critical event that maintains homeostatic levels of p53 in normal unstressed cells but primarily leads to p53 suppression and cell overgrowth in several Mdm2-overexpressed or amplified types of cancers. Our identification of ERβ as a novel target of Mdm2 is in line with the emerging concept that Mdm2 contributes to tumor development through additional p53-independent mechanisms. As such, other Mdm2 substrates such as Rb, E2F1, FoxO3a and androgen receptor, have recently been described and associated to cell proliferation and tumorigenesis [36]. Mdm2 ligase activity is regulated by multisite phosphorylation of which Ser-166, 186 and 188 are included in a cluster that is responsive to growth factor/mitogenic signals [23]. We show that whereas Ser-166 seems dispensable, Ser-186 and 188 are essential in mediating the recruitment of Mdm2 to ERβ in response to Akt activation, mostly by inducing a relocalization of Mdm2 towards the nucleus. Such Akt-dependent nuclear shuttling of Mdm2 has been reported to involve Ser-166 and 186 in order to regulate p53 function [24]. This illustrates a common mechanism for Mdm2 availability shared by p53 and ERβ, and possibly other substrates, although some site selectivity seems to be required.

The increased interaction between ERβ and Mdm2 by the PI3-K/Akt pathway was potentiated by CBP, revealing a stabilizing effect of CBP on ERβ/Mdm2 complex formation. In addition to ERβ Ser-255 which participates in this interaction, the phosphorylation of CBP at Thr-1872 was essential to incite the interaction between ERβ and Mdm2, and also promote its own recruitment of Mdm2, providing a prominent role of Thr-1872 for ERβ/CBP/Mdm2 complex assembly. However, we found that Tyr-658 of the KIX domain, as opposed to Thr-1872 containing CH_3 domain of CBP, was directly implicated in contacting Mdm2 in these conditions, suggesting that an interdomain communication triggered by Akt is needed to confer a proper conformation of CBP to recruit phosphorylated Mdm2. Structural studies of CBP KIX domain have revealed a shallow hydrophobic groove stabilized by Tyr-658 which mediates interaction with phosphorylated CREB [26]. Whether such mechanism can also be envisioned for the recruitment of phosphorylated Mdm2 remains to be determined, but our data remain consistent with the reported recruitment of Mdm2 to p300 in p53 degradation, although the structural determinants may differ [37]. With our recent findings that Thr-1872 was identified as an Akt targeted site which dictates CBP intrinsic transcriptional activation potential in response to the PI3-K/Akt pathway [10], this study provides a functional relationship between the transcriptional activity of CBP and its ability to mediate protein degradation. The indispensable use of specific Akt sites of each component in order to maximize ERβ/CBP/Mdm2 assembly, leads us to propose

a mechanism by which an intersite phosphorylation switch regulates ERβ turnover in Akt-activated breast cancer cells. In this model presented in Fig. 7, ErbB2/ErbB3 receptor activation by heregulin-β provides the incoming Akt signal that mediates, in a concerted manner, Mdm2 nuclear translocation and activation, CBP phosphorylation and recruitment of Mdm2, and their interaction with ERβ through its negatively charged hinge region. Such assembly then promotes ERβ ubiquitination and degradation by the 26S proteasome pathway, resulting in a marked decrease in ERβ and enhanced proliferation of breast cancer cells.

Whereas ERα is considered the dominant subtype and correlates with most of the prognostic factors in breast cancers, the role of ERβ remains unclear. Overexpression of ErbB receptors is a frequent event in breast cancer and downstream signaling events that crosstalk with the ER pathway are strongly associated to endocrine resistance [4,38–40], a major obstacle in breast cancer treatment. In that respect, we showed that phosphorylation of ERβ by MAPK/Erk signaling pathway impacts its hormone-independent AF-1 transcriptional activity [7,8,21,41]. However, recent studies have added to the clinical value of ERβ in hormone-dependent cancers such as breast [42], ovarian [43] and prostate cancers [44], providing ERβ with antiproliferative functions. Our findings that increasing ERβ levels by Mdm2 knockdown resulted in MCF-7 growth retardation in the presence of heregulin-β are consistent with such activity of ERβ under pro-apoptotic conditions and bring important implications for the

pathophysiological assessment of breast tumors. Indeed, as ERα levels were less affected compared to ERβ in such setting, this raises the interesting possibility that Mdm2 activity can modulate the ERα/ERβ ratio in positive breast cancer cells. Mdm2 overexpression or amplification is a common event in breast cancer, and a higher ERα/ERβ ratio was reported in breast tumors compared to normal tissues, and was associated with an invasive phenotype [45–48]. Differential signaling between ERα and ERβ subtypes has also been demonstrated with estrogen and tamoxifen at the AP-1 response element in ER target genes [41,49], indicating that the ratio of ERα/ERβ is important in determining the response to selective ER modulators. More recently, increasing ERβ expression induced adhesion protein expression resulting in a reduced migration potential of breast cancer cells [50] and ERβ knockdown increased the growth of MCF-7 cells [51]. Although still debated, these studies raise the possibility that ERβ may possess tumor suppressor-like activity, where loss of ERβ could encourage tumorigenesis, and illustrate an unmet medical need to develop novel therapeutic strategies and tools to define the role of ERβ in cancer cell biology.

Our study identifies a novel mechanism that regulates ERβ function through an Akt-mediated phosphorylation switch involving CBP, which dictates ERβ activity and turnover to growth factor signaling pathways. The identification of ERβ as a novel target of Mdm2 also provides an opportunity of considering both as correlated biomarkers to

predict for a more successful indication of therapeutic responses and outcome of ER-positive tumors.

Materials and Methods

Plasmid Constructs—Expression of pCMX plasmids coding for ERα, ERβ, CBP, ErbB2, ErbB3, and luciferase reporter constructs vitA_2-ERE-tkLuc and UAStkLuc have been described previously [10]. The mouse ERβ Ser-255, human ERβ Asp-236 and Glu-237, and CBP Thr-1872 to alanine mutants were generated by PCR mutagenesis using *Pfu* polymerase (Stratagene) or *Pwo* DNA polymerase (Roche Diagnostics). All constructs were verified by automated sequencing. Expression plasmids coding for the constitutively active p110α catalytic subunit and the dominant negative p85α Δi-SH2-N subunit of PI3-K were a kind gift from J. Downward. The coding region of mouse Mdm2, generously provided by G. Ferbeyre, was inserted into respectively pCMX-HA and pCMX-Gal4 to produce N-terminal tagged constructs. The Mdm2 C462A ligase-deficient mutant, and the S166A (A1) and S186, 188A (A2) mutants were generated by mutagenesis as above. The CBP functional subdomains were obtained by PCR amplification and fused in-frame with the VP16 activation domain.

Cell Culture, DNA Transfection, and Luciferase Assay—Human embryonic kidney 293T cells and human breast cancer MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% and 10% fetal bovine serum (FBS) respectively. For transient transfection, cells were seeded in phenol red-free DMEM supplemented with 5% charcoal dextran-treated FBS and plasmid constructs were

introduced using the calcium phosphate precipitation method as described [10]. Typically, cells were transfected with 1.5 μg of DNA per well, containing 400 ng of reporter plasmid, 100 ng of receptor expression vector, 200 ng of CMX-βgal, 100 ng each of PI3-K, Akt and Mdm2 expression vectors, and 30 ng of CBP plasmid when indicated. After 5-8 h, the medium was changed and cells were treated with 10 nM estradiol (E_2; Sigma) for 16-20 h or left untreated. Cells were then harvested and luciferase assays were performed in duplicate from at least three independent experiments, and values were expressed as relative light units normalized to the β-galactosidase activity of each sample as previously described [10].

Western Analysis and Immunoprecipitation Assay— To determine ER protein levels, Western analysis was carried out in MCF-7 cells, or by transfecting 293T cells with HA-tagged or untagged ERα or ERβ (wt or mutants). Cells were then treated with 50 ng/ml heregulin-β1 (R&D System), 10 μM E_2 and/or 1 μM proteasome inhibitor MG-132 (Enzo Life Sciences) for 16h and harvested as described [10]. Immunoblotting was performed using specific antibodies to ERα and ERβ (Santa Cruz Biotechnology) or anti-HA antibody (12CA5), and signals revealed by chemiluminescence using appropriate horseradish peroxidase-conjugated secondary antibodies. In each experiment, total protein content was normalized using an anti-β-actin antibody (Novus Biologicals). Western analysis of phosphorylated and total Mdm2 was performed using respectively a monoclonal antibody against phosphorylated Ser-166 of Mdm2 (Cell

Signaling Technology) and an anti-Mdm2 polyclonal antibody (Santa Cruz Biotechnology). To analyze ERβ/Mdm2 interaction, coimmunoprecipitation analysis was performed in cells transfected with wild type or mutated ERβ (myc-tagged) in the presence of HA-tagged Mdm2 (wild-type and mutant). Immunoprecipitation of ERβ was performed as described [21], except that NaCl concentration was raised to 0.7 M and no SDS was added in the lysis buffer. The anti-HA antibody (Roche Diagnostics) was used for immunoblotting.

Ubiquitination of ERβ - To analyze the ubiquitinated forms of ERβ, 293T cells were transfected with human or mouse ERβ (wild type or mutated) in the presence or absence (negative control) of an HA-tagged ubiquitin encoding plasmid [21]. Plasmids for CBP (wt or T1872A), Mdm2 (wt or C462A), and PI3-K p110α were also added as indicated. Immunoprecipitation of ERβ and blotting with an anti-HA antibody were as described [21].

Cycloheximide Chase- 293T cells were transiently transfected with plasmids expressing HA-tagged wild type or S255A ERβ in absence or presence of PI3-K p110α and/or CBP plasmids. At 12 h after transfection, cycloheximide (Sigma) was added at a concentration of 50 μM and cells were lysed for Western analysis at the indicated time points. Each signal intensity, derived from three separate experiments, was quantified using an image analyzer (Alpha Innotech, San Leandro, CA) and expressed relative to β-actin levels.

Preparation of Nuclear and Cytoplasmic Extracts - Nuclear and cytoplasmic extracts were prepared by resuspending cells in hypotonic buffer as previously described [21]. The content of selective markers for nuclear (anti-nucleolin; Stressgen Bioreagents) and cytoplasmic (anti-β-actin; Novus Biologicals) compartments were tested by immunoblotting to ensure for the qualitative purity of the prepared fractions. Coimmunoprecipitation of ERβ and Mdm2 was achieved as described above using an anti-HA antibody (Roche Diagnostics) and an anti-myc (9E10) antibody.

Cell proliferation assay - Cell proliferation was measured by using the [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl] tetrazolium bromide (MTT) assay essentially as described [9]. Briefly, MCF-7 cells were seeded at low density in phenol red-free DMEM supplemented with dextran charcoal-treated serum in 24-well plates. The following day (day 0), treatments with 50 ng/ml heregulin-β and/or 10nM E_2 were started and added in fresh medium every subsequent day. For growth measurements, 0.5 mg/ml MTT was added to cells during 1–2 h, followed by extraction with 0.04 M HCl in 2-propanol. Absorbance was determined by spectrophotometry at 570 nm. All samples were assayed in triplicate from three to four independent experiments.

RNA Interference- Lentiviral knockdown of Mdm2 was performed essentially as described [41,52]. Small hairpin RNA duplexes targeting the sequence GGAATTTAGACAACCTGAA of human Mdm2 (shMdm2) were inserted in pLenti vector, a kind gift of C. Beauséjour. The shCBP encoding plasmid was kindly provided by C. Massaad. Viral particles were produced in 293T cells as described [41,52], and

used to infect MCF-7 cells. Mdm2 and CBP efficient knockdown were monitored by Western analysis (data not shown). A shRNA targeting luciferase (shLuc) was used as a negative control.

Acknowledgments

We thank members of the laboratory for critical reading and useful comments. M.S. is supported by a doctoral award from the FHSJ (Fondation de l'Hôpital Ste-Justine), N.P. by the FRSQ (Fonds de la Recherche en Santé du Québec) and the FHSJ, and K.S. by the GRUM (Groupe de Recherche sur le Médicament de l'Université de Montréal), the FHSJ and the CIHR (Canadian Institutes of Health Research). A.T. is a New Investigator of the CIHR. This work was supported by grants from the CIHR, the NSERC (Natural Sciences and Engineering Research Council of Canada), the Cancer Research Society Inc., and the Canadian Foundation for Innovation.

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Figure legends

Figure 1. ErbB2/ErbB3 receptors signal ERβ degradation by the 26S proteasome through the PI3-K/Akt pathway.

(A) Human 293T cells were transiently transfected with mouse ERβ in the presence or absence of plasmids encoding ErbB2, ErbB3, CBP, and p85α dominant negative form of PI3-K. Cells were then treated with 50ng/ml heregulin-β or left untreated for 16 h and harvested for Western analysis using an anti-ERβ antibody. Increasing concentrations of 26S proteasome inhibitor MG132 (1 and 10μM) were also used to treat cells. Loading was monitored with β-actin for each sample. **(B)** Similar as in (A) except that constitutive p110α PI3-K and Akt plasmids were used to trigger Akt activation. MG-132 was used at 1μM. **(C)** Western analysis similar as in (B). Cells were treated or not with 10 nM estradiol (E2) for 16 h. **(D)** 293T cells were transfected with an ERα expression plasmid in the same conditions as in (A), and harvested for Western analysis using an anti-ERα antibody. **(E)** Cycloheximide chase experiment in 293T cells expressing ERβ in absence or presence of CBP and p110α PI3-K plasmids. Cells were treated with 50μM cycloheximide (CHX) and lysed at the indicated time points for Western analysis. Results are normalized to β-actin content and expressed as a percentage of change of time zero, which was set at 100%.

Figure 2. Serine 255 in the hinge region regulates ERβ proteasomal degradation by the PI3-K/Akt pathway.

(A) Western analysis of 293T cells transfected with ERβ S255A mutant, in the presence or absence of ErbB2, ErbB3, CBP, and p85α PI3-K plasmids. Cells were treated with 50ng/ml heregulin-β or left untreated for 16 h. β-actin was monitored for protein loading. **(B)** Similar as in (A) except that a p110α PI3-K plasmid was used. **(C)** Cycloheximide chase experiment in cells expressing the ERβ S255A mutant in absence or presence of CBP and p110α PI3-K. Cells were treated with 50μM cycloheximide and lysed at the indicated time points for Western analysis. Results are normalized to β-actin content and expressed as a percentage of change of time zero, which was set at 100%. **(D)** Validation of cell fractionation into cytoplasmic (C), soluble nuclear (N) and nuclear matrix (M) protein extracts by Western analysis using antibodies against nucleolin and β-actin markers. **(E)** Western analysis of HA-tagged wild type and S255A mutated ERβ in fractionated cell compartments. Transfected 293 cells were fractionated into cytoplasmic, soluble nuclear and nuclear matrix preparations as in (D), and analyzed by Western using an anti-HA antibody. An Akt-unresponsive T1872 CBP mutant was also used in transfection.

Figure 3. Activation of the E3 ubiquitin ligase Mdm2 by Akt promotes ERβ downregulation and transcriptional repression.

(A) 293T cells transfected with increasing concentrations of Mdm2 exhibit a decrease in ERβ levels as determined by Western analysis. The E3 ligase-deficient C462A mutant of Mdm2 was also tested. β-actin was monitored for control loading. **(B)** Similar as in (A) except that the S255A ERβ plasmid was used. **(C)** The transcriptional activity of wild type and S255A mutated ERβ was determined by luciferase reporter assay using an ERE$_{tk}$Luc reporter. 293 cells were transfected with or without the indicated plasmids and treated with vehicle or 10nM E2 for 16 h. Cells were then harvested for luciferase activity measurements, and values normalized to β-galactosidase activity and expressed as fold response compared with untreated cells set at 1.0. **(D)** The PI3-K/Akt induces endogenous Mdm2 phosphorylation in 293T cells. Cells were transfected with the respective plasmids for ErbB2/ErbB3, constitutive p110α and dominant negative p85α PI3-K as indicated, and then treated or not with 50ng/ml heregulin-β and increasing concentrations (5 and 10 nM) of the PI3-K inhibitor LY294,002 for 20 min. Mdm2 phosphorylation was monitored using a specific anti-phospho Mdm2. **(E)** Downregulation of ERβ is dependent upon Ser186/188 of Mdm2. Western analysis of HA-tagged wild type and S255A mutated ERβ in response to expression of Mdm2 mutants S166A (A1), and S186/188A (A2), compared to wild type. The p110α PI3-K was used to constitutively activate Akt. Samples were analyzed by Western blot with an anti-HA antibody and protein loading was monitored with β-actin.

Figure 4. Mdm2 interacts with ERβ and promotes its ubiquitination following Akt activation.

(A) Mdm2 coimmunoprecipitates with ERβ. 293T cells were transfected with HA-tagged ERβ in absence or presence of myc-tagged Mdm2, along with CBP and PI3-K constructs as indicated. Immunoprecipitation (IP) was carried out with an antibody specific to ERβ and Mdm2 was detected by immunoblotting analysis (IB) using an anti-myc antibody. ERβ was monitored in each sample using an anti-HA antibody. An HA-tagged S255A ERβ was also tested in the same conditions, as well as the ligase-deficient C462A Mdm2. **(B)** Ser186/188 of Mdm2 is required to mediate interaction with ERβ. Immunoprecipitation assay similar as in (A) to test the ability of Mdm2 S186/188A mutant compared to wild type in response to PI3-K/Akt activation. **(C)** Nuclear coimmunoprecipitation assay on isolated nuclei of cells expressing wild type, S166A (A1) and S186/188A (A2) Mdm2 in the presence of ERβ, CBP and p110α PI3-K. Immunoprecipitation was carried out using an Mdm2 antibody and immunoblot with an ERβ antibody. Mdm2 content was normalized in each nuclear fraction. **(D)** Mdm2 mediates the ubiquitination of ERβ in response to Akt activation. 293T cells were transfected with myc-ERβ in the presence of HA-ubiquitin, and ubiquitination of ERβ was detected by immunoblotting using an HA antibody on immunoprecipitated ERβ extracts. CBP (wt and T1872A) and P110α PI3-K were added as indicated. Knockdown of specific targets was achieved by lentiviral infection using shCBP or shMdm2 RNAs,

with shLuc (luciferase) used as a negative control. ERβ content was normalized in each sample using a Myc antibody. **(E)** Ser-255 is needed to achieve maximal ERβ ubiquitination under Akt activation. Ubiquitination assay performed similarly as in (D) of wild type and S255A myc-tagged ERβ in response to CBP and PI3-K/Akt activation.

Figure 5. The Thr-1872 Akt site of CBP is required to mediate Mdm2 recruitment and ERβ degradation.

(A) Mdm2 interacts with CBP in an Akt-dependent manner. One-hybrid assay using Gal4-Mdm2 fusion in 293T cells transfected with a UAStkLuc reporter. CBP (wt and T1872A) and PI3-K/Akt plasmids were added to measure their effect on luciferase reporter activity. Values are normalized to β-galactosidase activity and expressed as fold response compared with control cells set at 1.0. **(B)** CBP Thr-1872 is involved in ERβ degradation by the PI3-K/Akt pathway. Cycloheximide chase experiment in 293T cells expressing ERβ. The effect of wt and T1872A CBP were monitored on ERβ levels following treatment with 50μM cycloheximide. Cells were lysed at the indicated time points for Western analysis. Results are normalized to β-actin content and expressed as a percentage of change of time zero, which was set at 100%. **(C)** The interaction of CBP with Mdm2 requires Tyr-658 of the KIX domain. Two-hybrid assay of cells transfected with Gal4-Mdm2 and VP16-fused CBP subregions representing various functional domains (depicted on top). Luciferase activity was measured on a UAStkLuc reporter in response to activation of the PI3-K/Akt pathway. Results are normalized to β-

galactosidase activity and expressed as fold response relative to empty VP16 construct set at 1.0. **(D)** Ser-186/188 of Mdm2 are required to coimmunoprecipitate CBP. Immunoprecipitation of CBP and CBP T1872 was carried out in 293T cells with an antibody specific to CBP and samples were immunoblotted for the presence of myc-Mdm2 or -S186/188A (A2) mutant using a myc antibody. CBP content was normalized by Western analysis in each sample.

Figure 6. The Akt-induced activation of Mdm2 by heregulin-β promotes human ERβ degradation and increases proliferation of breast cancer cells.

(A) Human ERβ is degraded by CBP and the PI3-K/Akt pathway upon activation of ErbB2/ErbB3. Western analysis of ERβ in 293T cells transfected with ErbB2, ErbB3, CBP, and p85α PI3-K constructs as indicated. Cells were treated with vehicle or 50ng/ml heregulin-β in presence or absence of increasing concentrations (1 and 10μM) MG-132. β-actin was used to monitor loading **(B)** CBP and the PI3-K/Akt pathway promotes human ERβ ubiquitination by Mdm2. 293T cells were transfected with myc-ERβ in the presence of HA-ubiquitin, and ubiquitination of ERβ was detected by immunoblotting using an HA antibody on immunoprecipitated ERβ extracts. CBP (wt and T1872A) and P110α PI3-K were added as indicated. ERβ content was normalized in each sample using a Myc antibody. The ligase-defective C462A mutant of Mdm2 was also tested. **(C)** A negatively charged hinge region mediates human ERβ degradation to the PI3-K/Akt pathway. Western analysis of 293T cells expressing wild type, D236A

(D/A) or D236A/E237A (DE/AA) mutants of ERβ. CBP (wt or 1872A) and p110α PI3-K plasmids were also added as indicated. β-actin was used to control loading. **(D)** Mdm2 supports the growth of breast cancer cells by heregulin-β. MTT proliferation assay was done on human breast cancer MCF-7 cells treated or not with 50ng/ml heregulin-β starting at day 0 and maintained over a period of 4 days. Cells were harvested each day for MTT reduction colorimetric assay. Results are expressed as percent change from untreated cells set at 100% and represent mean values of triplicate measurements from three to four independent experiments. Lentiviral infections with shLuc or shMdm2 were carried out 24 h prior to treatments (day -1). **(E)** Heregulin-β promotes ERβ degradation by Mdm2 in MCF-7 cells. Western analysis of ERα and ERβ in MCF-7 cells treated or not with 50ng/ml heregulin-β was performed as in (D). Samples were collected at day 4 of treatments and analyzed with respectively an ERα or ERβ antibody. Total Mdm2 content was also monitored with an antibody against Mdm2. β-actin was used to control loading. Lentiviral infections or shLuc and shMdm2 constructs were carried out as in (D).

Figure 7. A schematic model of ERβ degradation by the 26S proteasome involving an Akt-concerted intersite phosphorylation switch.

Activation of ErbB2/ErbB3 receptors by heregulin-β signals the PI3-K/Akt pathway that induces a concerted effort of interacting partners to target ERβ to the 26S proteasome system of degradation. These include the E3 ligase Mdm2 which following Akt

activation translocates to the nucleus and interact with ERβ in a process involving Ser-186 and -188 Akt sites and coactivator CBP. CBP acts as a scaffold protein to further enhance and stabilize the Mdm2/ERβ complex through phosphorylation of Thr-1872, an Akt-targeted site essential for CBP intrinsic response and recruiting ability toward ERβ and Mdm2. The phosphorylation of mouse ERβ Ser-255 by Akt provides a negatively charged environment within the hinge region which accordingly exists in human ERβ with corresponding Asp-236 and conserved Glu-237, and facilitates Mdm2 recruitment in Akt-activated cells. Such assembly then promotes ERβ ubiquitination and degradation by the 26S proteasome pathway, resulting in a marked decrease in ERβ and enhanced proliferation of breast cancer cells in response to mitogenic/survival signals.

FIGURES

Fig. 1

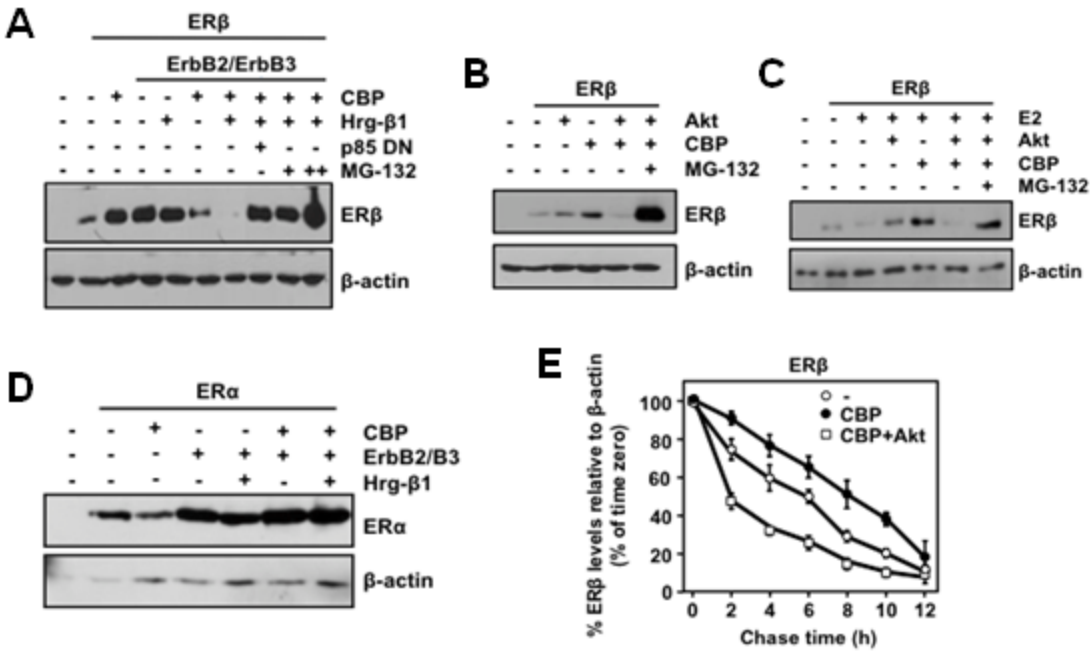

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Fig. 2

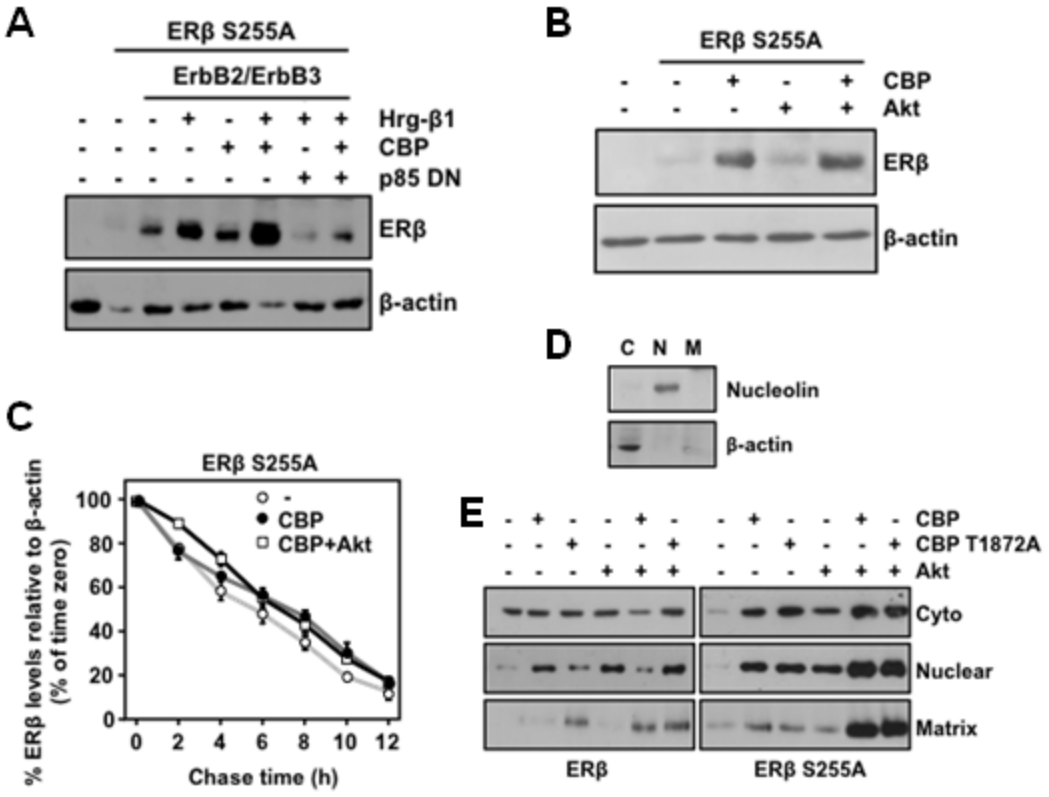

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Fig. 3

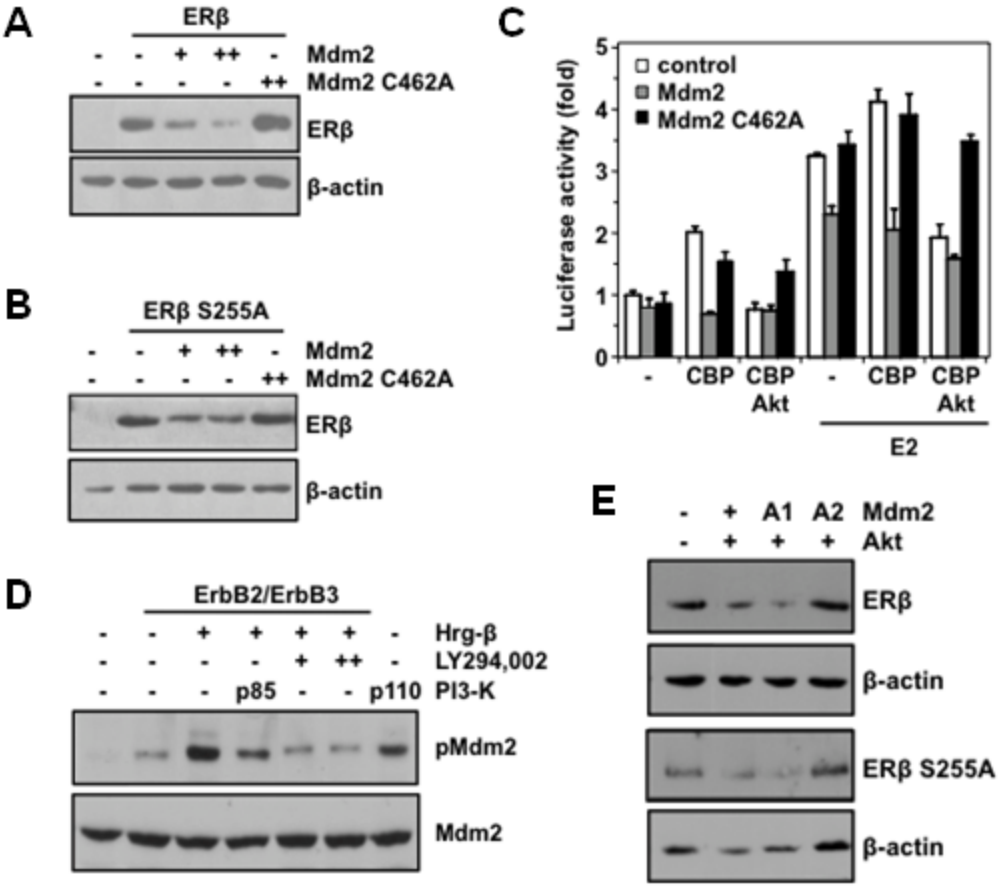

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Fig. 4

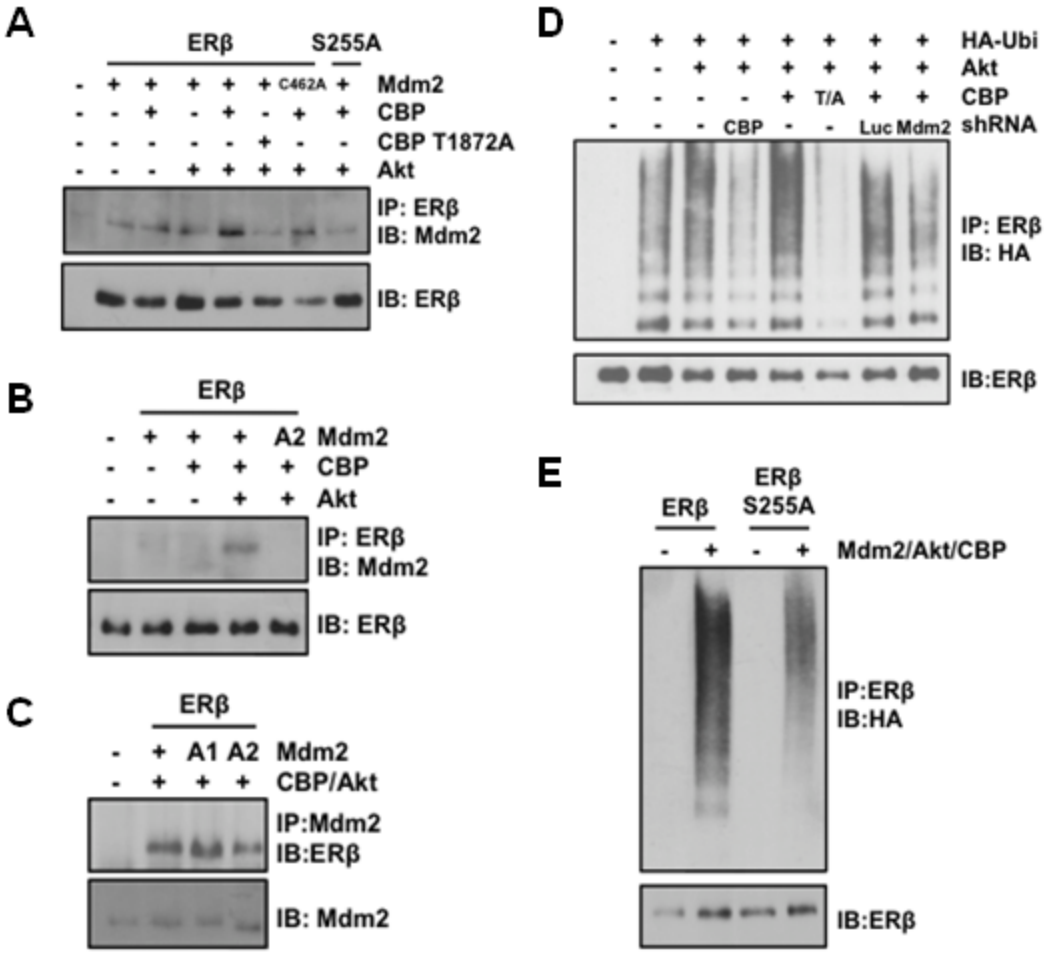

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Fig. 5

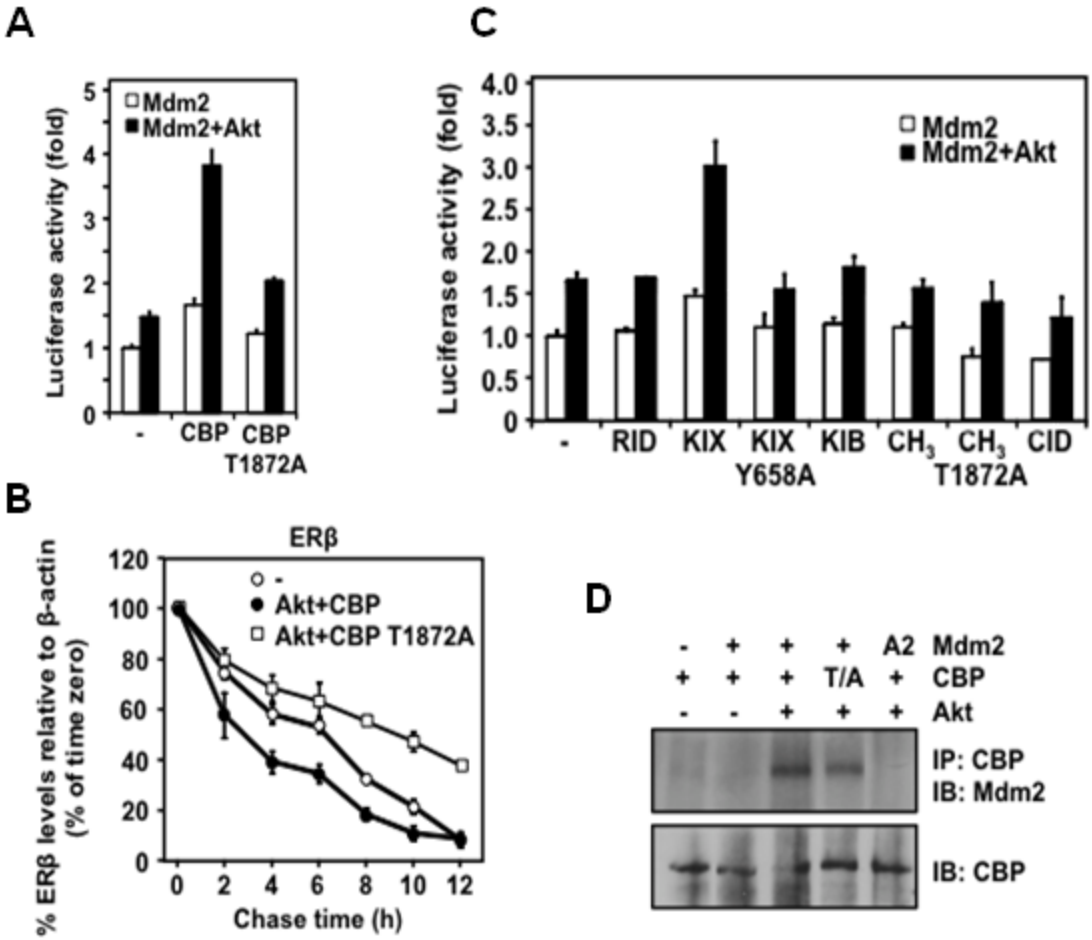

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Fig. 6

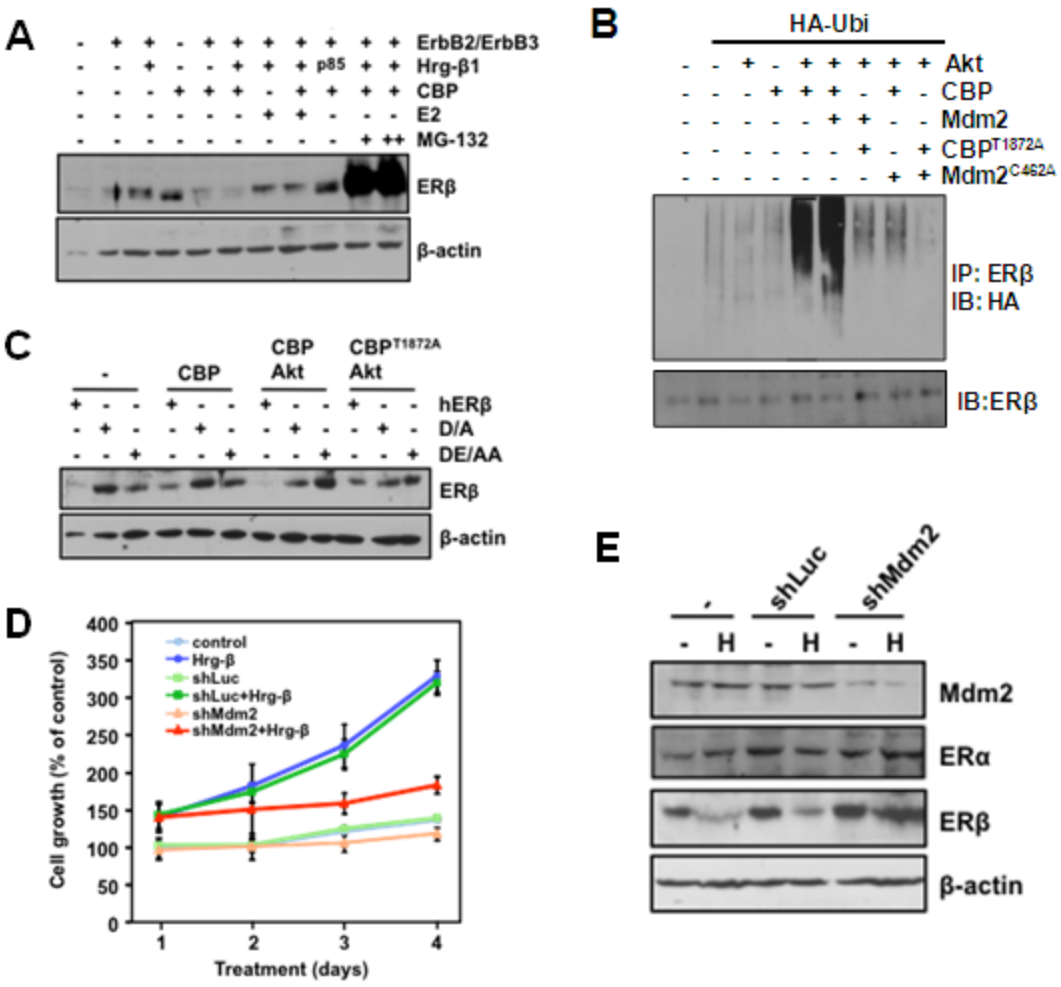

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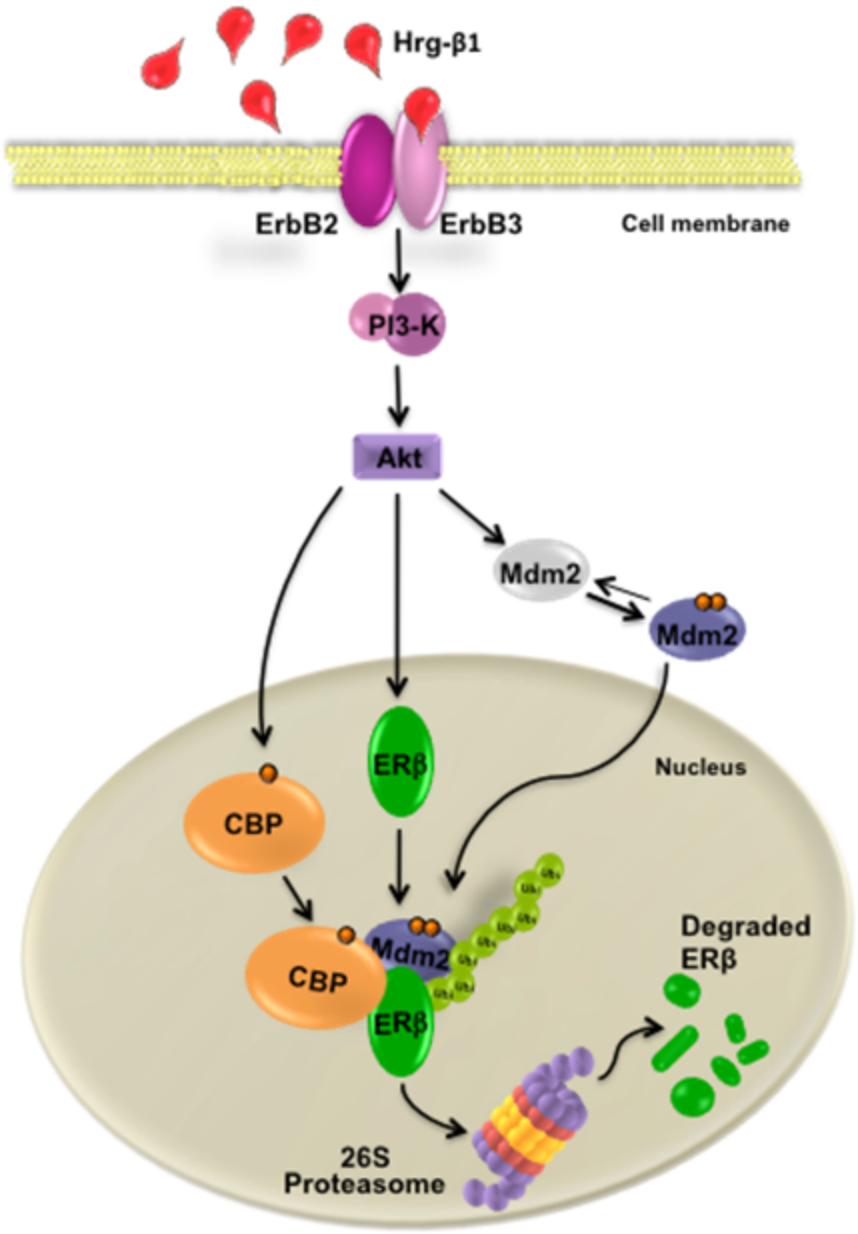
Fig. 7



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Supplementary figure S1
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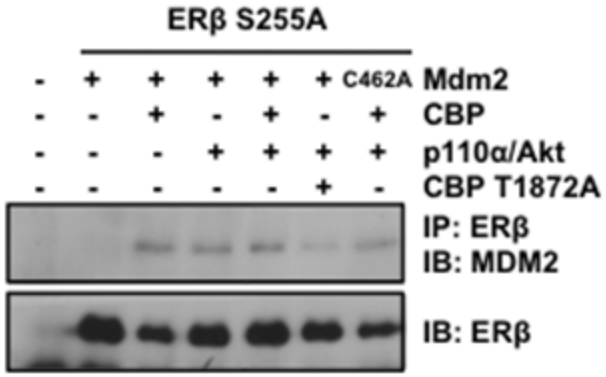

Fig.S1. Mutation of Ser-255 of ERβ prevented the additive effects of CBP and Akt on Mdm2 reruitment.
293T cells were transfected with HA-tagged mouse ERβ S255A mutant with or without myc-Mdm2, myc-Mdm2 ligase dead C462A, CBP, and CBP Akt-defective T1872A constructs. To activate the Akt pathway, constitutive p110α PI3-K and Akt plasmids were added. Immunoprecipitation (IP) was carried out with an antibody specific to ERβ and Mdm2 analyzed by Western analysis (IB) using an anti-myc antibody. Total ERβ content was normalized with an HA antibody.

Supplementary figure S2
Sanchez et al.

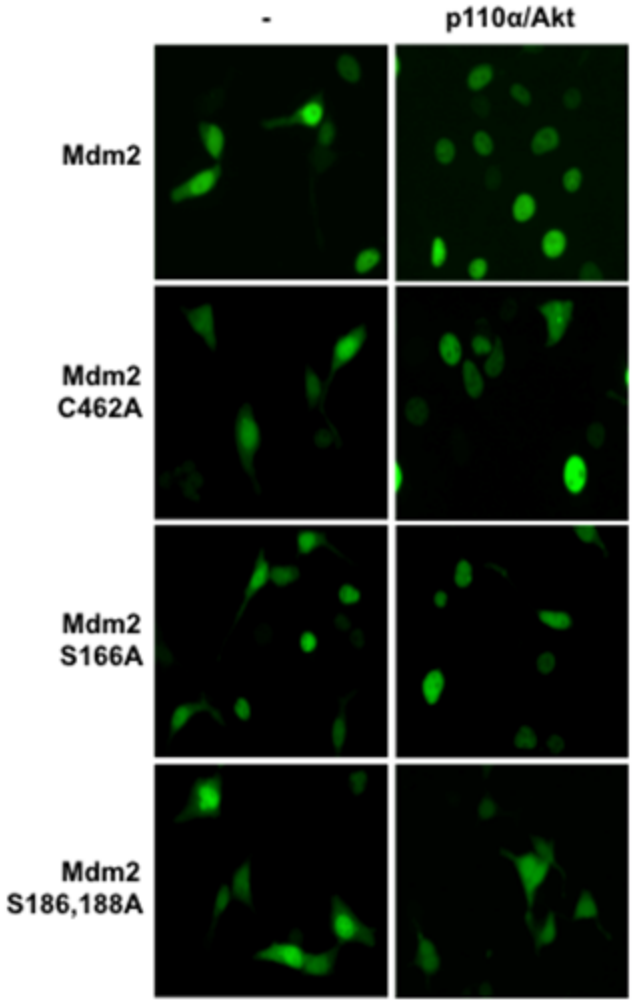

Fig.S2. Subnuclear localization of Mdm2 and mutants in response to the Akt pathway.
293-T cells were seeded on coverslips and transfected with YFP-fused expression plasmids for wt and mutated Mdm2. Akt was activated by cotransfecting cells with constitutive p110α PI3-K and Akt plasmids. Cells were examined in fluorescence with excitation/emission filters of 480/535 nm.

Supplementary figure S3
Sanchez et al.

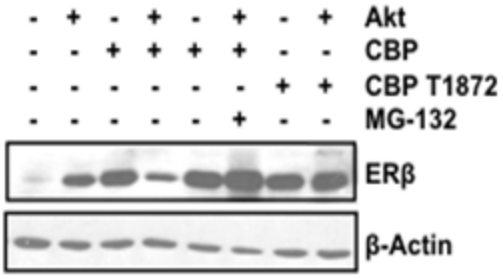

Fig.S3. CBP Thr-1872 is required in ERβ downregulation by the Akt pathway.
293T cells were transfected with ERβ in presence or absence of wt and T1872A mutated CBP. Western analysis was performed using an ERβ antibody. Control loading was monitored with β-actin. Proteasome inhibitor MG-132 was used at 1μM

Supplementary figure S4
Sanchez et al.

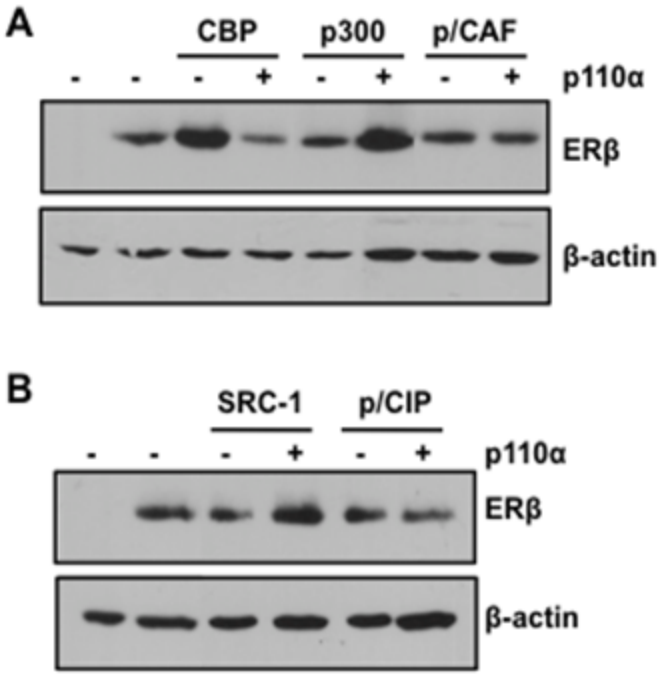

Fig.S4 Regulation of ERβ levels by nuclear receptor coactivators in response to Akt activation.
(A) and (B) 293-T cells were transfected with HA-tagged ERβ in the presence of plasmids encoding coactivators CBP, p300, p/CAF, SRC-1 or p/CIP. Akt was activated by adding constitutive p110α PI3-K construct. ERβ levels were monitored using an HA antibody and protein loading with a β-actin antibody.

CHAPTER 3: GENERAL DISCUSSION, PERSPECTIVES AND CONCLUSIONS

1 ErbB2/ErbB3regulation of ERβ

One of the major contributions of the work presented here is the unraveling of a previously unknown mechanism which differentially regulates ERα and ERβ and provides a greater comprehension of ER regulation during the activation of ErbB receptor signaling. ErbB2/ErbB3 partnering has been described to be the most powerful combination within the family of ErbBs observed in many poor-prognostic cancers (Karunagaran et al., 1996). In fact, studies have demonstrated that ErbB2/ErbB3 constitutive signaling correlates with the development of resistance to anti-hormone treatment in ER positive cell lines labeling them aggressive tumors. The mechanisms leading to the eventual loss of ERs hormone-responsiveness following ErbB deregulation is still not clear, however the results exposed in this thesis suggest a potential mechanism by which a shift in ERα/ERβ ratio can affect ER-positive cell proliferation in the absence of estrogen and therefore possibly lead to a hormone-independent state of proliferation, a severe outcome of prolonged anti-estrogen treatment in hormone-dependent cancers.

One of our main objectives was to examine the behavior of ER subtypes following the activation of ErbB2/ErbB3 by growth factors. The first article presented in this thesis reveals an event by which ERα and ERβ are selectively regulated due to the activation of certain kinase pathways and the presence of coregulatory complexes. We sought to investigate how the selectivity in responses was established between ERα and ERβ.

We found that subtype specificity relies on the amino acid sequence of nuclear receptors. Although the AF-1 was initially thought to be involved, our studies identify the hinge domain as a discriminatory region of nuclear receptor response to cellular events. On the premises of these results our next objective was to identify the mechanisms involved in the differential regulation of ERβ and ERα. We uncovered that ERβ unlike ERα was targeted towards the ubiquitin-proteasomal machinery following ErbB activation. Decrease in the levels of ERβ correlated with an increase in the proliferation of breast cancer cells treated with the ErbB3 ligand, HRG-β1, demonstrating that the ERα/ERβ ratio is an important parameter which can define the proliferation potential of breast cancer cell lines.

1.1 Activation of ErbB2/ErbB3 in hormone-independent cancers

Endocrine therapy is the predominant treatment option for women diagnosed with ER-positive breast cancer. Yet, high levels of ER-expressing tumors lead to endocrine therapy resistance. Overexpression of the ErbB family of growth factor receptors has been associated with resistance to endocrine therapy. In fact, overexpression of ErbB3 has been observed in 17% of breast tumors (Witton et al., 2003; Naidu et al., 1998) while ErbB2 is amplified in 20-30% of breast tumors and correlates with increased proliferation, higher metastatic potential, accelerated relapse and poor patient prognosis (Slamon et al., 1987; Ross and Fletcher, 1998). Although ErbB2 can dimerize with any of the three other ErbB members, it preferentially heterodimerizes with ErbB3 creating a powerful and prevalent signal transducer accounted in several breast cancers (Introduction section 5.3) (Karunagaran et al., 1996; Citri et al., 2003). Stimulation of ErbBs leads to the activation of particular signaling pathways determined by the ligand and receptor homo/hetero dimerization (Figure 20) (Introduction section 5.2).

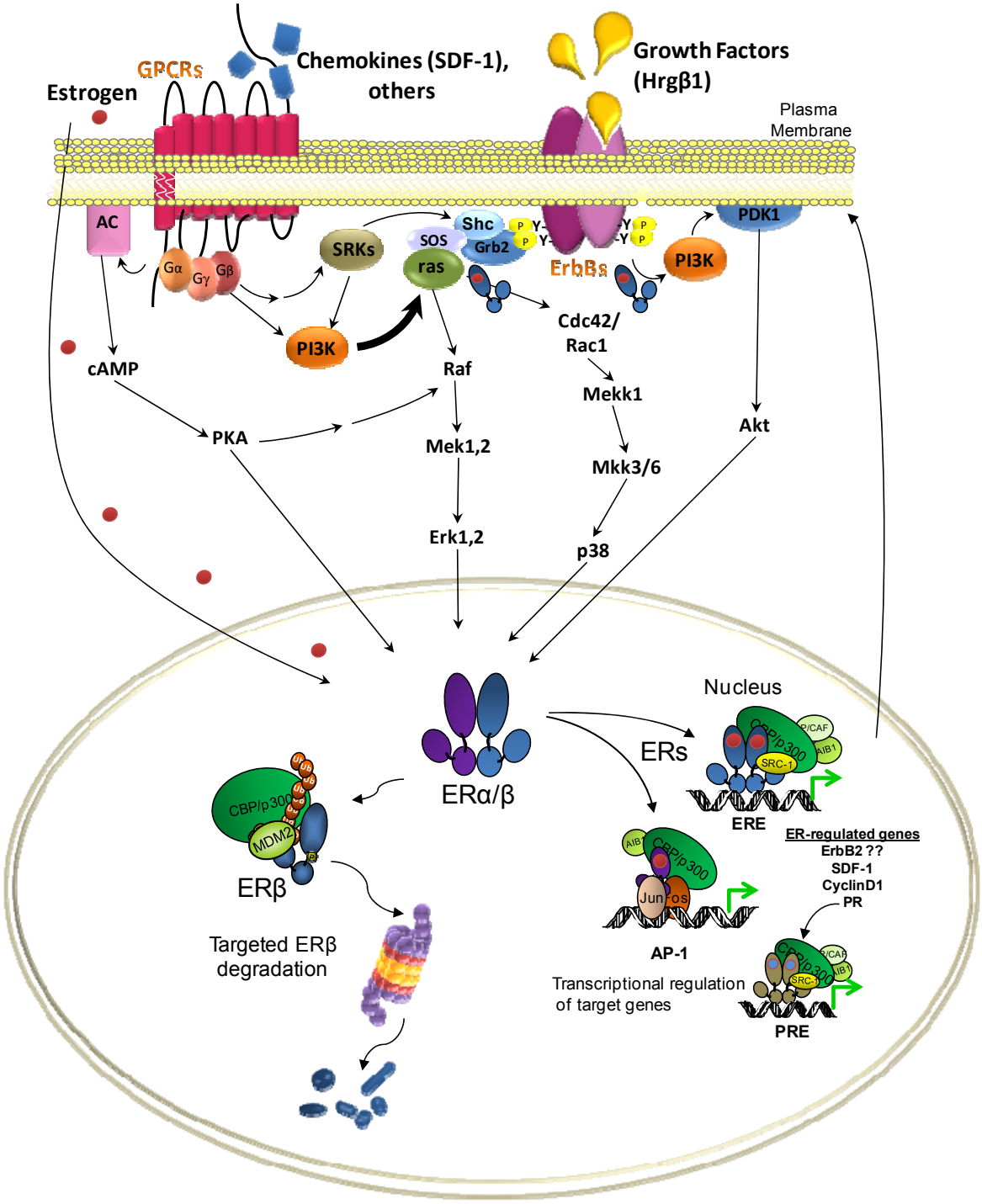

Figure 20 Summary of signaling pathways described in the introductory chapters, unraveled by our studies regulating the transcriptional activity (classical and non-classical) and the non-genomic activities of ERα and ERβ following membrane receptor activation, with a particular emphasis on ErbB2 (dark pink)/ErbB3 (light pink) RTK activation. Figure was adapted from (Sanchez et al., 2009).

1.1.1 Intracellular pathways regulating ERs activity

The regulation of downstream factors (such as ERs) not only depends on cellular content but also which extracellular cues are translated to intracellular signaling pathways (Figure 20). Interaction of growth factors, such as Hrgβ1, with ErbB3 promotes heterodimerization with ErbB2 which has been associated with the activation of the PI3K/Akt pathway (Motoyama et al., 2002; Hutcheson et al., 2007). Similarly we observed that Hrgβ1 was able to activate ErbB2/ErbB3 in our model of transfected 293T cells which caused the activation of the PI3K/Akt pathway. It is however important to consider that ErbB2 can equally activate MAPK pathways and such activation has been shown to stimulate the transcriptional activity of ERα and ERβ by targeting specific sites within the N-terminal, AF-1 domain of both receptors (Figure 20). However results from our first study revealed a down-regulation of ERβ transcriptional activity that was ligand-dependent and independent, suggesting that estrogen cannot relieve the inhibitory effect of the PI3K/Akt pathway, following HRGβ1 treatment. Although both pathways can be activated, Akt and PI3K mutants (containing a dead kinase activity) and PI3K inhibitor LY23213 completely abrogated the observed effects, strongly suggesting that the outcome observed for ERβ was primarily induced by the PI3K/Akt pathway.

Nonetheless ERα was activated under these conditions, suggesting that Akt signaling could positively regulate this subtype. In addition we do not exclude the possible participation of the MAPK pathway which our laboratory observed (Sauvé et al., 2009; St Laurent et al., 2005a) to be activated by ErbB2/ErbB3, yet we stipulate that the PI3K/Akt pathway is the predominant activated pathway as inactivation of the MAPK pathway did not completely relieve the transcriptional repression by the activation of the ErbB2/ErbB3 pathway (St Laurent et al., 2005a).

It would be interesting to examine if ErbB2 does indeed participate in the degradation of ERβ as previous results demonstrate that specifically blocking its signaling capacities does partially relieve the inhibition on ERβ (St Laurent et al., 2005a). This could imply that possibly other signaling pathways could participate with Akt to inhibit the activity of ERβ whilst still promote transcriptional activation through ERα.

1.2 Downregulation of ERβ is intensified by CBP

Downregulation of ERβ by the activation of Akt was further decreased in the presence of CBP. Activation of intracellular kinase signaling cascades leads to the phosphorylation of several cytoplasmic and nuclear targets which control cell fate. The decrease observed in the transcriptional activity of ERβ whether ligand-dependent and /or independent was more pronounced by the presence of CBP. CBP and its heterologue p300 are functional integrators of multiple signal transduction pathways and diverse transcription factors compete with each other to interact with a limiting amount of CBP/p300 within the cell (Lemasson and Nyborg, 2001; McKay and Cidlowski, 2000). However CBP/p300 can regulate diametrically opposite processes, from cell proliferation to growth arrest. They are required for the transcription of oncogenic transcription factors contributing to cell growth, transformation and development (Goodman and Smolik, 2000). Yet they can act as tumour-suppressors by increasing the expression of apoptotic genes such as the ones controlled by BRCA1 and p53 (Kung et al., 2000; Pao et al., 2000; Gu and Roeder, 1997). The ability of CBP/p300 to serve as mediators for cell proliferation of growth arrest has been proposed to be highly dependent on cellular environment (Goodman and Smolik, 2000).

1.2.1 The power of switching on and off transcription by CBP

CBP is recognized as a coactivator to transcription factors, however reports show that CBP or its homolog, p300, can negatively affect the activity of transcription factors by switching from one promoter to another. Competition between different transcription factors for CBP/p300 plays a role in the coordination of gene expression in response to

signaling (Kamei et al., 1996). Indeed phosphorylation of CBP/p300 by the IκB Kinase complex, IKKα, potentiates its coactivating function on the NF-κB promoter leading to the inhibition of p53 transcriptional regulation (Huang et al., 2007) as a result of a decrease in the association of CBP from p53 following IKKα phosphorylation. This also indicates that different signaling pathways can interfere with one another and modulate the availability of CBP for a particular transcriptional complex via posttranslational modifications (Chapter 7). In agreement with this concept, it was shown that CBP methylation by CARM1 causes a transcriptional switch from CREB-regulated to nuclear hormone receptors regulated gene-expression (Xu et al., 2001). In fact several posttranslational modifications of CBP have been reported to regulate its activity. SUMOylation of p300 by Ubc9 (Girdwood et al., 2003) has the ability of inhibiting the coactivating activity of p300 by the recruitment of HDAC complexes. Phosphorylation of CBP/p300 within its C-terminal domain (figure 16) amplifies its HAT activity (Introduction, section 6.1.2) stimulating the transcriptional activity of substrates during neuronal differentiation (Ait-Si-Ali et al., 1999). In addition Akt was shown to target p300 phosphorylation at Ser-1384 which was capable of increasing its HAT activity, contributing to inflammatory gene expression by TNF-α (Huang and Chen, 2005).

We observed that the presence of CBP was required to inhibit the activity of ERβ. Although we detected an increase in the activation of CBP's coactivating potential (Chapter 2, section 1, figure 5) there was a marked decrease in the transcriptional activation of ERβ, which initially lead us to believe that CBP was possibly recruited to other promoters resulting in a downregulation of the activity of ERβ. Coimmunoprecipitation assays reflected a decrease of the interaction between ERβ and CBP when the Akt pathway was activated. The treatment of estrogen was not capable of altering the interaction, substantiating our initial results that ligand-binding could not activate ERβ during Akt signaling. In order to support the hypothesis of a switch mechanism by CBP following Akt activation, we looked at the steady-state levels of both CBP and ERβ. CBP levels were not

affected by the activation of Akt following Hrβ1 treatment (chapter 2, section 1, Figure 6C). Akt is effectively a positive regulator of CBP. It is able to interact with p300 within the C-terminal domain where the Akt consensus phosphorylation site is present (1459-1892 aa) and maintain steady-state levels of the coactivator (Chen et al., 2004). However we observed a decrease in the levels of ERβ, which we discovered to be subtype specific, as ERα levels were not affected. Our experiments in chapter 2, section 2, demonstrate that in fact the decrease of the levels of ERβ is due to the targeted degradation of receptor.

Therefore it seems apparent that CBP could play two distinct roles on each ER subtype. It could promote degradation of ERβ while simultaneously activate ERα. However considering the low expression levels of CBP and results demonstrating the requirement of CBP to degrade ERβ, in a context where both are expressed, we could speculate that possibly p300, which is equally targeted by Akt (Chen et al., 2004), could participate in the transcriptional activation of ERα. We looked at other coactivators, such as p300, to observe if they were also capable of regulating negatively ERβ. However not only did p300 not induce a downregulation of steady-state levels of ERβ but other coactivators of the SRC family did not decrease the levels of ERβ either, strongly pointing out a specific role for CBP in the downregulation of ERβ. However, it would be interesting to verify if both p300 and CBP are recruited to ERα-bound promoters, by Chromatin Immunoprecipitation assays, giving us an indication of simultaneous behaviour of coactivators following Akt activation.

1.2.2 ER regulation following post-translational regulation

Since the results demonstrated the requirement of CBP in Akt-mediated downregulation of ERβ was not that of a coactivator, we investigated other possibilities for the role of CBP. The first case reporting a role for acetylation in the control of protein stability was observed by the HAT activity of the coactivator PCAF on the function of E2F-1. PCAF-mediated acetylation of E2F-1 increased the stability of the protein (Ianari et al.,

2004; Martinez-Balbas et al., 2000). Although the underlying mechanisms remain unknown, we also observed a stabilisation of ERβ in the presence of CBP but in the absence of Akt activation (chapter 2, section 2, Fig 1). Other observations point to acetylation as a signal for protein degradation. In fact, both phosphorylation and acetylation collaborate to target the degradation of substrates. Indeed, both posttranslational modifications have been observed to be implicated in the regulation of protein degradation via the ubiquitin/proteasome pathway of the haematopoietic transcription factor GATA-1 (Kouzarides, 2000; Glickman and Ciechanover, 2002). Acetylation and MAPK phosphorylation were both required to trigger ubiquitin-proteasome targeted degradation of GATA-1 (Hernandez-Hernandez et al., 2006).

As our results indicate, the presence of CBP followed by the activation of Akt led to the targeted degradation of ERβ by the 26S proteasome complex, which was corroborated following the treatment of the proteasome inhibitor, MG-132 (chapter 2, section 2, figure 1A). In fact ERβ was strongly ubiquitinated following the activation of Akt in the presence of CBP. This HAT has previously been described to participate in the degradation of proteins such as E2F-1 following DNA-damage (Galbiati et al., 2005) and its HAT activity, although does not stimulate degradation, acetylates and inhibits the activity of STAT1 through the recruitment of HDAC3 (Kramer et al., 2009). We did not look at the levels of acetylation of ERβ in order to detect if its acetylation was required for its ubiquitination to take place. Mutation of the HAT domain within CBP or verifying by the levels of ERβ acetylation we could obtain further information on the role of CBP in the degradation of ERβ. We did observe however that an Akt-unresponsive CBP, lead to the recuperation of the levels of ERβ even during Akt activity. Also, HAT containing proteins could promote the interaction of acetylated substrates with E3-ligases (Introduction, section 7). The degradation of HIF-1α occurs after its interaction with pVHL, an E3-ligase complex, which mediates its ubiquitination (Ke and Costa, 2006). The acetylation of HIF-1α promoted the interaction with pVHL and its subsequent ubiquitination and degradation (Bilton et al.,

2006). A similar mechanism is observed with the SV40T antigen and the retinoblastoma tumour suppressor RB (Shimazu et al., 2006; Leduc et al., 2006).

1.2.3 A Ubiquitin-ligase activity for CBP?

New investigations are confirming the occurrence of enzymes harbouring both HAT and ubiquitination activities. Recent studies have shown that CBP/p300 can act as an E4-ligase and itself ubiquitinate substrates, such as p53, which proceeds to its degradation (Shi et al., 2009). The N-terminal of p300 and CBP can catalyse the poly-ubiquitination of p53 after the initial mono-ubiquitination performed by Mdm2. This E4 activity of p300 was also observed for another target of Mdm2, Tip60 (Col et al., 2005). Interestingly, although previous work demonstrated acetylation and ubiquitination relied upon one another, the E4 activity of p300 did not require its HAT function. However, PCAF, which in addition contains within its N-terminal an E4-ubiquitin ligase activity, partly relies on its HAT activity (Linares et al., 2007). Our results on ERβ ubiquitination (chapter 2, section 2, Figure 4E) do not eliminate the participation of CBP as an E4-ubiquitin ligase. Though the selective knockdown of CBP affected the general level of poly-ubiquitination of ERβ, residual poly-ubiquitination of the nuclear receptor still remained (chapter 2, section 2, Fig. 4E) the ubiquitination of ERβ in the presence of Akt-unresponsive CBP T1872A was almost entirely eliminated. Further investigations identifying CBP as an ERβ targeting-E4-ligase would be of interest as very limited information of enzymatic function for HATs is available.

In addition CBP/p300 has been observed to participate in the degradation of selective targets by recruiting the substrates directly to the proteasome. p53 turnover was found to be dependent on the E3-ligase activity of Mdm2 and the potential of CBP to interact with components of the 26S Proteasome complex (Zhu et al., 2001). In fact CBP can be a target of the proteasome (Brouillard and Cremisi, 2003; Poizat et al., 2000) and it has been observed to redistribute towards PML bodies for this function (St-Germain et al.,

2008). PML bodies could represent sites were ubiquitinated proteins are processed prior to proteasomal degradation as they were observed to contain proteasome complex components (Lallemand-Breitenbach et al., 2001). In fact a subpopulation of coactivator GRIP1 is able to localize to discrete nuclear foci containing PML bodies, CBP and enriched in components of the 26S proteasome which led to a decrease in the levels of GRIP1 (Baumann et al., 2001). In our first publication we observed that following Akt activation, CBP redistributed into nuclear foci, a hallmark of PML bodies. Although ERβ distribution did not adopt the identical distribution of CBP, its diffuse pattern does not refute this possibility either. By examining if ERβ effectively interacted with components of PML bodies following Akt activation we could conclude that CBP whether an E4-ligase or not, redistributes ERβ to sites of active degradation.

1.3 Mdm2 and CBP regulate the level of ERβ

Several studies have now established that optimal nuclear receptor transcriptional activity is achieved by a continuous receptor turnover. ERα and ERβ have been shown to be targets of the ubiquitin-proteasome pathway that can be estrogen-dependant and independent (Nawaz and O'Malley, 2004; Lonard et al., 2000; Nawaz et al., 1999; Reid et al., 2003; Picard et al., 2008; Tateishi et al., 2006). Ubiquitination of either ERα and/or ERβ can be carried out by several E3 ligases described in section 7.1. Activation of the PI3K/Akt pathway is known to stabilize and activate Mdm2. Phosphorylation of Mdm2 at Ser-166 and Ser-188 (considered the major site (Milne et al., 2004)) by Akt can decrease its ubiquitination and as a result is stabilized (Feng et al., 2004). Mdm2 stabilization has been shown to promote poly-ubiquitination and degradation of substrates such as p53 (Li et al., 2003a).

1.3.1 Mdm2 is activated by ErbB2/ErbB3

Akt activation by ErbBs targets the degradation of p53 by Mdm2 but this time targeting Ser-166 and Ser-186 (Zhou et al., 2001; Ogawara et al., 2002). Furthermore,

Ferreon *et al.* have described that CBP plays a role in the degradation of p53 in unstressed cells by functioning synergistically with Mdm2 (Ferreon et al., 2009). Several nuclear receptors are targets of Mdm2 induced degradation. GR activity is regulated by Mdm2 and is influenced by p53. In the presence of its ligand, GR is inhibited when Mdm2 is present during the cell's response to stress. Inhibition of GR is due to its increased polyubiquitination by Mdm2 which requires the presence of p53 (Sengupta and Wasylyk, 2001). Since the inhibition of GR is p53 dependent, this suggests that cells lacking p53, as it occurs in most cases during cancer development, Mdm2 might promote rather than suppress GR-mediated transcription, as observed for ER (Saji et al., 2001). The regulation of nuclear hormone receptors by Mdm2 has additional levels of complexity due to the cross-talk between the receptors. AR degradation is promoted through a sequential phosphorylation of AR by Akt and recruitment of Mdm2 into an AR-Akt-Mdm2 triplex, where AR ubiquitination is mediated through the E3-ubiquitn ligase activity of Mdm2 (Deep et al., 2008; Lin et al., 2002). In addition the AR receptor is also targeted towards the 26S proteasome complex in a mechanism that involves Mdm2 and HDAC1 (Gaughan et al., 2005). Therefore it appears that Akt signaling not only activates nuclear factors but it can also regulate their protein level within the cell.

1.3.2 Role of Mdm2 on ERs

Studies have shown that ERα is able to regulate the levels of Mdm2 (Suga et al., 2007). Reciprocally, Mdm2 can promote the degradation of ERα in a ligand-dependent and independent manner (Duong et al., 2007) which associates with the observation of a negative correlation between these two factors in breast cancer (Turbin et al., 2006). However others have demonstrated that Mdm2 can activate ERα transcription (Saji et al., 2001) therefore the cellular environment is crucial for the regulation of ERα. No studies so far have examined the potential regulation of ERβ by Mdm2. Mdm2 is an E3 ubiquitin-ligase which targets p53 towards ubiquitination (Michael and Oren, 2003). Low levels of

Mdm2 activity can induce mono-ubiquitination and nuclear export of p53, whereas high levels promote ubiquitination and nuclear degradation of p53 (Li et al., 2003a).

Results from our second manuscript show that Mdm2 can control the levels of ERβ. In fact, as observed in coimmunoprecipitation assays, mutation of Mdm2 at ser 186 and 188, rendering Mdm2 unresponsive to Akt, affect its ability to interact with ERβ. Mutation of these two sites affected the relocalisation of Mdm2 towards the nucleus, however examining the nuclear fractions; we observed that the mutants who localized to the nucleus were not capable of interacting as strongly with ERβ compared to the wild-type, demonstrated in chapter 2, section 2 figure 3. Therefore these sites are not only important for the activation and the relocalisation of Mdm2 towards the nucleus but also participate in the interaction with ERβ which lead to a disruption of ERβ protein levels.

1.3.2.1 Ser-166, a negative regulator of Mdm2 ?

Curiously, we observed that the mutation of Ser 166 of Mdm2, known to respond to Akt phosphorylation, unexpectedly maintained its potential to decrease the levels of ERβ. The MAPK pathway can also target ser-166, even in the absence of Akt activation (Malmlof et al., 2007). Studies have shown that activation of PI3K signaling pathway is able to reduce the activity of Mdm2 (Stommel and Wahl, 2004) by phosphorylation although specific sites were not explored. This suggests that in our model phosphorylation of Ser-166 by Akt (or possible MAPK through ErbB2) moderates the E3-ligase activity of Mdm2.

1.3.3 Mdm2 and CBP

Activation of the PI3K/Akt did not to promote a greater interaction between ERβ and Mdm2 which was reflected in the levels of ubiquitination of ERβ. Interestingly a dramatic increase in the ubiquitination of ERβ was observed following activation of Akt in the presence of CBP establishing the importance of CBP in the degradation of ERβ. CBP's

role in the degradation was further examined by creating a mutant that was non-responsive to Akt through the mutation of the threonine residue (Thr 1872) in the C-terminal bromo domain of CBP (figure 16). Results show that, not only are the levels of ERβ ubiquitination drastically reduced when CBP can no longer respond to Akt, but the interaction between Mdm2 and ERβ is affected as well, demonstrating that phosphorylation of ERβ by Akt is not sufficient to target its degradation (Chapter 2, section 2 Fig 1A and B). In fact the phosphorylation of CBP by Akt is required to trigger the degradation of ERβ. Furthermore the selective knockdown of CBP also resulted in a decrease in the ubiquitination of ERβ. However the knockdown of MDM2 did not decrease as dramatically the levels of ubiquitinated ERβ, signifying the possibility that other E3 ubiquitin-ligases might be involved. Indeed, E6-AP was recently uncovered to target ERβ degradation in a ligand-independent manner which relied on a MAPK-dependent pathway (Picard et al., 2008).

1.4 The hinge region- regulation of transcriptional activity

ERs are sensitive to post-translational-dependent events, especially in proliferation of tumours where their activity, ligand-dependent and independent is often deregulated. Several sites for ERα and ERβ have been described to increase their ligand-dependent as well as their ligand-independent activity (Figure 12 and 13). These sites were first described to be located within the N-terminal AF-1 domain for both receptors; however, further examination revealed that the DBD as well as the hinge region have been subject for ER control. Indeed, pak1 and PKA target ERα ser-305 (Bostner et al., 2010) promoting its transcriptional activation resulting in an increase in *cyclin D1* production (Balasenthil et al., 2004) and a decrease in its sensitivity to tamoxifen (Kok et al., 2010). Phosphorylation of Ser-305 prevents the acetylation of the neighbouring lysine residue K303 often mutated in breast cancer and leading to an increase in the sensitivity of ERα to E_2 (Fuqua et al., 2000). ERα's Thr-311, targeted by p38 promotes its nuclear localization and is linked to an increase in its activity (Lee and Bai, 2002). Moreover methylation, within the hinge domain

of ERα has led to a decrease in its turnover rate (Subramanian et al., 2008), suggesting that ERα can be regulated through modifications of its hinge region.

A recent study has reported the importance of the hinge region for both ERα and ERβ's transcriptional activity involving the AF-1 and AF-2 domains (Zwart et al., 2010). Indeed, the length and the composition of the hinge region can affect the capacity for transactivation for ERα however these sequences and modifications are not observed in the hinge region of ERβ. Results show that the interaction of ERα with SRC-1 together with the hinge region, allows full activation potential of ERα in both the presence of the ligand and in its absence. This occurrence is not observed for ERβ, which can be a reflection of the divergence within the AF-1 domain between both subtypes. Indeed the AF-1 domain of ERα is crucial for its E_2-dependent activity; however it is not the case for ERβ. We, as well as others, have observed that the removal of the N-terminal AF-1 domain of ERβ, strikingly improves its response to E_2.

For ERα, the hinge region has 43% identity from *Homo sapiens* to *Xenopus laevis*, and the hinge residues between K302 and S305, immediately before the start of the LBD, are approximately 85% conserved between *Homo sapiens* to *Xenopus laevi.* This higher conservation relative to the rest of the hinge region strongly implies that this region may be functionally important. In fact a study showed the importance of the hinge of ERα which is necessary for BRCA1 interaction promoting ERα mono-ubiquitination leading to a disruption of its activity (Eakin et al., 2007). In effect, mutation of K305 renders breast cancer cells hypersensitive for proliferation and has been observed in as many as 34% of premalignant breast tumours. The interaction of p300 with ERα and ERβ acetylated only the lysine residues 266 and 268 of ERα (Kobayashi et al., 2000b) which increased its affinity for DNA (Kim et al., 2006). Alignment sequences have shown that several nuclear receptors do conserve these two lysine residues; interestingly ERβ does not contain these lysine residues and is therefore not a substrate for p300 acetylation at the hinge region.

1.4.1 The hinge of ERβ

Realising the pressure under which the hinge region of ERs is subjected to in order to regulate the receptor, it was of great interest for us to observe that our results also revealed a role for the hinge region of ERβ. Most of our studies carried out in chapter 2, section 1 and 2 were achieved with the murine isoform of ERβ. Scanning through the sequence of murine and human ERβ revealed that only the murine isoform contained a perfect and unique consensus site for Akt phosphorylation. However the human isoform contains a partial Akt-consensus site where the serine residue has been replaced with an aspartic (236) which is followed by a glutamic acid (237) residue. Results from our first publication shows that Akt does phosphorylate serine 255 within the hinge region of mERβ (Chapter 2, section 1, Figure 2) and the mutation of this serine into an alanine decreased the potential for phosphorylation demonstrating that Akt can directly target ERβ. In fact results from our laboratory demonstrate that there is a direct interaction between Akt and ERβ following the activation of ErbB signaling pathway (data not published). In order to understand the impact of Akt phosphorylation on ERβ we looked at the transcriptional activity of the mutant mERβ S255A, following Akt activation by Hrgβ1 treatment. Surprisingly the transcriptional activity of the ERβ was fully recuperated following the mutation of this single site, suggesting that not only is the hinge region important in regulating the function of ERβ but in addition this site is of particular interest as, alone, it can determine the fate of the receptor given the proper physiological cue.

Due to the high degree of similarity of both biology and sequence between mouse and humans, the mouse genome has been receiving considerable attention as a tool for cross-species comparison (Battey et al., 1999). This strong similarity has raised doubts regarding the general usefulness of human-mouse sequences comparison for distinguishing functionally conserved features against a background of recently evolved sequences. In the case of ERβ, there is a substitution that has occurred in time which has resulted in a variation to the Akt responsive sequence in the hinge region. By examining the response of

the hERβ in chapter 2, section 2, we show that although an aspartic residue is present instead of a serine residue, its response to the proposed mechanism remains similar to that of mERβ. Mutation of the aspartic residue abrogated the decrease in steady-state levels of hERβ similar to mERβ suggesting that the negatively-charged environment created by the aspartic acid in wild-type hERβ is in all probability mimicking a phosphorylated site, therefore the activation of the PI3K/Akt pathway creates a similar environment as the one observed with mERβ leading to a decrease in its cellular content with a parallel increase in ubiquitination following PI3K/Akt activation in the presence of CBP (chapter 2, section 2, fig .6).

Given the importance of the hinge region, we examined how other nuclear receptors namely ERα, PR, GR, the three isoforms of ERR (α, β, γ) and PPARγ, respond to the activation of the PI3K-akt pathway in the presence of CBP. Our results revealed that only those receptors containing within their hinge a consensus site for Akt (GR and ERRβ) resulted in a decrease in their transcriptional activity following Akt activation in the presence of CBP (Chapter 2, section 1, Figure 7). In addition, this decrease occurred in the presence of their respective ligands, suggesting that the activation of the PI3K/Akt pathway was able to override the activation displayed by their respective ligand as observed with ERβ.

1.5 Influence of ERβ regulation by Akt and CBP in breast cancer cells

It is apparent now that the activation of ErbB2/ErbB3, which preferentially activates the PI3K/Akt pathway, targets the degradation of ERβ. Activated Akt has several targets, one of which is Mdm2, driving its relocalisation towards the nucleus. Akt can also target the phosphorylation of CBP which, in certain cases, allows for an increase in it coactivating potential as observed with ERα, PR and PPARγ. Results also demonstrate that all these events are subtype specific and while they lead to an acute degradation of ERβ that impact

its regulation ERα protein levels remain unaffected and its activity is on the other hand stimulated.

1.5.1 Hrgβ1 treatment of breast cancer cell lines

We next examined if these results were transposable to cells which endogenously expressed ERβ. We designed stable breast cancer cell lines which originally did not express either ER subtype, to express the mERβ or the mERα in order to discriminate the response of each receptor following the activation of the PI3K/Akt pathway. We proceeded to examine ERE-regulated genes by RT-PCR experiments which show that the treatment of cells with Hrgβ1, which do endogenously express ErbB2/ErbB3 resulted in a decrease in the ERE genes regulated by ERβ. Although targets for AP-1 were also available, the results were inconclusive. Nevertheless decreasing the amount of ERβ could potentially affect AP-1 regulated genes in a similar manner to ERE regulated genes. Our laboratory recently published that activation of CXCR4 by SDF-1, which activated the MAPK cascade, resulted in an upregulation of proximal ERE promoters-regulated *SDF-1* itself and *PS2* gene products as well as the *PR* gene. In addition, AP-1 genes were also looked at, under the treatment of tamoxifen, capable of activating ERβ. Similarly to ERE regulated genes, tamoxifen was capable of activating AP-1 regulated *cyclinD1* and *c-myc* genes (Sauvé et al., 2009). Since we observed a decrease in the genes known to be regulated by the activation of ERβ, we next examine the proliferation of breast cancer cell lines.

MCF-7 cells are known to express ERα, and although the expression of ERβ in this cell line is still under debate, several publications and our laboratory have been able to observe protein expression of hERβ. Similarly to mERβ, hERβ is also degraded following the activation of Akt and in the presence of CBP (Chapter 2, section 2, fig.6). The proliferation of MCF-7 treated with Hrgβ1 was observed. MCF-7 cells were strongly stimulated by the treatment of Hrgβ1 compared to untreated cells. However the selective knockdown of Mdm2, proposed to participate in the degradation of ERβ, dramatically

affected the rate of proliferation of Hrgβ1 treated MCF-7 cells. Preliminary western analysis showed that treatment of Hrgβ1on MCF-7 was capable of decreasing the levels of ERβ, which were restored following the knockdown of Mdm2. Measurements of the levels of ERα did remain unaffected. Since the decrease in Mdm2 levels could in fact promote apoptosis; we looked to see if the steady-state levels of p53 were affected, however we did not observe any difference when comparing p53 levels in Hrgβ1-treated MCF-7 vs. untreated-cells. This sets forth the premise that we are in fact seeing a regression in proliferation rather than an attempt to enter apoptosis. This proposes that the removal of ERβ creates a more prolific environment as opposed to its presence, which we can conclude that the presence of ERβ might very well regulate the activity ERα and therefore when the ratio of ERα/ERβ increases, we observe a greater proliferation, as observed in many cases of anti-hormone resistant cancers (Treeck et al., 2009).

There have been reports that have looked at how the ratio of ERα/ERβ can overturn an effect observed on ERα. Estrogen-induced MCF-7 survival during treatment with TNF can be reduced by the mere reinstatement of ERβ into these MCF-7 cells, or by treatment with the SERD, ICI 182,720 (Lewandowski et al., 2005). The presence of ERβ was able to reduce the overall activity of ERα and increase the levels of p21 protein, a target of p53. The ratio of ERα/ERβ expression in breast tumours is becoming a popular prognostic to evaluate the severity of the tumour. Covaleda *et al.* used T47D-ERβ cell model in which the levels of ERβ could be reduced by adding tetracycline. It was shown that the proliferative actions in the T47D-ERβ cells were mediated by the ERα, whereas ERβ was able to downregulate the effectiveness of ERα-induced proliferation (Covaleda et al., 2008). It cannot yet be concluded whether the inhibition via ERβ results in a reduced transcription of genes involved in cell division or that possibly nongenomic signal transduction pathways are induced as well (Figure 20). Addition of ERβ in ovarian cancer cell line SK-OV-3 reduced its proliferation following treatment with estrogen (Treeck et al., 2007). Similarly the expression of ERβ in MCF-7 treated or not with estrogen can have

differential impact on gene regulations (Chang et al., 2006). Expression of ERβ in breast cancer cell lines T47D reduced tumour growth and angiogenesis (Williams et al., 2008). Uncovering the contribution of ERβ in the regulation of breast tumour growth is important for the understanding and treatment of this disease.

2 Perspectives

Many reports have examined the clinicopathologic importance of ERβ expression in breast cancers (Esslimani-Sahla et al., 2004; Leygue et al., 1999) although a precise role for ERβ has yet to be identified, some common themes are emerging, including the association of ERβ expression with ERα, PR, and low tumour grade, factors usually associated with a better clinical outcome (Jarvinen et al., 2000; Skliris et al., 2001; Speirs et al., 1999b). However, many of these studies executed at the molecular level examine the impact of ERβ by incorporating ERβ into cell lines, which might not be an accurate representation of endogenous levels and therefore results could be misleading due to overexpression of ERβ.

In endometrial cancer, the development of a specific ERβ agonist caused regression of lesions in an experimentally induced model of endometriosis using human tissue (Harris et al., 2005) Endometriosis, characterized by the presence of endometrium-like tissue outside uterine cavity has been shown to express high level of ERβ and low levels of ERα. Selective knockdown of ERβ led to an increase in *ERα* expression and protein levels (Trukhacheva et al., 2009). Furthermore treatment of prostate cancer cell lines with a demethylating agent resulted in an increased expression of ERβ implying that methylation of ERβ is reversible and a tumour-stage specific process (Zhu et al., 2004). In agreement with the above observations, our results propose a mechanism whereby the levels of ERβ are important in the control of cellular proliferation and point to an critical regulatory region, not only within ERs but of other nuclear receptors as well which can help predict the response to the activation of specific (in this case the PI3K/Akt pathway) signaling events. However the results presented here can be considered as groundwork and further

examination of the pathways involved need to be addressed. First it would be of significant value to assign a physiological role to the hinge domain of ERβ in tumoural growth and secondly explore the molecular events which take place following a reduction in the levels of ERβ.

2.1 An anti-proliferative role for the hinge region

It would be of great interest to ascribe a functional anti-proliferative role to the hinge region of ERβ due to the discriminatory component within its sequence between ER subtypes particularly in ERα+/ERβ- tumours. In order to ascertain its role as a potential regulator of proliferation, I propose to directly observe the *in vivo* effect of incorporating a mutant ERβ harboring a point mutation at the equivalent site of ser-255 (in humans asp-236) by lentiviral vector injections into mice models harboring an induced tumour. As our model demonstrates the inability of estrogen to either pronounce or reverse the observed ERβ degradation, the use of ovariectomized mice would provide a relatively estrogen-free environment and tumour growth could be identified as hormone-independent. Therefore ovariectomized female nude mice would be xenografted with a breast cancer cell line characterized as ER+/ERβ-, ErbB2+/ErbB3+ (known to be very aggressive) subcutaneously. Once tumours reach a particular diameter mice would be randomised to receive vehicle, lentivirus sh-wt ERβ (possibly targeted towards degradation) or sh-ERβ (D236A) (possibly stabilized). Tumour growth would be assessed by caliper measurements. Should the hinge region have an effect we could see two possible outcomes: 1- either a regression of the tumour indicating a possible tumour-suppressor activity of ERβ or 2- deceleration of tumour proliferation as we observed in MCF-7 cells when levels of ERβ were reduced by treatment of Hrgβ1.

2.1.1 Tumour-suppressor activity

If we observe a regression of tumour size, we would investigate the possible event of cellular apoptosis. Visualizing and quantifying apoptosis on our tumour samples by DNA fragmentation (TUNEL assay to visualize apoptotic cells, and DNA laddering on agarose gel) and examining the prospect of ERβ binding to promoters of known apoptotic inducing markers as previously observed in the overexpression of ERβ in ERα positive cells, would suggest that signaling pathways activated by ErbB2/ErbB3 would activate ERβ (as observed in our first publication following mutation of the hinge region) and promote tumour regression.

2.1.2 Reduction in cellular proliferation

Should the tumour's rate of proliferation decrease as observed in MCF-7 cells in our second manuscript, we could envision the likelihood of ERβ having a negative role on ERα activity. In fact Chang *et al.* showed that ERβ bound the promoter region of ERα which contains a single ERE site but several AP-1/sp-1 sites, following treatment with E_2 as well as without E_2. The binding was only observed in endometriosis and not in normal endometrial cells. In addition, ERβ has been shown to affect the gene network regulated by ERα in breast cancer cells and ERα down-regulation was noted in the presence of high ERβ levels in MCF-7 (Chang et al., 2006). It would be of interest to verify by chromatin immunoprecipitation if the tumours from the xenografted mice showed evidence of a greater number of ERβ D236A mutants at ERα promoter region and if so which coregulatory complexes (corepressors) are involved in order to decrease the transcription of the ERα gene. Of course for either outcome aforementioned, we would look at the protein levels of ERα, ERβ and ERβ D236A to give us an indication of the environment of the tumour and to support our hypothesis of ERβ's role in breast cancer proliferation.

2.1.3 Possible cellular mechanisms at play

Having demonstrated that the activation of the PI3K/Akt pathway by ErbB2/ErbB3 leads to the targeted degradation of ERβ and the activation of ERα, then examining the molecular mechanisms which are at stake would aid in developing new methodologies to reinstate the expression of ERβ in order to decrease the ERα/ERβ ratio, a positive factor in breast cancer treatment. As such, developing a peptide derived from protein sequence, which would mask the aspartic acid 236 residue, could potentially reduce ERβ targeted degradation and reduce the proliferation potential. In addition it would also be of interest to understand the function of CBP during the degradation of ERβ. As mentioned previously in the discussion, we are still unsure whether CBP exerts an E4-ubiquitin ligase activity or acetylation of ERβ is a signal for its degradation. As for its ligase activity, it has been previously ascribed to the N-terminal, although neither CBP nor p300 exhibit clear or known consensus ligase sequences. Deletion mutants would provide information as to which region of CBP is involved and point mutation would determine which sites are important. Although inhibiting the function of CBP could be a possibility (chapter 8 HAT inhibitors), its vital role in every aspect of cell function implies that drug-specific delivery would be necessary in order to reduce non-specific effects on peripheral healthy tissues, therefore targeting its possible ligase activity and not its HAT activity might provide a more specific approach to inhibit ERβ degradation.

Lastly, most of the information and the propositions stated above involve the use of breast cancer cells. We have stated several times the importance of considering every aspect of cellular biology (level of proteins expressed, cell-type and promoter context) in order to provide a more accurate treatment towards developing hormone-independent cancers. On a long-term perspective we would examine the behavior of ERβ C236A in other hormone-independent cancers, such as in the endometrium, prostate and colon in order to establish whether our observations are tissue-specific or are a general mechanism established by ERβ.

Conclusion

Studies from rodent models have helped us to understand human physiology and propose treatments for human cancers. It is interesting to note that although both mice and humans are predisposed to spontaneous breast cancer; it appears that endometrial cancers are only spontaneous in humans (Anisimov et al., 2005). Although it is a great extrapolation, it would be interesting to investigate the possibility that, at least in the case of ERβ, the difference in sequence homology would bring a protective factor in mice as opposed to humans. Nevertheless we conclude that the measurement of ERα, ERβ, and additional proteins such ErbBs and coregulators in endocrine tumors complementing with studies regarding function regulation of these cellular components would provide important additional information not only for predicting cellular deregulation incidences but also anticipate better and personalized therapeutic responses.

CHAPTER 4: APPENDIX

Challenging Estrogen Receptor β with Phosphorylation

(Review published in Trends in Endocrinology and Metabolism)

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keywords: estrogen receptor; ERα; ERβ; growth factors; EGF; heregulins; receptor tyrosine kinase; EGFR; ErbB2/HER-2/Neu; ErbB3; AF-1; AF-2; MAPK; SRC-1; CBP/p300; CHIP; E6-AP; 26S proteasome; ubiquitin; SUMO

DISCLOSURE STATEMENT: The authors have no competing interests.

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Licensed content date	February 2010
Licensed content volume number	21
Licensed content issue number	2
Number of pages	7
Type of Use	reuse in a thesis/dissertation
Portion	full article
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Title of your thesis/dissertation	Growth Factor Activation of ErbB2/ErbB3 Signaling Pathways Regulate the Activity of Estrogen Receptors (ER)
Expected completion date	Oct 2010
Estimated size (number of pages)	300
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Review

Challenging estrogen receptor β with phosphorylation

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From classical gland-based endocrinology to nuclear hormone receptor biology, tremendous progress has been made in our understanding of hormone responses underlying cellular communication. Estrogen elicits a myriad of biological processes in reproductive and peripheral target tissues through its interaction with the estrogen receptors ERα and ERβ. However, our knowledge of estrogen-dependent and independent action has mainly focused on ERα, leaving the role of ERβ obscure. This review discusses our current understanding of ERβ function and the emerging role of intracellular signals that act upon and achieve estrogen-like effects through phosphorylation of ERβ protein. Improving our understanding of how cellular determinants impact estrogen receptor actions will likely lead to treatment strategies for related endocrine diseases affecting women's health.

The estrogen receptor ERβ – here and for what purpose?

The unexpected identification of a second estrogen receptor, termed ERβ, in the mid-1990s rapidly forced the scientific field to reassess the classical model of estrogen action [1]. Originally identified in rat, then in human and mouse [2–4], ERβ was shown to share obvious structural features and functional responses to estrogen with its elder homolog ERα, but also exhibited unique characteristics in its ability to respond to cellular signals, suggesting ERβ might perform similar as well as distinct functions compared to ERα.

In fact, studies characterizing ERβ structure and function by analyzing ligand binding, recruitment of transcriptional coregulators, interactions with cellular signaling and the pharmacology of selective estrogen receptor modulators (SERMs) among others, have increased our understanding of ERβ and helped distinguish its role(s) from ERα [5–7]. The characterization of mice lacking ERα and/or ERβ has also established that each receptor subtype has overlapping and unique roles *in vivo*, revealing the importance of these receptors in a variety of physiological processes including male and female sexual differentiation, fertility, ovarian function, bone formation and cardiovascular aspects (Box 1).

Over the years, the role played by ERβ in tissues has become less ambiguous; however, few studies have focused on modifications of ERβ that control its biological actions. Therefore, uncovering the molecular mechanisms regulating the activity of ERβ is paramount to understanding its role in cellular and biological events surrounding gene regulation.

Transcriptional regulation by ERβ

Activation of ERs by hormone is a multistep process relying on several events including dimerization, ligand binding, interaction with cofactors and DNA binding. It has also become increasingly clear that phosphorylation of specific sites on ERs can occur as part of both ligand-induced and ligand-independent activities. Mostly based on their distinct and poorly conserved distribution between the two isoforms, it is predicted that a selective use of these phosphorylation sites might contribute to regulate various aspects of ERβ function not always shared with ERα, in order to complement responses to ligands and/or provide on their own transcriptional potential.

ERβ phosphorylation

Activation of ER by signal transduction pathways, in the absence of ligand, was first suggested in the early 1990s when ovariectomized mice were treated with epidermal growth factor (EGF) leading to nuclear translocation of ER and transcriptional activation [8]. Since then, the pathways involved and the mechanisms supporting ligand-independent activation of ERα have become clearer although not fully characterized. The identification of ERβ, however, has added tremendous complexity in our understanding of potential mechanisms by which estrogen-dependent and -independent responses can be achieved.

It is now widely recognized that the activation of growth factor signaling cascades can promote hormone-like responses. Peptide growth factors of the EGF class of ligands, such as the neuregulins/heregulins and EGF itself, interact with cell surface receptors of the EGFR/ErbB family of receptor tyrosine kinases to initiate phosphorylation-coupled activation of protein kinases, such as MAPK/Erk, Jnk, p38 and PKB/Akt [9]. Consensus sites for these kinases have been functionally characterized within the N-terminal activation function-1 (AF-1) domain of ERα and ERβ (Box 2). Serines -104, -106 and -118 of ERα were shown to be phosphorylated by MAPK/Erk [10–12], whereas Ser-167 was described as a functional Akt site [13]. Our contribution has allowed the identification of serines -106 and -124 of ERβ as recruiting signals for transcriptional coactivators to mediate ERβ activation

1043-2760/$ – see front matter doi:10.1016/j.tem.2009.09.007

Box 1. ERα versus ERβ

In vitro studies have demonstrated redundancy in the roles of ERα and ERβ; however, tissue localization revealed their shared but also distinct expression patterns of each receptor [59], suggesting that specific roles are probably observed. ERα is expressed in many different tissues, including the female and male reproductive tract, skeletal and cardiac muscle, kidney, liver and hypothalamus. ERβ displays a more limited expression pattern and is predominantly observed in the ovary, prostate, testis, spleen, lung and areas of hypothalamus. Further evidence of distinct biological roles for the two isoforms is suggested by the phenotypes of ERα, ERβ and ERα/β knockout mice [60–63]. In the female αERKO mice, estrogen insensitivity leading to hypoplasia in the reproductive tract, hypergonadotropic hypergonadism, lack of pubertal mammary gland development and excess adipose tissue is observed. In αERKO males, testicular degeneration and epididymal dysfunction are the major phenotypes. αERKO and αβERKO females are infertile, and βERKO females are subfertile owing to inefficient ovarian function. An abnormal vascular function resulting in hypertension was also reported in βERKO mice [64]. None of these phenotypes have been linked with receptor phosphorylation owing to the current lack of appropriate mouse models.

Box 2. The AF-1 and AF-2 of ERβ – copartners for a shared cause

As with other nuclear receptors, the transcriptional competence of ERβ is mediated by two nonacidic activation domains, an activation function AF-1 located in the N-terminal region and a ligand-dependent AF-2 located in the C-terminus (Figure 1). The AF-2 domain is well characterized, with a highly conserved amphipathic α-helix (H12) essential to mediate hormone-dependent activation of transcription through interaction with cofactors that facilitate chromatin remodeling and transcription. AF-1 activation is more complex and variable and depends on a wide region consisting of several phosphorylation sites, mostly consensus Ser–Pro motifs for MAPK, which dictate ERα and ERβ responses to various kinase signaling pathways [4,10,11]. AF-1 activity also depends on the recruitment of coregulators that can be either similar or unique from those employed by AF-2 [32], providing AF-1 and AF-2 with synergistic potential in ligand-activated mechanisms and also with independent functions related to cell and promoter context [6]. Thus, AF-1 emerges as a key target for kinase-regulated events to achieve distinct as well as concerted actions with AF-2.

by EGF and oncogene ras [14,15], thereby providing a possible mechanism by which AF-1 mediates ERβ responses to ligand-independent signals. These studies therefore highlight a prominent role of receptor phosphorylation through which ligand-independent activation of ERβ can occur [16,17]. A schematic representation of AF-1 phosphorylation sites of human and mouse ERβ that regulate receptor activity and cofactor recruitment is shown in Figure 1, as well as the ones characterized in the hinge region and ligand-binding domain of ERβ.

Examples of genes regulated by ERβ in a ligand-independent manner have been reported. For example, ERβ has a role in regulating cyclooxygenase-2 (COX-2) in fetoplacental endothelial cells, even in the absence of estrogen [18]. Similarly, ligand-independent phosphorylation of ERβ is associated with increased promoter occupancy and gene expression of human telomerase reverse transcriptase, a major determinant in telomerase activity, preventing apoptosis of pancreatic cancer cells [19]. Among the possible mechanisms involved in ligand-independent activation of ERβ is the selective recruitment of steroid receptor coactivator-1 (SRC-1) and histone acetyl transferase CREB binding protein (CBP), following AF-1 phosphorylation in response to EGF [14,15]. Although both coactivators can also be efficiently recruited to ERα, this process is not enhanced through ERα phosphorylation, indicating that EGFR/ErbB activation signals might selectively affect each ER isoform. Moreover, the phosphorylation-initiated ERβ/SRC-1 complex is inefficiently disrupted by SERM tamoxifen in normal and cancer cells

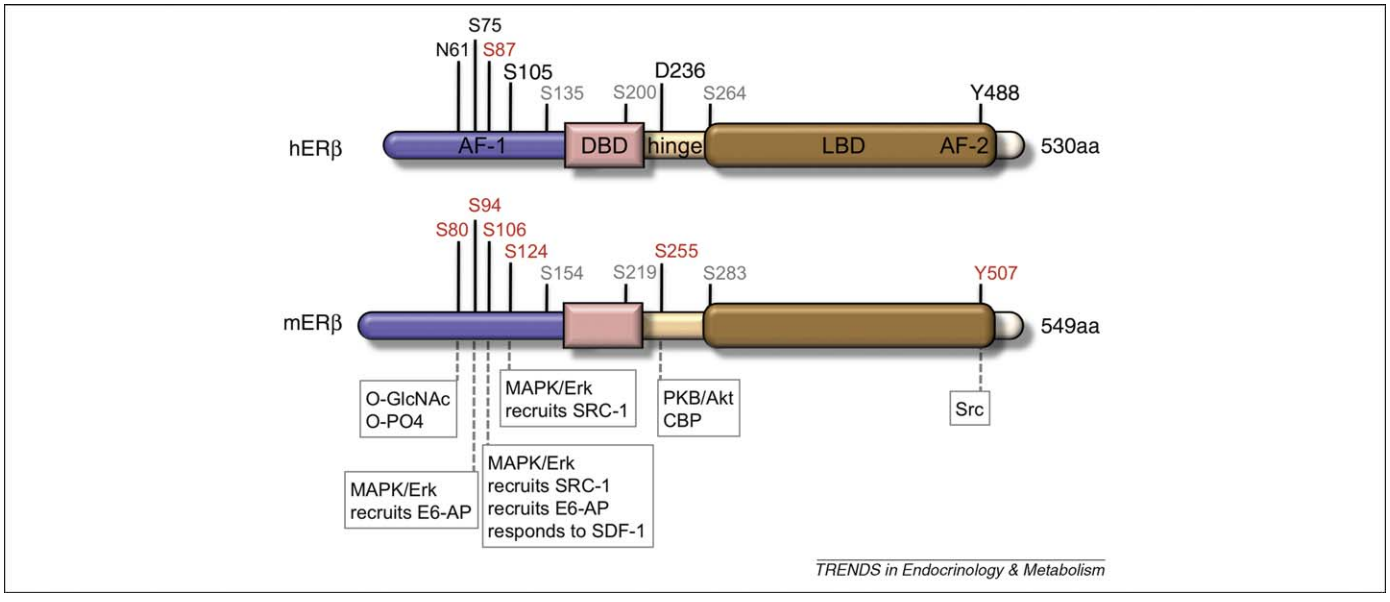

Figure 1. Targeting estrogen receptor β with phosphorylation. Schematic representation of modified amino acids in human ERβ with corresponding residues in mouse ERβ. The difference in numbering is due to a shorter N-terminal form of the human versus mouse isoform. Structural and functional domains of ERβ include the transcriptional activation functions AF-1 and AF-2, the DNA-binding domain (DBD), the hinge region and the ligand-binding domain (LBD). Experimentally supported phosphorylated residues (red) with their homologous sites (black) are shown, along with the putative kinase and recruiting cofactors involved. Other potential sites (gray) are derived from sequence homology with reported phosphorylated residues in ERα. References describing phosphorylated residues are as follows: S80 [44], S87 [29], S94 [39], S106 [14,29,39], S124 [14], S255 [16] and Y507 [17].

[14,20], suggesting that ERβ activation could occur in tumors in which therapeutic resistance ensues.

Despite its properties to behave as an intrinsic activation domain, the AF-1 of ERβ can also complement hormonal response with AF-2 function, as is the case with several nuclear receptors, including ERα (Box 2). Following estrogen treatment, the expression of TGFβ-inducible early gene (*TIEG*), an ERβ target, is shown to be dependent on the ability of AF-1 to recruit coactivators such as SRC-1 to initiate transcription [21]. Phosphorylation by the p38 pathway has also been linked to the transcriptional activation of ERβ in a ligand-dependent manner, although specific site(s) targeted by the p38 pathway have yet to be identified [22]. However, the outcome of ERβ phosphorylation by protein kinases in the presence of estrogen seems to differ according to cell types, promoter usage and the identity of the effector. For example, p38 pathway activation by the ErbB2/ErbB3 heterodimer, a combination often associated with SERM-resistant breast cancers, leads to repression of the hormonal response of ERβ in breast cancer cells through an impairment of AF-1 to facilitate recruitment of coactivators [23]. Interestingly, activation of ErbB2/ErbB3 induces phosphorylation of mouse ERβ at Ser-255, a site consensus for Akt (Figure 1), resulting in the disruption of CBP from liganded ERβ, providing a possible mechanism by which ERβ response to hormone is reduced by ErbB2/ErbB3 dimer activity [16]. Activation of protein kinase A (PKA) by increasing cAMP intracellular levels through adenylate cyclase activation with forskolin also stimulates ERα and ERβ activity through selective mechanisms involving a distinct receptor phosphorylation pattern, although neither the AF-1 nor cofactor requirement seem to be involved [24]. Therefore, the diversity of phosphorylated sites in ERβ, their targeting by various signaling pathways and their intrinsic potential to regulate cofactor usage has challenged us to understand how ERβ integrates numerous incoming cellular signals. An overview of these pathways is represented in Figure 2.

ERβ regulation by phosphorylation extends to the chemokine receptor family

Recently, the identification of chemokine receptors as novel signaling regulators of ERα and ERβ has added to the complexity by which ER-responsive gene transcription can be regulated by upstream cellular pathways. The chemokine receptor CXCR4 and its ligand, the stromal cell-derived factor SDF-1 (also referred to as CXCL12) have been widely associated with metastasis of several epithelial and hematopoietic cancers, including breast, prostate, ovary and

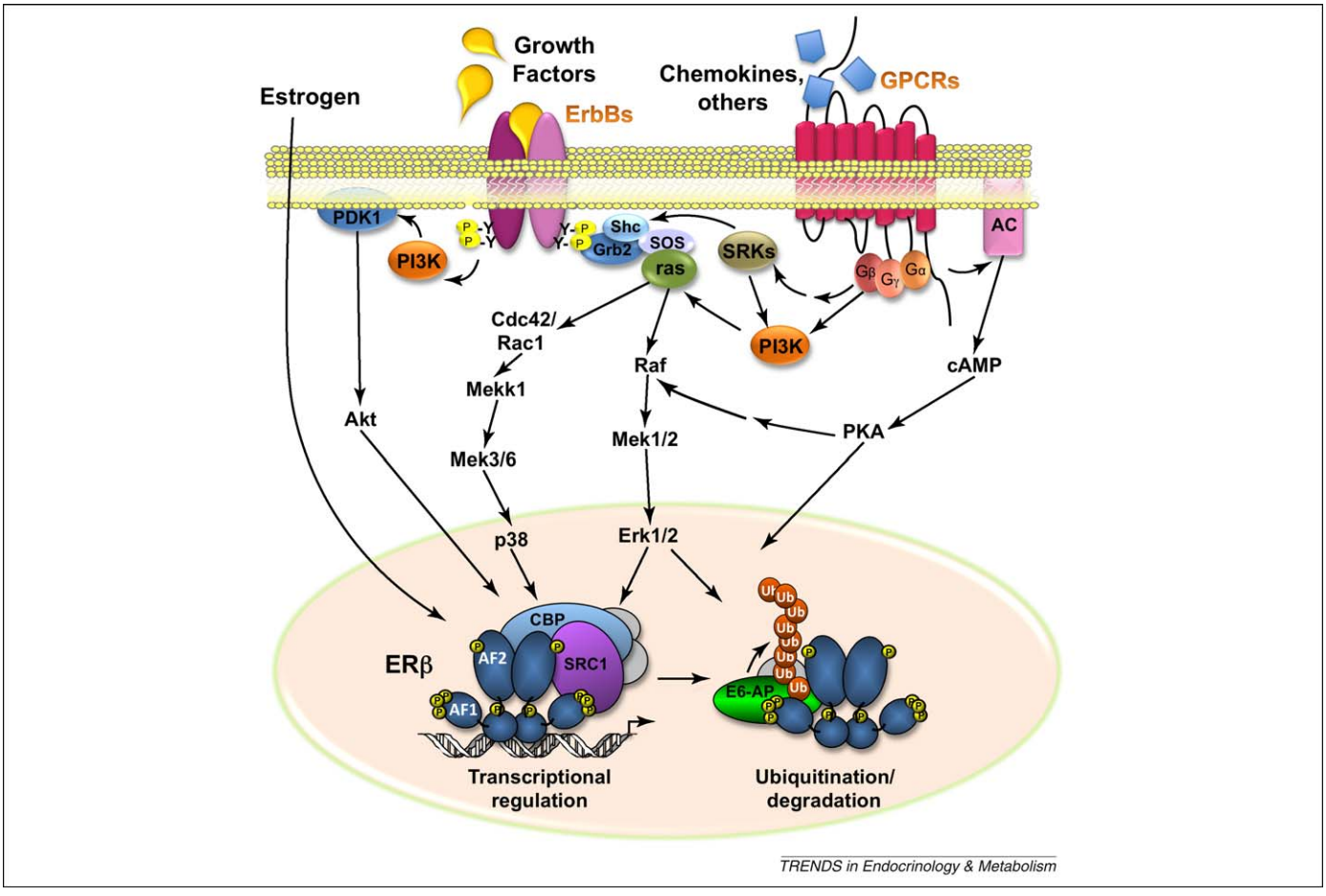

Figure 2. Signaling networks targeting ERβ. Overview of cellular signals regulating ERβ transcriptional competence and degradation. Depicted are kinase pathways elicited upon activation of growth factor receptors of the EGFR/ErbB family and of G-protein-coupled receptors such as CXCR4, which direct phosphorylation of ERβ. Such phosphorylation, as illustrated by yellow spheres labeled with the letter P, facilitates the recruitment of coactivators, such as SRC-1 and CBP, to promote transcription, and of E3 ubiquitin ligase E6-AP which favors ubiquitination of the receptor. The Erk pathway has been linked with both processes. SRKs, Src-related kinases; AC, adenylate cyclase; PDK1, 3-phosphoinositide-dependent protein kinase-1; PI3K, phosphatidylinositol 3-kinase.

lung cancers [25,26]. Initial studies in breast cancer cells expressing CXCR4 found that their chemotactic potential was involved in the dissemination of metastases to SDF-1 producing target tissues, such as lung, liver and bone marrow [27]. In line with a previous report that identified *SDF-1* as an estrogen-regulated gene in ERα-positive ovarian and breast cancer cells [28], we found that SDF-1 expression is not only increased by estrogen through ERα and ERβ, but this ER-produced SDF-1 in turn activates ERα and ERβ to enhance estrogen-regulated proliferating genes and growth of breast cancer cells [29]. In particular, ERβ activation by the CXCR4/SDF-1 axis relies on AF-1 Ser-87 phosphorylation by Erk (Figure 1), resulting in enhanced assembly of ERβ at both estrogen-responsive and AP-1 elements of targeted promoters, including SDF-1 itself. SDF-1-induced recruitment and activation of ERβ at AP-1 sites is insensitive to SERM tamoxifen, providing a way by which ERβ remains competent despite the presence of tamoxifen [29]. These findings establish an autologous regulation of SDF-1 through the ability of ERβ to increase gene expression in the context of enhanced CXCR4 activation (Figure 2), providing a mechanism by which the CXCR4/SDF-1 axis and ER (α and β) signaling pathways mutually contribute to a positive autocrine/paracrine feedback loop underpinning breast cancer progression. The ER/CXCR4 pathway also opens new perspectives as to how breast cancer cells might escape SERM-mediated growth blockade.

A little help for ERβ

As ERα and ERβ undergo phosphorylation through kinase-mediated pathways with subsequent effects on their ligand-independent transcriptional potential, it was predicted that phosphorylation might serve as a recruiting signal for coregulators. Candidate coregulators have been described to interact with ERα in a manner dependent on Ser-118 phosphorylation to support receptor activation, such as RNA helicase [30] and splicing factor SF3a120 [31]. It is of interest that AF-1 coactivators for ERα are not always shared with ERβ, at least based on phosphorylation-directed mechanisms. Indeed, originally identified as an AF-2 coactivator but also interacting with ERα AF-1 to strengthen hormonal response [32], we reported that SRC-1 was potently recruited to ERβ AF-1 following phosphorylation of Ser-106 and Ser-124 triggered by EGF or oncogene ras [14], a ligand-independent mechanism which also allowed for CBP recruitment [15]. This increased recruitment of SRC-1 and CBP has also been recently reported to mediate ligand-independent activation of ERβ by hypoxia inducible factor HIF-1α in hypoxic conditions [33]. In addition, a recent report describes ligand-independent activation of ERβ in ovarian granulosa cells by follicle-stimulating hormone which, by enhancing PKA signaling, triggers the recruitment of gonadotropin-inducible ovarian transcription factor-4, a bridging factor for components of the chromatin remodeling SWI/SNF complex [34].

To add further complexity to the interaction of ERβ with cellular pathways, recent evidence indicates that coregulators serve as points of convergence between ERs and growth factor signaling pathways by being targets of phosphorylation, an event which in most cases contributes to enhance their transcriptional potential [35]. Whether such modifications also serve as an interacting interface to recruit receptors and provide selectivity is highly considered, but triggering phosphorylation events that afford the receptor the ability to primarily recruit a coactivator and then benefit from its enhanced transcription potential would provide a means by which optimal transcriptional responses can be achieved through a shared use of incoming activating signals. Such a mechanism is likely to occur in the activation of unliganded ERβ by SRC-1 under enhanced Erk signaling as SRC-1 contains several consensus Erk phosphorylation sites that confer activation potential [14,36]. By contrast, a common upcoming signal that benefits coactivators might serve to attenuate receptor activity in certain conditions. For instance, although Akt activation confers an intrinsic activation potential to CBP through phosphorylation of Thr-1872, it reduces the ability of ERβ and the estrogen-related receptor ERRβ to benefit from this CBP activation owing to phosphorylation of a conserved Akt site in their respective hinge region [16]. In contrast, receptors missing this Akt site, such as ERα and peroxisome proliferator-activated receptor PPARγ, efficiently respond to CBP coactivation. Therefore, the availability and ability for second messenger signaling to regulate ERβ might account for the differences in ERβ action in various expressing cells and tissues, providing a coordinated and organized recruitment and activation of appropriate coactivators.

Our initial findings regarding the ability of ERβ AF-1 to recruit SRC-1 in a ligand-independent manner revealed another layer of regulation in the actions of ERβ [14]. For example, the repressive effect of ERβ on PPARγ activity in adipose tissue involves the titration of SRC-1 to ERβ in a ligand-independent manner, preventing SRC-1 from facilitating PPARγ activation and expression of adipogenic target genes, such as adipocyte protein 2 and adiponectin [37]. In addition, ERβ represses the transcriptional activity of the nuclear factor of activated T-cell (NFAT3) in a ligand-independent manner, resulting in downregulation of interleukin-2 promoter activity in breast and kidney cells [38]. Together, these studies underline the importance of ligand-independent and phosphorylation-mediated regulation of ERβ to coordinate the association and functionality of cofactors in order to affect its own or other transcription factor-signaling pathways.

A bigger wardrobe for ERβ

In addition to phosphorylation, other types of post-translational modifications influence ERβ function. Ubiquitination, for example, promotes ligand-induced degradation of ERβ by the 26S proteasome system [39,40], a process also characterized for ERα and essential to confer optimal response to estrogen through continuous receptor turnover [41]. The Carboxyl Terminus of Hsc70-Interacting Protein CHIP, a chaperone-dependent U-box E3 ubiquitin ligase, is essential for mediating ERβ degradation by estrogen through its interaction with the N-terminal end of ERβ [40]. This interaction switches off the transcriptional response of ERβ to hormone, in contrast to the C-terminal end which protects unliganded ERβ from degradation, suggesting that receptor degradation is involved in the

cessation of transcription. SUG1/TRIP1, a regulatory subunit of 26S proteasome, is also involved in ERβ degradation in a ligand-dependent manner [40,42]. We recently reported that ERβ phosphorylation also serves as a signal for receptor ubiquitination and degradation through the 26S proteasome. Indeed, the Angelman syndrome-associated protein E6-AP, a dual function steroid receptor coregulator/E3 ubiquitin ligase initially described to participate in the hormonal response of progesterone receptor and ERα [43], causes proteasome-mediated degradation of ERβ in a phosphorylation-dependent manner [39]. The recruitment of E6-AP to ERβ is induced by Erk phosphorylation of AF-1 Ser-94 and Ser-106, without a need for estrogen. Together with the ability of phosphorylated Ser-106 and Ser-124 to recruit SRC-1 under Erk activation [14], our findings illustrate a prominent role for a tight cluster of AF-1 phosphorylated residues (e.g. Ser-94, Ser-106 and Ser-124) that cooperate in generating signals to regulate the activation-degradation cycling of ERβ (Figure 1). Phosphorylation of Ser-80 of mouse ERβ has also been reported to regulate ERβ degradation through a competitive interplay with O-β-GlcNAcylation at the same site (Figure 1). O-GlcNAcylation of ERβ was predicted to prevent Ser-80 phosphorylation resulting in enhanced degradation and reduced activity of ERβ [44].

These mechanisms provide AF-1 domain with an important and intricate role in responding to signals that couple ERβ activity and degradation. Recently, SUMOylation, a process by which SUMO (small ubiquitin-like modifier) is covalently linked to target proteins, has emerged as a new modification of nuclear receptors and associated coregulators. For ERα, SUMO-1 modifications are linked with enhanced activity, a process probably also supported by SUMOylation of SRC-1 [45–47]. Although the functional significance of this modification remains to be elucidated, SUMOylation is emerging as an important and highly dynamic process that adds to the complexity of fine-tuning nuclear receptor functions. ERβ has not yet been described to be SUMOylated but it will be of interest to analyze whether and how this modification affects ERβ function in a similar manner.

ERβ, an asset or a threat

Although ERα is expressed in a majority of breast cancers, providing a strong predictive value and first line therapy using SERMs, the clinical significance of ERβ is still under debate [48–50]. Reductions in ERβ protein expression have been associated with the development of an invasive phenotype and poor survival rates during tamoxifen treatment, raising the possibility that loss of ERβ encourages tumorigenesis [51–53]. However, the positive association of ERβ with the proliferation marker Ki-67 might also support the role of ERβ in tumor proliferation [54–56]. The unfortunate development of acquired resistance to the SERM tamoxifen is a major clinical problem, and although tamoxifen has an initial benefit a majority of patients eventually relapse. These limitations illustrate the need to better understand the role of ERα and ERβ in resistance.

Preclinical and clinical studies revealed that upregulation of Erk signaling is associated with failure of endocrine therapy [57]. Although effective in blocking ER AF-2 function, tamoxifen does not prevent SRC-1 from activating ERβ in EGF-activated cells [14,20]. Additionally, in the presence of tamoxifen, SDF-1 maintains the ability to increase ERβ recruitment and expression of AP-1 regulated genes such as cyclin D1 and c-Myc [29], two genes commonly overexpressed in primary breast tumors and recognized markers of early steps in breast tumorigenesis [58]. The cellular mechanisms by which ER-positive tumor cells overcome anti-estrogen effects and exhibit excessive proliferation remain uncertain at present, but these findings are consistent with the possibility that the autocrine regulation and coupling between Erk-regulated pathways and ER signaling might function despite the presence of tamoxifen. Our observations that both processes require Erk activation to phosphorylate ERβ Ser-87 are consistent with a prominent role of the AF-1 region in sustaining transcription potential by ERβ. It is tempting to speculate that the ability of deregulated cellular signals to phosphorylate and upregulate AF-1 activity during tamoxifen exposure might supplant AF-2 as the primary route of ER activation in tamoxifen-resistant breast cancer cells. In consonance, the use of ERβ protein expression levels, complemented with its site-specific phosphorylation status, might represent a valuable biomarker in tumor screening which, along with ERα protein analysis, has the potential to indicate therapeutic responses and course/outcome of disease for breast cancer patients.

Concluding remarks

Phosphorylation emerges as an additional and significant determinant in regulating ERβ transcriptional competence in response to hormone and ligand-independent signals. With the extent of upstream signaling pathways involved, the diversity of sites being characterized and the high degree of homology of some of them with nuclear receptor isoforms, it is highly expected that additional and/or overlapping roles will be revealed for individual receptor functions. Although specific roles of several of these sites in ERβ have been unveiled, a tremendous task remains to functionally assign detailed mechanistic relationships and biological significance to each putative site in dictating ERβ function. Global gene expression profiling affected by specific site disruption and mouse knock-in models are necessary strategies to address target- and tissue-specific requirements for ERβ phosphorylation. Future studies are thus needed to further elucidate how ERβ exerts its varying effects.

Acknowledgments

We thank members of the laboratory for critical reading and useful comments. M.S. is supported by a doctoral award from the FHSJ (Fondation de l'Hôpital Ste-Justine), N.P. by the FRSQ (Fonds de la Recherche en Santé du Québec) and by the FHSJ, and K.S. by the GRUM (Groupe de Recherche sur le Médicament de l'Université de Montréal), the FHSJ and the CIHR (Canadian Institutes of Health Research). A.T. is a New Investigator of the CIHR. This work was supported by grants from the CIHR, the NSERC (Natural Sciences and Engineering Research Council of Canada), the Cancer Research Society Inc. and the Canadian Foundation for Innovation.

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